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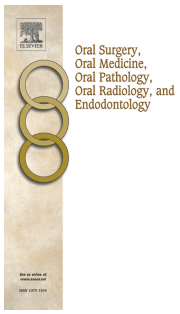

Immunohistochemical expression of K6, K8, K16, K17, K19, Maspin, Syndecan-1 (CD138), α-SMA and Ki-67 in ameloblastoma and ameloblastic carcinoma: diagnostic and prognostic correlations

Rima A. Safadi, BDS, MS, Basma F. Quda, BDS, MS, Huda M. Hammad, BDS, MS

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Immunohistochemical expression of K6, K8, K16, K17, K19, Maspin, Syndecan-1 (CD138), α-SMA and Ki-67 in ameloblastoma and ameloblastic carcinoma: diagnostic and prognostic correlations

Rima A. Safadi, BDS, MS [a 1], Basma F. Quda[b 2], BDS, MS, Huda M. Hammad BDS, MS[c]

[a]: Associate Professor, Faculty of Dentistry, Jordan University of Science and Technology, P.O Box: 3030, Irbid 22110, Jordan. Tel: 009627201000

[1]: "Current address": College of Dentistry, King Saud bin Abdul Aziz University for Health Sciences. P.O Box: 22490, mail code: 1243, Riyadh 11426, Kingdom of Saudi Arabia. Tele: 009668011111, ext: 14094, 14090, 95726. Mobile: 00966556443539
Email address: safadir@ksau-hs.edu.sa; rsafadi@just.edu.jo

[b]: Master Student at: Jordan University of Science and Technology, P.O Box3030, Irbid 22110, Jordan. Tele: 009627201000

[2]: "current address": Oral Medicine Specialist at Ministry of Health P.O Box 357, Zarqa11831, Jordan. Tel: 00962772077426
Email address: qudahbasma@yahoo.com

[c.] Associate Professor, Faculty of Dentistry, Jordan University of Science and Technology, P.O Box: 3030, Irbid 22110, Jordan. Tel: 009627201000
Email address: hmhammad@just.edu.jo

Author of Correspondence:
Rima A. Safadi
College of Dentistry, King Saud bin Abdul Aziz University for Health Sciences
P.O Box: 22490, mail code: 1243, Riyadh 11426, Kingdom of Saudi Arabia.
Tele: 009668011111, ext:14094, 14090, 95726.
Mobile: 00966556443539; 00966504457762
Email: safadir@ksau-hs.edu.sa
rsafadi@just.edu.jo

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Abstract

Objective. To identify cut off values of markers that correlate with the histopathologic diagnosis of ameloblastic carcinoma (AC) and/or the increased recurrence potential of ameloblastoma (AB). **Study Design**. Immunhistochemical expressions (IHCE) of nine selected markers were investigated in 18 non-recurrent ameloblastomas (NRAB), 6 recurrent ameloblastomas (RAB) and 5 ACs.

Results. No significant differences in IHCE of K6, K8, K16, K17, K18, K19, maspin and syndecan-1 were observed among study groups. α-SMA positive area in central epithelial cells significantly differentiated between AB and AC ($P = 0.017$; t-test). Ki-67 score significantly differentiated between AB and AC ($P < 0.005$; t-test) and between AC and RAB ($P = 0.015$; ANOVA/Post Hoc.

Conclusions. Ki-67 score of 75 cells/HPF, (ROC curve) is a potential indicator of AC. Clinical recurrence of AB may be predicted by α-SMA expression pattern. Syndecan1 and α-SMA may indicate a higher aggressive potential of AB when expressed in the stroma.

INTRODUCTION

Ameloblastoma (AB) is a benign but locally aggressive odontogenic tumor with variable clinico-pathologic behavior and tendency for recurrence.[1] Ameloblastic carcinoma (AC) is a malignant epithelial odontogenic tumor which may arise *de novo* or within an existing ameloblastoma. [2] Areas resembling ameloblastoma may still be seen with changes in pattern and variable cytologic features of malignancy. Good tissue sampling and adequate histologic evaluation to detect these cytologic changes are essential for correct diagnosis. [3] However, challenging cases of AC that exhibit subtle carcinomatous changes or limited amounts of biopsied tissue are occasionally encountered, making it difficult to confirm the diagnosis. In addition, some “atypical ameloblastomas” or “proliferative ameloblastomas” exhibit basilar hyperplasia and increased mitotic indices without sufficient histologic evidence of malignancy.[4] In these cases, the need for adjuvant histologic markers to confirm the diagnosis is obvious. [5]

In our biopsy service, diagnostic challenge has been met with two "atypical ameloblastoma" cases, which histopathologically showed no frank malignancy features, but clinically, there were multiple recurrences despite appropriate surgical treatment. This triggered a review of the current literature to select immunohistochemical marker/markers of diagnostic help. Different markers were available; some correlated with the diagnosis of AC, however, no cut off values have been published that would have offered some guidance. Thus, the given diagnosis for those lesions was "clinically aggressive ameloblastoma".

The literature is not elaborative in this area; not too many case reports of AC are cited. [6, 7-10] The same can be said about comparative immunohistochemical studies about corroborating the diagnosis of AC. [11-16] As for the prediction of the aggressive clinical behavior of ameloblastoma, the literature is mainly confined to suggestions of proteins of potential correlation with tumor invasiveness. [17, 18] The literature was searched to select markers of relevance to be further investigated in our study, and K6, K8, K16, K17, K19, maspin, syndecan-1 (CD138), α-SMA and Ki-67 were selected.

