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RESEARCH ARTICLE

EXPRESSION OF MIDKINE AND KI-67 PROTEINS IN AMELOBLASTOMA VERSUS AMELOBLASTIC CARCINOMA ACCORDING TO THEIR CLINICOPATHOLOGIC PARAMETERS.

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Key words:-
Ameloblastomas, Ameloblastic carcinoma, Midkine, Ki-67.

Abstract

Aims: To correlate between Midkine and Ki-67 expression in ameloblastoma and ameloblastic carcinoma and their relation to clinicopathological parameters.

Methods: This study was carried out on 27 cases of ameloblastoma and ameloblastic carcinoma. Archival blocks were obtained from Pathology Department, Faculty of Medicine, Assuit University. Paraffin sections of tumor tissue from all cases were submitted for routine haematoxylin and eosin stain and immunohistochemistry using Midkine and Ki-67 monoclonal antibodies. Immunostaining was evaluated using area fraction and the results were analyzed statistically. P value ≤ 0.05 was considered significant.

Result: Among 27 cases, 11 were females and 16 were males with ratio 1:1.4. Age ranged from 12 to 64 years old with a mean age 34.2 years. Ten lesions were located in maxilla and 17 were located in mandible with ratio 1: 1.7. No statistically significant in midkine or Ki-67 expression was found in relation to patient age, gender or tumor site. However, there was a significant difference in their expression between ameloblastoma and ameloblastic carcinoma. Moreover, There was a significant positive correlation between midkine and Ki-67 expression in the studied cases ($p = 0.000$).

Conclusion: Evaluation of Midkine and Ki-67 expression can provide information about clinical behavior of the tumor. There is a significant positive correlation between midkine and Ki-67 expression in ameloblastoma and it is not dependent on clinical or tumor criteria.

Introduction:-

Ameloblastoma (AB) is a relatively rare odontogenic epithelial tumor as it represents approximately 1 % of all jaw tumors. Although rare, ameloblastoma is considered the second most common odontogenic tumor after keratocystic odontogenic tumor (Niranjan & Shaikh, 2014). Ameloblastoma may arise from developing enamel organ, epithelial cell rest of dental lamina, epithelial lining of odontogenic cysts or basal cells of oral epithelium (Amaral et al., 2012). It is a benign tumor but has aggressive characteristics such as persistent growth and local invasion to

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surrounding structures (Intapa, 2017) with a high rate of recurrence (Shaikhi et al., 2012). In 2005, World Health Organization (WHO) classified ameloblastoma into multicystic ameloblastoma (MCA), extra-osseous/peripheral, desmoplastic, and unicystic ameloblastoma (UCA) (Barnes et al., 2005).

Ameloblastic carcinoma (AC) is a rare malignant odontogenic tumor. It is more commonly located in the mandible and shows clear cytological features of malignancy with high tendency for recurrences (Shaikhi et al., 2012). According to WHO classification, ameloblastoma is considered malignant when metastasizes in spite of having a benign histological appearance. Moreover, ameloblastoma with cytological atypia is termed ameloblastic carcinoma even if metastasis is absent (Barnes et al., 2005).

Ki-67 antigen is a classic cellular proliferation marker which has been widely used in the diagnostic research (Bologna-Molina et al., 2013). Ki-67 is expressed in all phases of active cell cycle (G1, S, G2 and M phase) by proliferating cells, but it is absent in resting (G0) phase (Abdel-Aziz & Amin, 2012). The fact of its absence in quiescent cells (G0 phase) makes Ki-67 an excellent marker for determining the cellular proliferation and growth rate (Bologna-Molina et al., 2013).

