ORIGINAL ARTICLE

Role of AgNOR Staining in Histological Grading of Ameloblastoma

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ABSTRACT

Objectives: To compare the different histological variants of ameloblastoma in terms of proliferative activity, using the histochemical method of AgNORs and to grade different variants of ameloblastoma.

Methods: It was a cross sectional study of 50 surgical specimens, collected by non probability convenient sampling technique, of ameloblastoma were selected from different hospitals of Lahore (Mayo Hospital, Punjab Dental Hospital, Fatima Memorial Hospital, Services Hospital and Ghurki Hospital). All variants of ameloblastoma (histological variants), all ages and both genders were recruited. Putrefied and unfixed tissues and all the cases underwent decalcification for the presence of bone were excluded from the study. The duration of study was one year from 15-07-2009.to 14-07-2010. All the selected samples were fixed in 10% neutral formalin and processed for initial screening by hematoxylin and eosin in the Department of Pathology, King Edward Medical University, Lahore. These sections were initially reviewed by two pathologists by examination of several hematoxylin and eosin-stained tumour slides (mean, 2.5 slides per tumour; range, 2–8) and a consensus diagnosis was obtained.

Results: Overall mean age of the patients was 39.9±15.1 years with a range from 12 to 80 years. In the present study, there were 37 (76%) males and 13 (25%) females. When mean AgNOR values studied among 4 study groups, the mean AgNOR was highest for acanthomatous i.e. 3.15±0.30 and lowest for follicular variant 1.39±0.82. Comparison of mAgNOR was done by using ANOVA and it was calculated that the mean mAgNOR was different in 4 groups, p value <0.001. When association of mAgNOR cutoff value of 3.0 was seen in histological variants, it was observed that the association exists (p=0.003). The cases with mAgNOR >3.0 were only in desmoplastic and acanthomatous groups. Similarly, the association of pAgNOR >5.0 was significant with histological variants (P<0.001). Here all cases of desmoplastic and acanthomatous were having pAgNOR >5.0. Association of size was significant with histological variants (P=0.001). Desmoplastic variants were having +1 and +2 size while acanthomatous were having +2 size only. There were few cases in follicular that had size +1. Similar results were found for association of dispersion with histological variants with p value <0.001.

Conclusion: AgNOR staining technique can be useful in histopathological grading of ameloblastoma. Quantitative and qualitative evaluation of silver nitrate staining reflect more malignant potential in acanthomatous and desmoplastic variants than in follicular and plexiform varieties.

Keywords: AgNOR, Ameloblastoma, Histological grading

INTRODUCTION

Ameloblastoma is the commonest oral tumour arising from odontogenic epithelium. Its relative frequency equals the combined frequency of all other odontogenic tumours, excluding odontomas. They are usually benign but are biologically characterized by local recurrences following treatment.[1-4] Clinical and histological features of ameloblastoma are intriguingly contradictory, paradoxical and incongruent, if its benign histological aspect along with destructive clinical behaviour is considered.[1,5]

Nucleolar organizer regions [NORs] are loops of DNA that transcribe to ribosomal RNA. They can be visualized as intranuclear black dots by histochemical staining with a colloid silver solution. Silver stained nucleolar proteins (AgNORs) have been counted in a variety of jaw bone tumours.[6]

Histological variants of ameloblastoma have different outcome. Follicular ameloblastoma is considered to be more common than plexiform ameloblastoma, as it showed smaller AgNOR area and higher AgNOR number. The combination of counting the number and measuring the area of AgNOR dots showed a significant overall difference between AgNOR profiles of follicular and plexiform variants of ameloblastoma, indicating that the AGNOR count might help in determining malignancy, evaluating the effect of chemotherapy, and deciding the prognosis.[6-8]

SUBJECTS AND METHODS

It was a cross sectional study on 50 surgical specimens, collected by non probability convenient sampling technique, of ameloblastoma were selected from different hospitals of Lahore (Mayo Hospital, Punjab

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Dental Hospital, Fatima Memorial Hospital, Services Hospital and Ghurki Hospital). All histological variants of ameloblastoma, among all ages and both genders were recruited. Putrefied and unfixed tissues and all the cases underwent decalcification for the presences of bone were excluded from the study. The duration of study was one year from 15-07-2009.to 14-07-2010. All the selected samples were fixed in 10% neutral formalin and processed for initial screening by hematoxylin and eosin in the Department of Pathology, King Edward Medical University, Lahore. These sections were initially reviewed by two pathologists by examination of several hematoxylin and eosin-stained tumour slides (mean, 2.5 slides per tumour; range, 2–8) and a consensus diagnosis was obtained.

A modified AgNOR staining method was performed on paraffin-embedded tissues. AgNOR staining was performed by mixing 2 volumes of 50% silver nitrate with one volume of 2% gelatin and 1% formic acid, followed by immediate incubation of the slides in this mixture for 30 min. Nuclei stained light yellow and the outline of nuclei and cells was clearly visible. The AgNORs visualized as brown-black discrete dots of variable size within the nuclei. AgNORs were counted in one hundred cells in each specimen, and size variation and dispersion was also recorded. Mean AgNOR (mAgNOR) and percentage of AgNOR (pAgNOR) was calculated. mAgNOR count of more than 3.5 was suggestive of high malignant potential. Three µm sections were obtained, and the AgNOR technique was used according to Ploton et al[9] and further modified by Bukhari et al[10] with additional small modifications. To briefly describe the method, after the deparaffinization and rehydration processes, the slides were washed in running deionized water for 5 min. The slides were subjected to the silver staining. AgNOR staining was scored independently by two expert Surgical Pathologists. Intraobserver agreement was assessed and a substantial agreement was found between the observers.