Twenty types of intermediate filaments, keratins, found in the cytoskeleton of epithelial cells play vital role in dynamic cellular changes like mitosis, the post mitotic period, cell movement, and differentiation. [19] K8 and K19 are expressed in normal odontogenic epithelium as well as in ameloblastoma. [20] K6, K16, and K17 are induced upon stress, injury, or inflammation and in epithelial tumors. [21-23]

Maspin, is a member of the serine protease inhibitor (serpin) superfamily which has antiprotease and tumor metastasis-suppressing activities.[24] The nuclear reaction of maspin immunostain may be due to binding directly with the tumor suppressor protein, p53.[24]

Syndecan-1 is a tumor inhibitor which is highly expressed in fibroblasts and epithelial cells.[25] Changes in Syndecan-1 expression reflect changes in behavior, shape, growth, migration and cytoskeletal organization of the cells.[26] Decreased syndecan-1 expression in head and neck squamous cell carcinoma has been shown to correlate with poor prognosis.[27]

The immunohistochemical expression of alpha smooth muscle actin (α-SMA), a marker of myofibroblasts, has been reported in a few studies of AB and AC.[28, 29] A positive link has been suggested between the number of myofibroblasts present in the stroma and the aggressive behavior of odontogenic tumors.[30] Myofibroblasts may facilitate invasion by expression and secretion of cytokines, growth factors, extracellular matrix molecules,

adhesion molecules and their receptors, which enhance epithelial-mesenchymal interactions.[31]

Ki-67 is a nuclear protein that is present during all active phases of the cell cycle.[32] It has been accepted as a marker of proliferative activity which is superior to proliferating cell nuclear antigen in measuring proliferative activity of tumors.[33, 34] Many tumors that highly express Ki-67 antigen exhibit poor survival rates.[35, 36]

This study has three main aims: first; to identify and suggest a cut off value of a marker/markers that will have practical implications in the histopathologic diagnosis of "atypical ameloblastoma" or AC with subtle carcinomatous changes. Second; to identify and propose a marker or markers that may be used to predict aggressive clinical behavior of otherwise benign looking ameloblastoma. Third; to apply an automated or semiautomated method in analysis of immunostains to arrive at objective results. The markers included in this study were: K6, K8, K16, K17, K19, maspin, syndecan-1, alpha-SMA, and Ki-67.

MATERIALS AND METHODS

Case selection

The research was approved by the IRB committee/Jordan University of Science and Technology number 58/2011.

The archives of King Abdullah University Hospital, Jordan University of Science and Technology, Irbid, Jordan, were searched for cases of AB and AC. Two other tertiary referral centers in Jordan were searched for cases of ameloblastic carcinoma (Al-Basheer Hospital, Ministry of Health and King Hussein Cancer Center).

Thirty four AB and five AC cases were retrieved. AB was grouped as recurrent (RAB) and non-recurrent (NRAB). RAB inclusion criteria were: surgical resection as the method of treatment, the availability of rebiopsy that was confirmed as recurrent tumor with follow up period of at least 5 years. Six cases of AB fulfilled the criteria for RAB and eighteen were NRABs. Ten AB cases did not fulfill the criteria of follow up and were excluded. RAB group included specimens of the primary lesion that subsequently recurred. Therefore, RAB and NRAB cases represent different cohorts of patients.

The microslides of retrieved cases were re-evaluated by a certified oral and maxillofacial pathologist to confirm that histopathologic features are consistent with the WHO Histological Classification of Tumours.[1]

Immunohistochemical procedure

Four micrometer-thick sections were cut from the 29 formalin-fixed paraffin-embedded tissue blocks and mounted on vectabond-coated and charged glass microslides (DiaPath, SuperFrost Plus, 060SFP, Italy). Tissue sections were dewaxed and rehydrated in preparation for avidin-biotin complex immunostaining method. Immunoreactivity of the tissue was enhanced by immersion of the sections in an antigen retrieval solution (Reveal Decloaker 10X, Biocare Medical, USA) in an autoclave for 7 minutes, at a temperature of 121ºC and a pressure of 1.5 Bar. After that, sections were allowed to cool down overnight in the antigen retrieval solution. Non-specific stickiness of proteins in the tissue was blocked by a non-specific protein block (Protein block, Biogenex, San Ramon, CA, USA), applied to each microslide for 20 minutes at room temperature.

Immunohistochemistry was performed by the standard method, using horseradish peroxidase (HRP)-diaminobenzidine (DAB) detection kits using (Dako EnVision™+ Dual Link System-HRP, diaminobenzidine solution (Dako, Glostrup, Denmark) and counterstained with Mayer's hematoxylin.

The immunohistochemical procedure was performed using an automated stainer (Autostainer *Plus,* Dako cytomation, Denmark). Sections stained in each run were from different study groups. All primary antibodies were diluted at 1:150 and used at room temperature for 1 hour incubation period. The primary antibodies used from Dako, Glostrup, Denmark were:

monoclonal Mouse Anti-Human CD138, Clone MI15, Monoclonal Mouse Anti-Human Ki-67 Antigen, Clone MIB-1, Mouse Monoclonal Anti-Human α-smooth muscle actin and Mouse Monoclonal Anti-keratin 17. Primary antibodies from Biogenex, California, USA were: Rabbit Monoclonal Anti-keratin 6, Mouse Monoclonal Anti-keratin 8, Rabbit Monoclonal Anti-keratin 16, and Mouse Monoclonal Anti-keratin 19 while Rabbit Anti-Maspin Polyclonal Antibody (H-130) was obtained from Santa Cruz Biotechnology Inc., Santa Cruz, California, USA.