Midkine (MK) is a 13-kDa small heparin-binding growth factor (Hodeib et al., 2017). It was found as a product of retinoic acid gene and located at chromosome 11p11.2 (Scheper et al., 2012). MK is highly expressed in the mid-gestation period. Despite of its high expression during embryogenesis, MK is not detectable in healthy adults (Jono & Ando, 2010). However, many studies showed high expression of MK in various types of carcinomas in a tissue type-independent manner (Kadomatsu & Muramatsu, 2004). In some tumors, MK expression is induced early in the pre-cancerous stage (Muramatsu et al., 1991; Ikematsu et al., 2000). Moreover, MK plays a significant role in carcinogenesis related activities, such as proliferation, fibrinolysis, cell migration, cell survival, anti-apoptosis, mitogenesis, transformation, and angiogenesis (Kadomatsu, 2005; Jono & Ando, 2010).

The purpose of this study is to assess and correlate between MK and Ki-67 protein expression in ameloblastoma and ameloblastic carcinoma and their association with different clinicopathologic parameters.

Material and Methods:-

This study was approved by the review board of the Ethics Committee of Faculty of Dentistry, Minia University.

Specimen Selection:-

Twenty seven formalin-fixed and paraffin-embedded specimens were included in the study, which were previously diagnosed as ameloblastoma and ameloblastic carcinoma. Clinical records for the patients were reviewed and information was gathered regarding age, gender and location of the tumor. The specimens were collected from archives of Pathology Department, Faculty of Medicine, Assuit University. Each case was coded and patient's name was not shown for ethical reasons.

To confirm the diagnosis, 5 μm thick sections were cut and mounted on glass slides; sections were stained with haematoxylin and eosin stain and examined by light microscope. According to the histopathological criteria and based on WHO classification, slides were classified into ameloblastic carcinoma (Figure 1) and ameloblastoma which was subdivided into multicystic (Figure 2) and unicystic type (Figure 3).

Immunohistochemistry:-

For all specimens, 3-μm sections were cut and mounted on positively charged glass slides. The cutting sections were de-waxed in xylene and rehydrated in graded alcohol. Blocking of endogenous peroxidase activity was done by applying 3% hydrogen peroxide for 5 minutes at room temperature, then the slides were washed by phosphate buffer solution. For antigen retrieval, we used Envision^{TM} FLEX Target Retrieval solution, high pH (pH 9) (Dako, Denmark) with concentration 1:50. Using a heater, we preheated the solution to 65° C and then the slides were immersed in it and incubated for 20 minutes at 97°C. Two primary antibodies were used, flex monoclonal mouse anti-human ready to use ki-67 antibody (clone MIB-1, IS626, Ready to use, Dako, Denmark) and monoclonal mouse anti-human MK primary antibody (clone A-9, sc-46701, Santa Cruz Biotechnology Inc., CA) with a dilution 1/100. The slides were incubated at 4°C overnight. Next day, the slides were incubated with goat secondary antibody (HRP) for 20 minutes. A 3, 3-diaminobenzidine- H_2O (Dako, Denmark) substrate was used to visualize the reaction. Finally, the sections were counterstained with Mayer's hematoxylin. All steps were performed according to manufacture instruction guide. Proper positive and negative controls were used. Tonsil tissue was used as positive

control for Ki-67 and human duodenum tissues for MK (according to manufacture recommendation), while negative controls were obtained by omission of the primary antibodies, which were substituted with 1% phosphate buffered saline (PBS).

Assessment:-
For immunostaining analysis, five photomicrographs for higher immunopositive areas for each case were captured using a digital camera mounted on the light microscope (Leica, Germany) at a magnification of ×400. Images were then transferred to the computer system for analysis using image analysis software (Image J, 1.41a, NIH, USA). MK and Ki-67 immunoreactivity was evaluated using area fraction (percentage of immunopositive area to the total area of the microscope field).

Statistical Analysis:-
The mean area fraction for each case was calculated and used for statistical analysis. The data was stored, analyzed and graphics were done by The Statistical Program SPSS for Windows version 22. The statistical tests performed included Mann–Whitney test, Kruskal–Wallis test and Spearman correlation factor. The results were considered significant for $p \leq 0.05$.

Result:-

Clinico-pathological Characteristics:-
A total of 25 cases of ameloblastomas and 2 cases of ameloblastic carcinoma were identified. Regarding gender, 16 were males (59.3%) and 11 were females (40.7%) with ratio 1.4:1and mean age of 34.2 years (range 12-64 years old). Ten lesions were located in the maxilla (37%) and seventeen in the mandible (63%) with ratio 1: 1.7. Based on clinico-pathologic criteria, the 25 ameloblastomas were classified as multicystic (14) and unicystic (11) (Table 1).

Immunohistochemical Results:-
Positive brownish immunostaining of MK (Figures 4, 5) and Ki-67 (Figures 6, 7) was found in all cases of AB and AC. MK immunoreaction was more prominent in the cytoplasm of columnar epithelial cells in the peripheral cell layer. Ki-67 showed positive nuclear immunoreaction in peripheral cell layer. Using area fraction method, the expression of MK in ameloblastoma ranged between (13.00% - 18.36%) with mean value of (16.47% ±1.45%). Ki-67 Expression ranged between (3.30% - 7.99%) with mean value of (5.37% ±1.45%) (Table 2).

No significant difference in MK expression was found in relation to age ($p = 0.06$), gender ($p = 0.154$) or site of tumor ($p = 0.2$). Similarly for Ki-67 expression, no significant difference was found in relation to age ($p = 0.191$), gender ($p = 0.297$) or site of tumor ($p = 0.977$).

Kruskal-Wallis test revealed a significant difference between MK or Ki-67 expression in AB and AC, with higher expression in AC, and no significant difference between UCA and MCA was found regarding both MK and Ki-67 expression (Table 3).

Spearman correlation analysis revealed a significant positive correlation between MK and Ki-67 expression in ameloblastoma and ameloblastic carcinoma ($p = 0.000$) (Figure 8).

Table 1:- Demographic data of studied cases.

Type	N	Age (Years)		Gender			Location of tumor		
		Range	Average	Female	Male	Ratio	Maxilla	Mandible	Ratio
MCA	14	12-64	38.7	6	8	1:1.3	5	9	1:1.8
UCA	11	17-47	28.2	5	6	1:1.2	3	8	1:2.6
AC	2	24,47	35.5	0	2	-	2	0	-

MCA: Multicystic ameloblastoma, UCA: Unicystic ameloblastoma, AC: Ameloblastic carcinoma

Table 2:- Descriptive Statistics showing mean and SD for MK and Ki-67 expression.

Type		N	Mean	Std. Deviation	Minimum	Maximum
Unicystic	MK	11	16.53345	1.386297	14.000	18.135
	Ki-67	11	4.83827	.969312	3.360	6.182
Multicystic	MK	14	16.41436	1.549393	13.000	18.360
	Ki-67	14	5.79371	1.649959	3.300	7.990
Carcinoma	MK	2	29.77200	2.146776	28.254	31.290
	Ki-67	2	26.45600	1.572605	25.344	27.568

Table 3:- Comparison between MK and ki67 expression among different groups using Kruskal-Wallis test.

Type	N	MK		Ki-67	
		Mean	p value	Mean	p value
Unicystic	11	16.533455	0.622	4.838273	0.079
Multicystic	14	16.414357		5.793714	
Multicystic	14	16.414357	0.026	5.793714	0.025
Carcinoma	2	29.772000		26.45600	
Unicystic	11	16.533455	0.030	4.838273	0.029
Carcinoma	2	29.772000		26.456000	

Correlation is significant when p value ≤ 0.05.