Negative control tissue section in each immunohistochemical run was incubated with non-specific mouse IgG (Biogenex, California, USA) at 1:150 dilution replacing the primary antibody as a negative control. Positive controls for keratins were prepared from squamous cell carcinoma tissue known to express study markers. For Maspin, Syndican, α-SMA and Ki-67 positive control was internal. The stained sections were thoroughly examined using a light microscope at different magnifications. Representative fields were digitized for each immunostain at a ×10 magnification, except for Ki-67, where five high power fields were digitized at ×40.

Image analysis

Digitized images of maspin, syndecan-1, α-SMA, K6, K8, K16, K17 and K19 immunostained sections were analyzed using color deconvolution software plugged in ImageJ, by which the positive epithelial area was measured as well as the total epithelial area in the analyzed field. ImageJ is a public domain Java image processing program developed at the National Institute of Health (Bethesda, Maryland, USA). Method of color deconvolution analysis is detailed in a previously published article. [37]

For Ki-67 analysis, positive nuclei were counted on the PC screen in each of the five high power fields then the average number of positive nuclei was considered as the Ki-67 score.

Statistical analysis

Data were entered into the Statistical Package for Social Science (SPSS) program, version 15, (SPSS Inc., Chicago, IL, USA). The positive epithelial area was divided by the total epithelial area in the field to calculate the percentage. ANOVA/ Post Hoc/Tukey method was used to conduct multiple comparisons. Paired comparisons between AB (as a whole group) and AC were conducted using independent samples t-test. Spearman's correlation coefficient was used to identify the correlation between the statistically significant markers. Receiver Operating Characteristic (ROC) curve was carried out to measure the area under the curve and the cut off values of the significant markers that can discriminate between AB and AC.

RESULTS

Twenty four cases of AB and five ACs were included in this study. Six cases of AB fulfilled the inclusion criteria of RAB while 18 were NRAB. The age range of patients with NRAB was 13 to 78 years with an average of 37.05 years. Half of the patients were females. All of the eighteen NRAB occurred in the mandible. Considering RAB group, the age ranged from 20 to 60 years

with an average age of 38 years. All patients were females having all of their lesions occurring in the mandible. For AC the age ranged from 23 to 62 years with an average of 53.2 years. Three patients were females and two were males. Four out of five AC cases had their lesions occurring in the maxilla while only in one case the lesion occurred in the mandible. Follow up periods ranged from 5 to 18 years for all ameloblastomas (RAB and NRAB).For AC group, one case was of 17 years follow up and 4 were with periods of 1 to 2 years.

Positively stained cells consistently showed cytoplasmic brown staining for all study markers except for Ki-67 where the staining was nuclear, and for maspin where the staining was both cytoplasmic and nuclear. The connective tissue stroma was negative except for syndecan-1 and α-SMA. All negative controls did not show any brown stain.

Number of positive cases and patterns of expression

No specific trend in the number of positive cases or staining patterns of keratins were observed in RAB, NRAB or AC groups (not presented in tables). K6 was positive in 38% (7 out of 18) of NRABs, 83% (5 out of 6) of RABs and 40% (2 out of 5) of AC. K8 positivity was seen in 77% (14 out of 18) of NRABs, 66% (4 out of 6) of RABs and 100% (5 out of 5) of ACs. In K16, all cases of NRAB, RAB and AC were positive. K17 was positive in 83% (15 out of 18) of NRABs, 100 % (6 out of 6) of RABs and 100% (5 out of 5) of ACs. K19 stained sections were positive in 66% (12 out of 18) of NRABs, 83% (5 out of 6) RABs and 40% (2 out of 5) of ACs. K6, K8, K17 and K19 were all expressed more in stellate reticulum cells (SRCs) compared to peripheral columnar cells (PCCs), **(Figures 1, 2, 3: a-e)**.

All study cases expressed positive reactions to maspin, being expressed more in PCCs compared to SRCs. For syndecan-1 (CD138), the tissue positivity was mainly in SRCs **(Figures 1f-g, 2f-g, 3f- g)**. For α-SMA, RABs and ACs showed different staining patterns compared to NRABs. α-SMA was expressed in the SRCs in RABs and ACs (50% (3 out of 6) and 80% (4 out of 5), respectively), while it was only expressed in PCCs in 27% of NRABs (5 out of 18), **(Figures 1h, 2h and Figure 3h-i)**. For Ki-67, positively stained nuclei were mostly located in the PCC layer. The number of positive cases was highest in AC followed by RAB. In AC, Ki-67 showed a wider distribution that included SRCs in addition to PCCs **(Figure 4a-c)**.