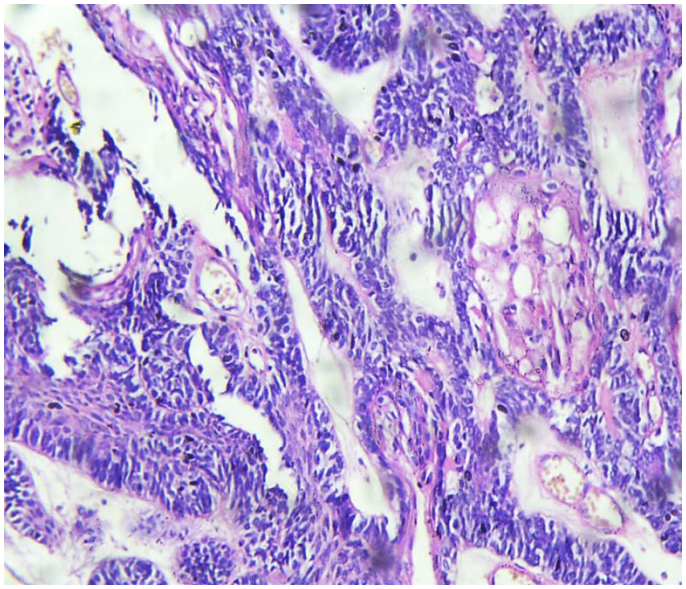

Figure 1:- Photomicrograph of AC showing ctologic atypia (H&E 400X).

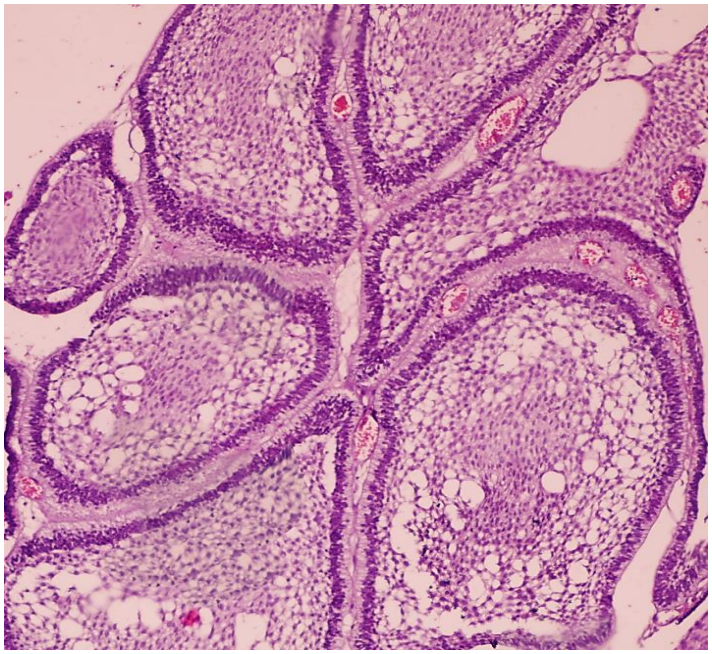

Figure 2:- Photomicrograph of MCA (follicular type) showing proliferating islands of odontogenic epithelium. The epithelial lining shows palisading and reverse polarization of nuclei of the peripheral cells (H&E 100X).

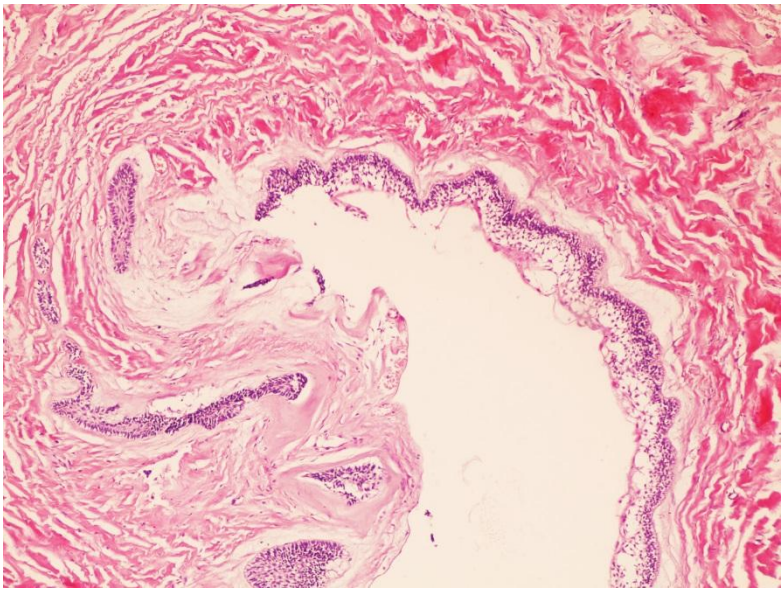

Figure 3:- Photomicrograph of UCA (mural type) showing ameloblastomatous epithelial lining the "cyst" wall (H&E 100X).

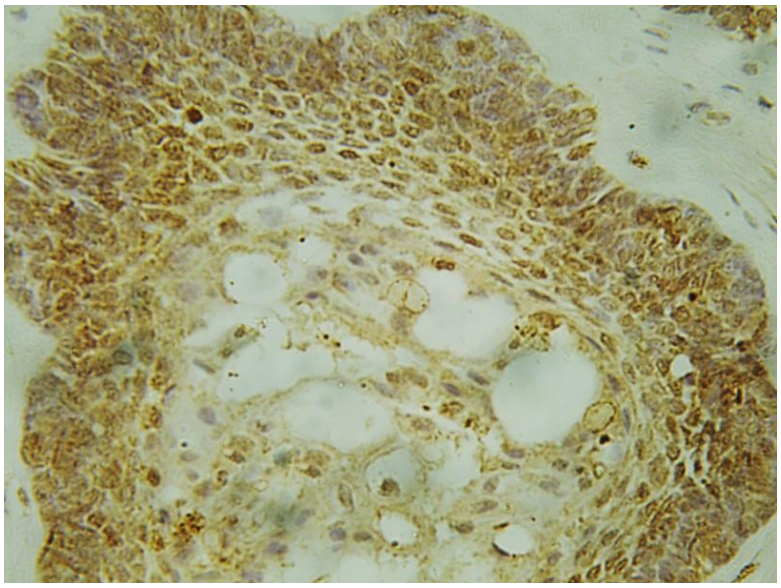

Figure 4:- Photomicrograph showing positive cytoplasmic immunoreaction for midkine in ameloblastoma (400X).

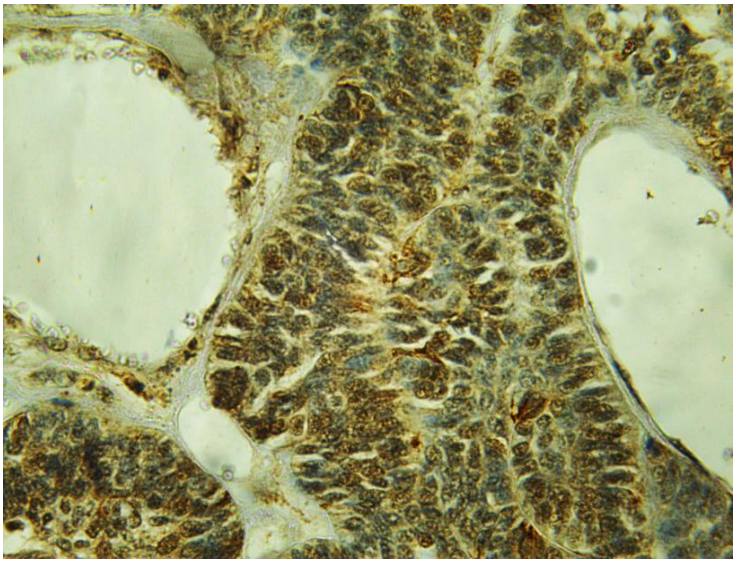

Figure 5:- Photomicrograph showing positive cytoplasmic immunoreaction for midkine in ameloblastic carcinoma (400X).