Image analysis results of study markers:

(Table I) presents the immunohistochemical expression profile of study markers in each case of all study groups as well as the mean percentages of positive epithelial areas and/or scores. The mean percentage of positive epithelial areas of K6 and K19 were less in AC compared to NRAB and RAB. The mean percentages of maspin and syndecan-1 (CD138) positive areas in the three study groups had close values. When multiple comparisons of the mean positive areas of all study markers were conducted; keratins, maspin, and syndecan-1 positive epithelial areas showed no significant differences among study groups ($P > 0.05$; ANOVA test, data not in tables). Only Ki-67 and α-SMA significantly differentiated among study groups, ($P = 0.004$ and $P = 0.002$, respectively; ANOVA test, data not in tables).

(Table II) presents paired comparisons between study groups using ANOVA/Post Hoc/Tukey test. α-SMA positive areas were not significantly different between RAB and NRAB groups, although they had a higher mean in the recurrent variant, nor it was different between RAB and AC (P = 0.99 and 0.14, respectively; ANOVA/ Post Hoc). However, the difference was statistically significant between AC and NRAB (P = 0.05; ANOVA/Post Hoc) and between AB as a whole group and AC (P = 0.017; student t test, data not in tables, results confirmed by Mann-Whitney U test with a P value of 0.013). Unlike α-SMA, Ki-67 score was significantly higher in AC compared to RAB (P = 0.015; ANOVA/ Post Hoc) as well as in AC compared with AB (P < 0.005; student t test, results confirmed by Mann-Whitney U test with a P value of 0.015; data not in tables**)**. Ki-67 scores of NRAB and RAB were not significantly different (P = 0.95; ANOVA/ Post Hoc**)**.

Cut off values for α-SMA and Ki-67 expression:

The area under the ROC curve was 0.833 and 0.917 for α-SMA and Ki-67, respectively. The best cut off values for the α-SMA area and Ki-67 score that gave the highest sensitivity and, at the same time, the highest specificity were 0.19 and 75, respectively. This provides a sensitivity of 80% with 100% specificity for α-SMA and 80 % sensitivity with a specificity of 95 % for Ki-67 score, to favor a diagnosis of AC (**Figures 5 and 6)**. Spearman's correlation coefficient showed that Ki-67 and α-SMA are highly and significantly positively correlated (Spearman's correlation coefficient = 0.51, P = 0.012), and are potentially competitive variables in terms of corroborating ameloblastic carcinoma. This correlation was evident in 3 out of the 5 AC cases in which α-SMA epithelial positivity was noticed along with increased Ki-67 expression (**Table I,** immunoprofile of AC cases).

DISCUSSION

Diagnostic difficulty is sometimes encountered with AB of unusually aggressive behavior, and in differentiating it from AC, which is a rare odontogenic tumor. Only about 92 cases of AC have been reported in the literature. [38] To our knowledge, the numbers of AC cases included in several studies ranged from one to a maximum of thirteen, further reflecting this rarity.[11-16] To overcome the inherent rarity of this malignant odontogenic tumor, we searched the archives of the four main tertiary referral centers in Jordan. Only five cases were found; all were included in this study.

To identify and suggest a cut off value of a marker/markers that will have practical implications in corroborating the diagnosis of AC especially those cases with subtle carcinomatous changes, and to identify and propose a marker/markers that may be used to predict aggressive clinical behavior of otherwise benign looking AB, the pattern and positive area of immunohistochemical expressions of a panel of cytokeratins (K6, K8, K16, K17, K19), as well as maspin, syndecan-1 (CD138), α-SMA and Ki-67 were investigated and analyzed using computerized image analysis. For the first time, cut off values of Ki-67 score rather than labeling indices were proposed, as a practical way that is familiar to the histopathologist.

The markers of potential diagnostic and prognostic values in this study were α-SMA and Ki-67, which statistically significantly differentiated between AB and AC, whereas keratins 6, 8, 16, 17 and 19, maspin and syndecan-1 did not show significant differences among the study groups, neither in pattern nor in positive area percentage.

α-SMA differentiated between AC and AB (as a whole group); however, no significant difference in α-SMA expression was present between RAB and AC. The inference that can be made from this result is that α-SMA positivity in the central cells of a specific case of ameloblastoma has two implications; first, if combined with appropriate histopathologic features and/or increased mitoses, it supports the diagnosis of AC. Alternatively, if histopathologic features are not convincing of a diagnosis of carcinoma, it may indicate an increased potential for clinical aggressiveness, which warrants labeling or commenting on such a lesion as “ameloblastoma with increased potential for clinical aggressiveness” or "with unusual increased recurrence potential".

Expression of α-SMA was seen in the SRCs of 80% of AC cases compared to none of those of the NRABs. The expression of this myofibroblastic marker in the central cells of the epithelial islands has been suggested to be a marker of carcinomatous change.[6, 11,] In addition to reactivity of SRCs, all cases of AC in our study showed strong positive connective tissue reaction for α-SMA which has been also correlated with increased invasiveness and aggressiveness. [30, 39]

Ki-67 was expressed mainly in PCCs of NRABs, RABs and ACs, which is in accordance with the literature.[13, 40, 41] The peripheral layer in AB is cuboidal to columnar, which cytologically resembles the basal cell layer in oral squamous epithelium with higher proliferative potential compared to the suprabasal SRCs which have a lower proliferative potential compared[14] However, in AC, Ki-67 expression extended to SRCs, reflecting the increase in proliferative potential of SRCs, a feature which is not seen RAB or the NRAB cases of this study.