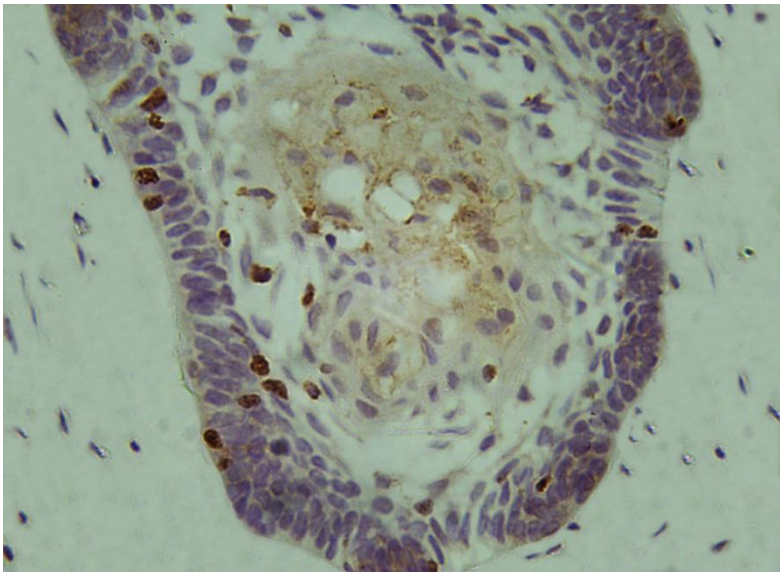

Figure 6:- Photomicrograph showing positive nuclear immunoreaction for Ki-67 in ameloblastoma (400X).

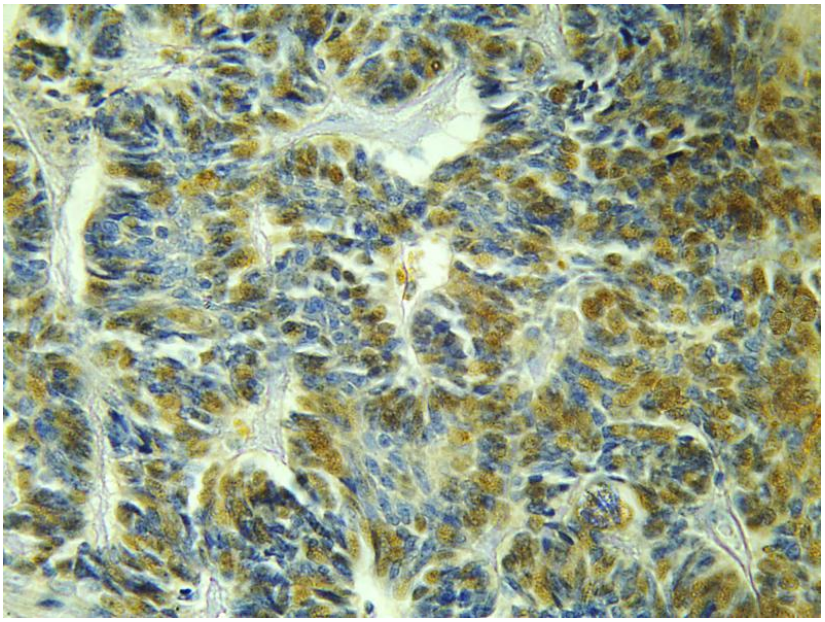

Figure 7:- Photomicrograph showing positive nuclear immunoreaction for Ki-67 in ameloblastic carcinomas (400X).