Our results propose that the clinical aggressiveness with potential for multiple recurrences of AB may not necessarily be reflected by a high Ki-67 score, since there was no statistically significant difference between RAB and NRAB. Therefore, it is suggested that other factors may play a role in the increased recurrence potential of AB, such as the increased α-SMA expression discussed above.

In our study, the histopathologic diagnosis of AC was correlated with a Ki-67 score of 75 cell/HPF. This score would be potentially helpful when considering challenging cases of AB, the “atypical” type as referred to by Slater, [42] that show atypical histopathologic features, but not frank malignancy. As indicated by Florescu *et al.,*[40] despite the fact that ACs show low numbers of mitotic figures, the number of cells capable of proliferation is very large, [40] raising the need for a reasonable adjuvant marker to reflect this hidden potential.

Ki-67 cut off values obtained from the ROC curve of this study, indicate that Ki-67 score is a very good test to differentiate among the studied groups, as the area under the curve approximates 1.0. ROC curve is a statistical method commonly used to predict certain diseases using specific cut off values with the highest specificity and sensitivity.[43, 44]

The rest of the studied markers showed no significant differences in the distribution of the mean positive areas of K6, K8, K16, K17, K19 and maspin among the study groups. The variable staining patterns among the constituent cells of the study groups is in accordance with what is mentioned in the literature. Ong'uti *et al.* [45] reported that there was weak expression for K8 in suprabasal and SRCs of AB, while Chen *et al.* [46] found strong to moderate K8 immunoreactivity within epithelial cells of AB. Fukumashi *et al.* [20] reported that K8 and K19, which are markers of odontogenic epithelium, reacted positively with the constituting cells in all types of AB.

The expression of syndecan-1 (CD138) was slightly lower in RAB and AC compared to NRAB. Similarly, Bologna- Molina *et al.* found a decreased expression of syndecan-1 in the epithelial component of AC. [27] Interestingly, the connective tissue of all cases of AC expressed syndecan-1, which may indicate increased local aggressive behavior. [15]

In conclusion, Ki-67 score of 75 cells/HPF is a potential indicator of AC. Clinical recurrence of otherwise benign AB may be predicted by pattern and extent of α-SMA expression.

Syndecan-1 may be potentially helpful in indicating a higher aggressive potential when expressed in the connective tissue stroma. Our study provides baseline data for future studies to compare the results in terms of cut off values of Ki-67. Further collaborative studies are encouraged to overcome the inherent rarity of AC.

Statement of Clinical relevance

A cut off value of 75 Ki- 67 positive cells /HPF is suggested to support the diagnosis of ameloblastic carcinoma, α-SMA pattern is suggested to indicate increased recurrence potential of ameloblastoma.

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Fig. 1. Colored photomicrographs of immunostained sections of all study markers in a NRAB case. Original images taken at 200X magnification. **(a)** K6 positive focal expression, **(b)** K8 positive expression more in SRC, **(c)** K16 positive diffuse expression, **(d)** K17 positive expression, **(e)** K19 positive focal expression, more in SRC (*A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM01158)*, **(f)** Maspin positive expression, note positive cytoplasmic and nuclear staining, **(g)** Syndecan-1 positive expression, more in SRC (*A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM01159)*, **(h)** α-SMA negative expression, note positive blood vessels walls (*A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM01160)*.

Fig. 2. Colored photomicrographs of immunostained sections of all study markers in a RAB case. Original images taken at 200X magnification. **(a)** K6 negative expression, **(b)** K8 positive expression (*A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM01161)*, **(c)** K16 positive expression, **(d)** K17 positive expression, **(e)** K19 negative expression, **(f)** Maspin expression, **(g)** Syndecan-1 expression (*A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM01162)*, **(h)** α-SMA positive expression, note positive expression in SRC of epithelial islands (*A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM01163)*.

Fig. 3. Colored photomicrographs of immunostained sections of all study markers in an AC case. Original images taken at 200X magnification. **(a)** K6 focal positive expression (*A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM01165)*, **(b)** K8 positive expression more in SRC, **(c)** K16 positive diffuse expression (*A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM01166)*, **(d)** K17 positive diffuse expression, **(e)** K19 positive focal expression, **(f)** Maspin expression, **(g)** Syndecan-1 positive epithelial and connective tissue expression (*A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM01167)*, **(h)** α-SMA negative expression except for blood vessels walls (*A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM01168)*, **(i)** an example of positive α-SMA expression in another AC case, note positive epithelial and connective tissue expression (*A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM01169)*.

Fig. 4. Colored photomicrographs of Ki-67 immunostained sections, originally taken at 200X. **(a)** NRAB showing scattered positive nuclear staining in basal cells (*A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM01170)*, **(b)** RAB showing positive nuclear staining in PCC and few scattered positive nuclei of SRC (*A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM01171)* and **(c)** AC showing diffuse positive nuclear staining in PCC and SRC (*A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM01172)*

Fig. 5. Area under the curve of the ROC test for α-SMA (0.833). The best cut off values of α-SMA that gave highest sensitivity (80%) and simultaneous highest specificity (100%) is 0.19 as presented in curve coordinates.

Fig. 6. Area under the curve of the ROC test for Ki-67 score (0.917). The best cut off values of Ki-67 score that gave highest sensitivity (80%) and simultaneous highest specificity (95%) is 75 as presented in curve coordinates.

Table I: Immunoprofile of all study cases and mean percentage of all study markers in the groups of: non recurrent ameloblastoma, recurrent ameloblastoma and ameloblastic carcinoma

Group	Non Recurrent Ameloblastoma																			Recurrent Ameloblastoma							Ameloblastic Carcinoma					
Case/ marker	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	**Mean %**	1	2	3	4	5	6	**Mean %**	1	2	3	4	5	**Mean %**
K6	*	+	+	-	-	-	-	-	*	*	-	-	-	+	+	-	-	+	**8.72**	+	+	*	-	+	-	**8.83**	-	-	-	+	+	**3.6**
K8	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	**18.89**	+	*	0	+	+	-	**8.83**	+	-	+	+	+	**16.8**
K16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	**45.22**	+	+	*	+	+	+	**39.17**	+	+	+	+	+	**51.2**
K17	+	+	*	+	-	-	+	*	+	+	+	+	+	+	+	+	+	+	**18**	+	+	*	+	+	-	**22.17**	+	-	+	*	+	**18**
K19	+	-	-	+	+	*	-	+	+	+	+	+	+	+	+	+	-	-	**21.72**	+	+	+	+	+	-	**20.67**	-	-	-	+	+	**12.2**
Maspin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	**59.11**	+	+	+	+	+	+	**51.67**	+	+	+	+	+	**53.2**
Syndican	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*	+	**39.06**	+	+	+	+	+	+	**31.33**	+	+	-	+	+	**33**
SMA	-	-	-	-	-	-	-	-	-	*	-	-	-	+	*	-	*	+	**3.11**	-	-	-	+	+	+	**15.67**	+	+	+	-	-	**26.4**
Ki67	22	70.4	72.4	63	11	0	0	0	40	42	37.2	39.8	3	68.2	42.6	91.5	43	39.8	**38**	19.6	29.6	83	16	106.	67	**42**	112.2	52.6	115.4	154.75	78.3	**103**

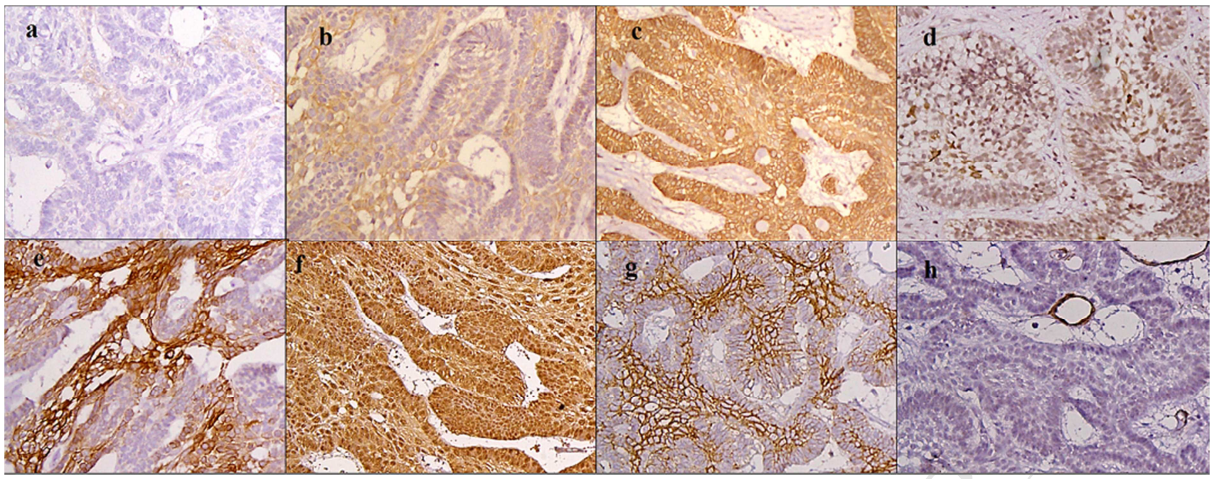
*=positive area is less than 10%

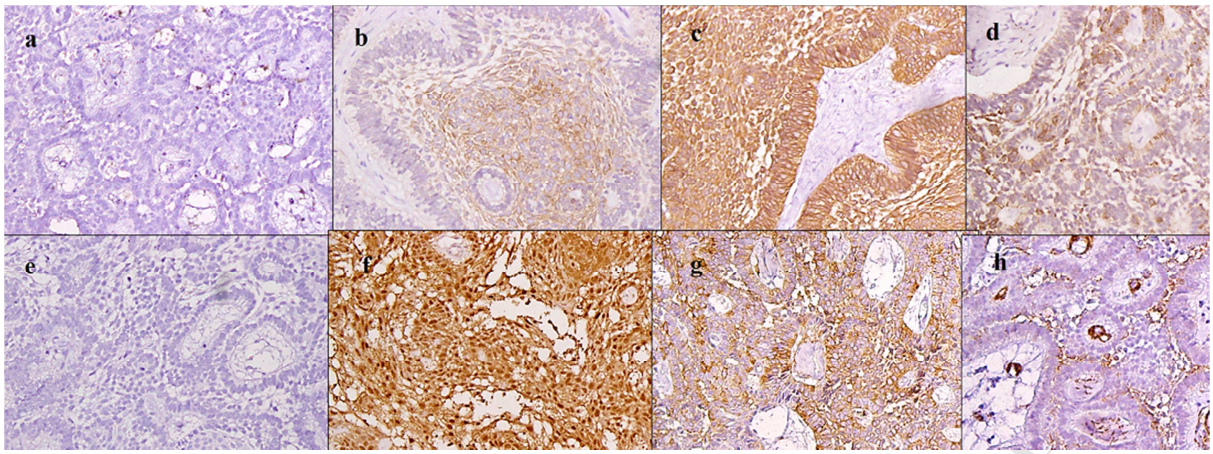
Table II: Paired multiple comparisons of mean percentages of positive areas of Keratins (6, 8, 16, 17 and 19), Maspin, Syndican 1, α-SMA and Ki-67 among study groups using ANOVA/ Post Hoc test.