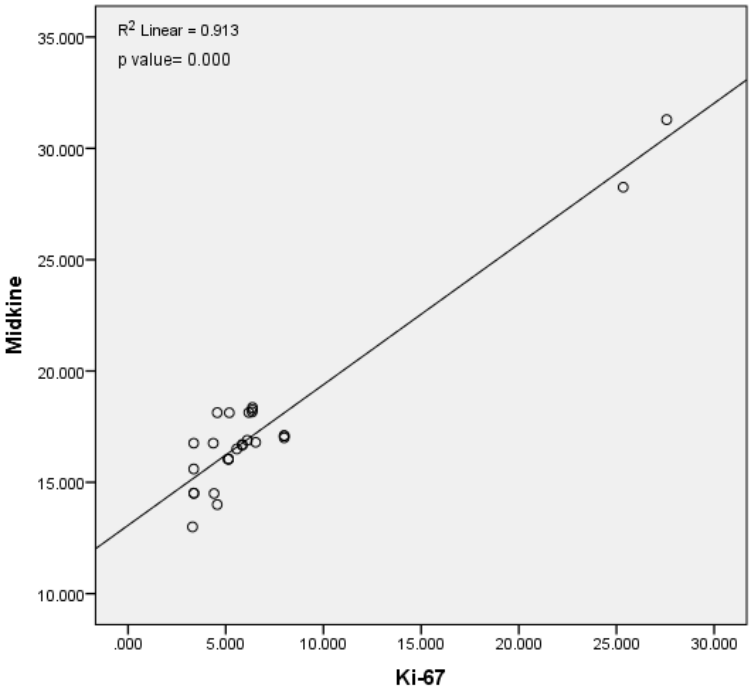

Figure 8:- Scatter plot showing significant positive correlation between MK and Ki-67 in ameloblastoma and ameloblastic carcinoma ($r^2 = 0.913$, $p = 0.000$).

Discussion:-

Immunohistochemistry is an important tool that can help to determine the biological differences in the behaviors of different tumors (Carreon-Burciaga et al., 2015). The usefulness of a marker for tumor diagnosis must be tested for each tumor type and application. Only those markers that have proven to be useful in practice should be considered (Bologna-Molina et al., 2013).

Midkine is a multifunctional peptide which, together with pleiotrophin (PTN), forms a structurally distinct family of heparin-binding growth factors. MK is highly expressed during embryogenesis and then becomes undetectable. It only re-appears in the body as a part of the pathogenesis of diseases (Jono & Ando, 2010). This includes inflammatory lesions, neurodegenerative diseases and cancers. MK expression is induced in various types of carcinomas including esophageal, gastric, gall bladder, pancreas, colorectal, breast, lung carcinomas, and Wilms' tumors at a high level (Kadomatsu & Muramatsu, 2004).

Ki-67 is one of the most widely used diagnostic tools for various types of neoplasms (Bologna-Molina et al., 2013) and trustworthy proliferation markers (Amaral et al., 2012). Ki-67 has been used to determine the proliferating activity and consequently to predict the biological and clinical behaviors of many tumors, including ameloblastomas (Sandra et al., 2001).

Although many studies have evaluated the expression of MK and Ki-67 in odontogenic tumors, none of these studies tried to find if there is a correlation between the expression of these two markers in relation to clinical characteristics of the tumors and this was the main target of the present study.

All studied cases of ameloblastoma and ameloblastic carcinoma showed MK expression was more prominent in the cytoplasm of columnar epithelial cells in the peripheral cell layer. This staining pattern is in accordance with that reported by Sandra et al. and Fujita et al. who found that MK expression is mainly in the outer layer of ameloblast-like cells (Sandra et al., 2004; Fujita et al., 2008). On the other hand, Scheper et al. reported that MK immunostaining was mainly in the stellate reticulum-like cells, followed by staining observed in both the stellate reticulum-like cells and the peripheral columnar ameloblast-like cells (Scheper et al., 2012). These differences may be due to different histologic subtypes of ameloblastoma.

Moreover, no identified relation was found between MK expression and age, gender or site of the tumor. This has also been reported by Sandra et al. (Sandra et al., 2004) and Scheper et al. (Scheper et al., 2012).

We assessed the difference in MK expression between the unicystic, multicystic and ameloblastic carcinoma cases. We found that there is no statistically significant difference in MK expression between unicystic and multicystic cases, while there is a statistical significant difference between carcinoma and both unicystic and multicystic ameloblastoma. This may be due to aggressiveness of the malignant tumor. Different results have been reported by other studies. Sandra et al. (Sandra et al., 2004) and Scheper et al. (Scheper et al., 2012) reported a significant difference in MK expression between multicystic and unicystic types of ameloblastoma. Fujita et al, reported no significant difference between benign odontogenic tumors and their malignant counterparts (Fujita et al., 2008). This could be due to the different techniques that are adopted by each study and its variation.