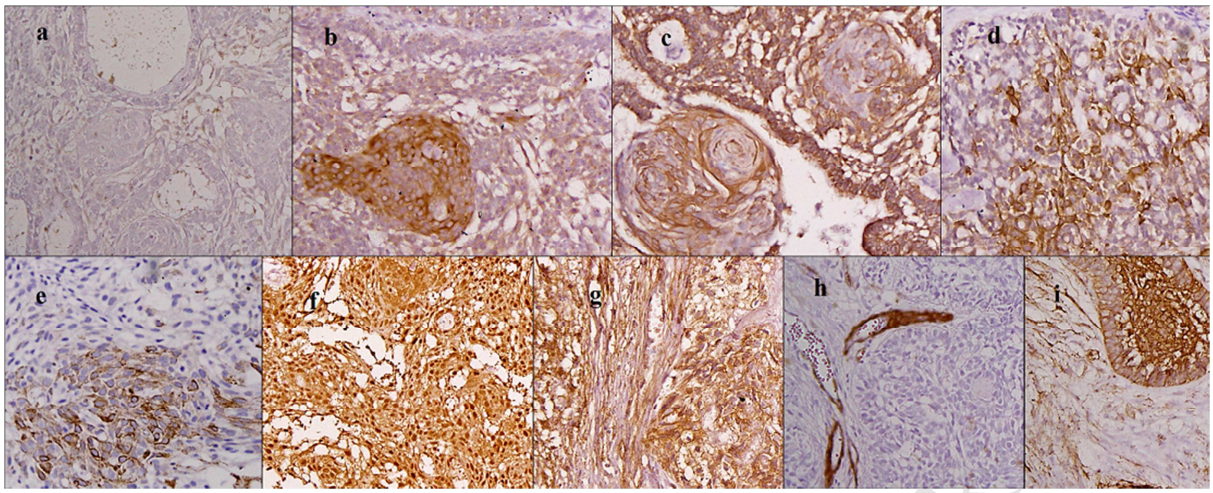
Marker expressed	Type of lesion	Compared with	Mean Difference	P value	95% Confidence Interval	
					Upper Bound	Lower Bound
K6	Non recurrent Ameloblastoma	Ameloblastic carcinoma	.051	.64	-.089	.191
		Recurrent ameloblastoma	-.001	1.0	-.132	.130
	Ameloblastic carcinoma	Recurrent Ameloblastoma	-.052	.72	-.220	.116
K8	Non recurrent Ameloblastoma	Ameloblastic carcinoma	.020	.95	-.154	.196
		Recurrent ameloblastoma	.100	.29	-.062	.263
	Ameloblastic carcinoma	Recurrent Ameloblastoma	.079	.61	-.130	.289
K16	Non recurrent Ameloblastoma	Ameloblastic carcinoma	-.059	.71	-.247	.128
		Recurrent ameloblastoma	.060	.67	-.114	.235
	Ameloblastic carcinoma	Recurrent Ameloblastoma	.120	.39	-.104	.345
K17	Non recurrent Ameloblastoma	Ameloblastic carcinoma	.000	1.0	-.182	.182
		Recurrent ameloblastoma	-.041	.81	-.211	.128
	Ameloblastic carcinoma	Recurrent Ameloblastoma	-.041	.88	-.260	.176
K19	Non recurrent Ameloblastoma	Ameloblastic carcinoma	.095	.64	-.169	.359
		Recurrent ameloblastoma	.010	.99	-.235	.257
	Ameloblastic carcinoma	Recurrent Ameloblastoma	-.084	.78	-.401	.231
Maspin	Non recurrent Ameloblastoma	Ameloblastic carcinoma	.059	.75	-.145	-.145
		Recurrent ameloblastoma	.074	.60	-.116	-.116
	Ameloblastic carcinoma	Recurrent Ameloblastoma	.015	.98	-.229	-.229
Syndican CD138	Non recurrent Ameloblastoma	Ameloblastic carcinoma	.060	.70	-.128	-.128
		Recurrent ameloblastoma	.077	.52	-.098	-.098
	Ameloblastic carcinoma	Recurrent Ameloblastoma	.016	.98	-.209	-.209
α-SMA	Non recurrent	Ameloblastic	-3.10	.05*	-6.23	-6.23

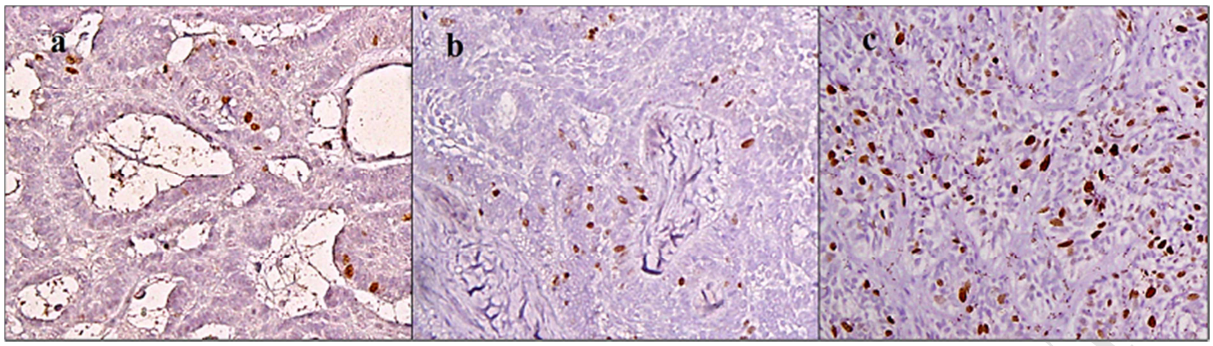
	Ameloblastoma	carcinoma				
		Recurrent ameloblastoma	-.125	.99	-3.04	-3.04
	Ameloblastic carcinoma	Recurrent Ameloblastoma	2.97	.14	-.777	-.777
Ki-67	Non recurrent Ameloblastoma	Ameloblastic carcinoma	-64.5	.002*	-105.9	-105.9
		Recurrent ameloblastoma	-4.37	.95	-42.9	-42.9
	Ameloblastic carcinoma	Recurrent Ameloblastoma	60.1	.015*	10.5	10.5

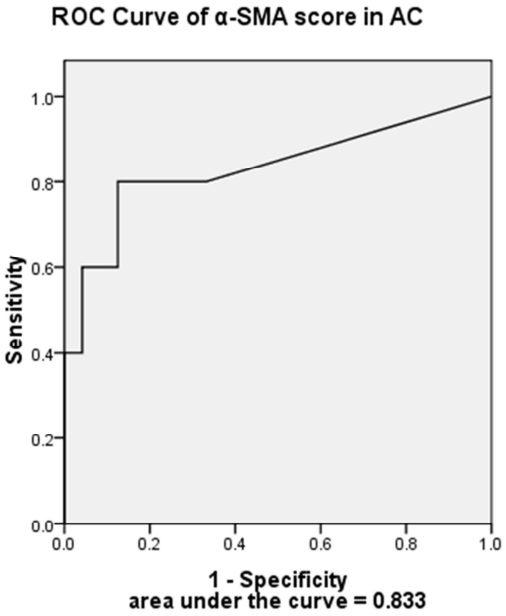
- Statistically significantly different





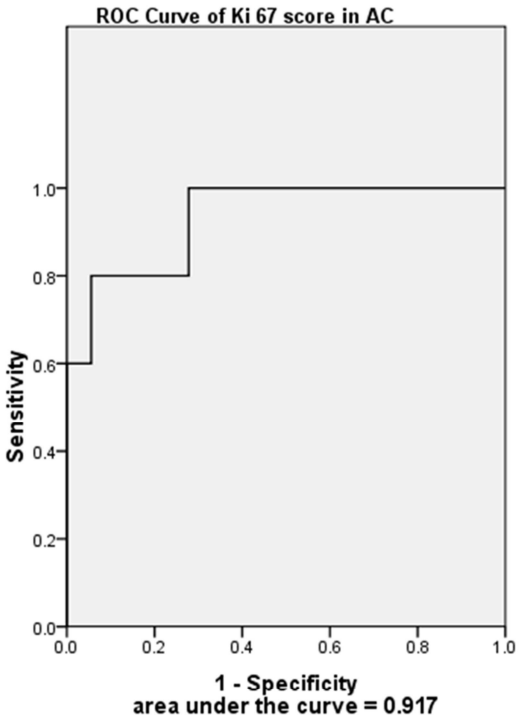






Coordinates of the Curve

α-SMA positive area in AC	Sensitivity	1 - Specificity
-1.0000-	1.000	1.000
.0100	.800	.333
.0400	.800	.292
.0950	.800	.250
.1500	.800	.208
.1750	.800	.167
.1900	**.800**	**.125**
.2100	.600	.125
.2450	.600	.083
.3100	.600	.042
.4000	.400	.042
.5350	.400	.000
7.5600	.200	.000
15.5000	.000	.000



Coordinates of the Curve

Ki67 score in AC	Sensitivity	1 - Specificity
-1.0000-	1.000	1.000
1.5000	1.000	.833
7.0000	1.000	.778
16.5000	1.000	.722
29.6000	1.000	.667
38.5000	1.000	.611
39.9000	1.000	.500
41.0000	1.000	.444
42.3000	1.000	.389
42.8000	1.000	.333
47.8000	1.000	.278
57.8000	.800	.278
65.6000	.800	.222
69.3000	.800	.167
71.4000	.800	.111
75.3500	**.800**	**.056**
84.9000	.600	.056
101.8500	.600	.000
113.8000	.400	.000
135.0750	.200	.000
155.7500	.000	.000

Statement of Clinical Relevance:

This study is of practical diagnostic aid to the histopathologist in predicting aggressive clinical behavior of ameloblastoma and in confirming a diagnosis of ameloblastic carcinoma, especially when carcinomatous changes are subtle.