Our study cases showed nuclear expression of Ki-67 in the epithelial cells in the peripheral or basal cell layer. It is similar to the staining pattern reported by Bologna-Molina et al. who found that Ki-67 expression in AB and AC was mainly in the nucleus of cells corresponding to the basal layer (Bologna-Molina et al., 2013). Also, Carreon-Burciaga et al. reported that expression of Ki-67 in AB and AC was predominant in higher density areas and in peripheral cells with columnar morphology (Carreon-Burciaga et al., 2015). Gadbail et al. observed staining predominantly in peripheral cells of tumor islands of both UCA and MCA (Gadbail et al., 2012). This could explain the locally infiltrating growth of ABs.

On the other hand, Florescu et al. reported that the cells showing nuclear reaction to Ki-67 were not only located predominantly in the peripheral area of tumor islands, but staining was also present in the central stellate reticulum like cells and they mentioned that there is a significant difference in Ki-67 expression between central cells and peripheral cells of tumor islands, peripheral areas being considered proliferative areas (Florescu et al., 2012).

Ki-67 expression was found to be higher in MCA than in UCA but without significant difference. It is totally similar to the findings of Gadbail et al. who reported non-significant difference in Ki-67 labeling indices between UCA and MCA with higher expression in MCA (Gadbail et al., 2012). On the contrary Meer et al. reported that unicystic ameloblastomas showed statistically significantly higher Ki-67 labeling indices than the solid variants (Meer et al., 2003). A possible reason for the inconsistent results might be the difference in the morphology of the tumors, with the multicystic lesions providing large follicles or plexiform sheets for analysis, whereas only a thin lining is available in the unicystic cases. Another possible explanation might be the difference in methodology, especially the counting protocol, used as reported by Meer et al. (Meer et al., 2003).

A statistically significant difference in Ki-67 expression was found when comparing AC with UCA and MCA suggesting a more aggressive biological behavior that is characteristic of malignancy. It is in accordance with what was reported by Yoon et al., Bologna Molina et al. and Carreon-Burciaga et al. as they demonstrated that Ki-67 expression is higher in AC than in AB (Yoon et al., 2011; Bologna-Molina et al., 2013; Carreon-Burciaga et al., 2015).

Statistical analysis revealed non-significant difference in Ki-67 expression in relation to age, gender and site of the tumor. This is similar to what has been reported by Carreon-Burciaga et al. (Carreon-Burciaga et al., 2015).

We used Spearman rank correlation analysis for assessing the correlation between MK and Ki-67 expression and we found a strong significant positive correlation between MK and Ki-67 expression in ameloblastoma and ameloblastic carcinoma ($r^2 = 0.913$, $p = 0.000$). Regarding AC, although Spearman rank correlation analysis reveal a significant positive correlation between MK and Ki-67 expression in AC, we can't draw any definite conclusions in this correlation as our study contain a very small number of carcinoma cases. So we can't make a reliable correlation from only these data.

Conclusions:-

In conclusion, we found that MK and Ki-67 expression are higher in AC than AB, so evaluation of MK or Ki-67 expression can provide information about aggressiveness of the tumor. We also conclude that the clinical features of ameloblastoma do not directly affect tumor cell proliferation or MK expression. Moreover, we found that there is a significant positive correlation between MK and Ki-67 expression in ameloblastoma and ameloblastic carcinoma.

Recommendations:-

Further studies with higher number of cases are required to get more reliable statistical results regarding the correlation between MK and Ki-67 in ameloblastoma and ameloblastic carcinoma. Also, it is better in the further studies to include a full clinical data together with a long follow-up period in order to correlate the expression of MK and Ki-67 with the tumor behavior.

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