The grading of size variation and distribution of AgNORs was performed by latest published criteria. Two investigators without knowledge of the method of AgNOR stain, tumour type, grade, stage, or disease outcome, performed the AgNOR counts, size and distribution. Two counts were performed. The first count was the mean number of AgNORs in 100 tumour nuclei (mAgNOR). The second count was the percentage of nuclei exhibiting five or more AgNOR granules/nucleus/100 cells called proliferative index (pAgNOR). This count was believed to represent proliferative activity. Tumours having a pAgNOR count of 8% or more were considered to display high proliferative activity.[10] AgNOR proliferative index (pAgNOR) was calculated as percentage of cells with five or more AgNOR dot. AgNOR Dots Size Variation: 0 = More or less uniform, 1+ =Two different sizes, 2+ = More than two different sizes (but not those of 3+), 3+ = All grades and sizes including too minute to be counted. AgNOR Dots Dispersion: 0 = Limited to the nucleoli, 1+ = Occasional dispersion outside the nucleoli, 2+=Moderate dispersion outside the nucleoli, 3+= Widely dispersed throughout the nucleus.

RESULTS

Overall mean age of the patients was 39.9±15.1 years with a range from 12 to 80 years. In the present study, there were 37 (76%) males and 13 (25%) females. 30 males were having follicular histopathological variant, 3 had plexiform varieties, while 2 had desmoplastic type and 2 were having acanthomatous ameloblastoma. Moreover, there were 10 females having follicular and 1 each having plexiform, desmoplastic and acanthomatous varieties (Table 1). When mean AgNOR values studied among 4 study groups, the mean AgNOR was highest for acanthomatous i.e. 3.15±0.30 and lowest for follicular variant 1.39±0.82. Comparison of mAgNOR was done by using ANOVA and it was calculated that the mean mAgNOR was different in 4 groups, p value <0.001 (Table 1). When association of mAgNOR cutoff value of 3.0 was seen in histological variants, it was observed that the association exists (p=0.003). The cases with mAgNOR >3.0 were only in desmoplastic and acanthomatous groups (Table 2). Similarly, the association of pAgNOR >5.0 was significant with histological variants (P<0.001). Here all cases of desmoplastic and acanthomatous were having pAgNOR >5.0 (Table 2). Association of size was significant with histological variants p value 0.001. Desmoplastic were having +1 and +2 size while acanthomatous were having +2 size only. There were few cases in follicular that had size +1 (Table 3). Similar results were found for association of dispersion with histological variants with p value <0.001 (Table 4). It was observed that majority of the patients (90%) were having tumour in mandible while 10% were having in maxilla (Table 5). According to our results, if mAgNOR was less than 3, pAgNOR was less than 5%, size and dispersion were zero. Such variants were placed in low grade. However, if mAgNOR was more than 3, pAgNOR was more than 5%, size and dispersion were more than zero. Such variants were placed in high grade variants (Table 6). Thus with the help of AgNOR index, DA and AA were categorized as high grade varieties while FA and PA were placed in low grade categories (Table 7). The pain was recorded in 42.86% cases of follicular type, 66.7% in desmoplastic and 100% in acanthomatous varieties and none in plexiform type. (Fig 1). Histology and AgNORs dots size and dispersion shown in figure 2, A, B, C and D.

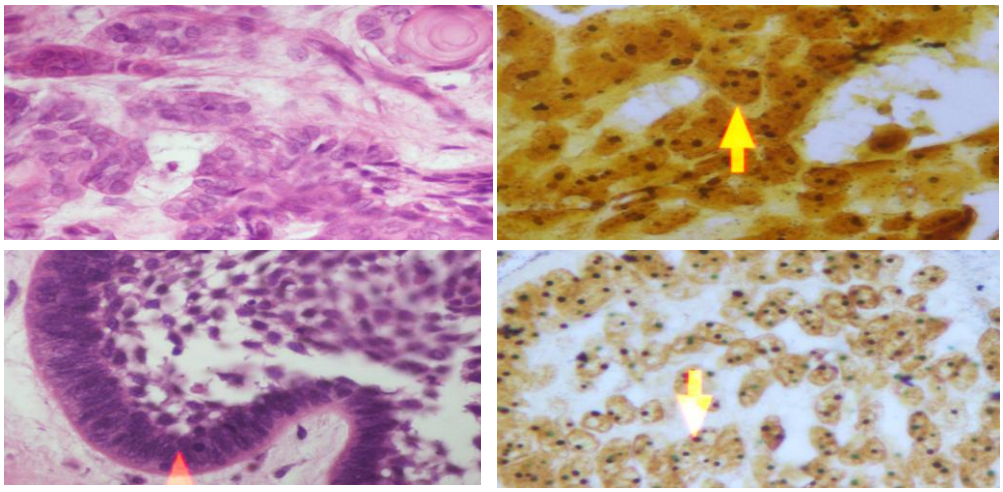

Fig. 2: Photomicrographs A. Desmoplastic variety of ameloblastoma (H&E X40); B. AgNOR staining of desmoplastic variant of ameloblastoma showing 4-5 intra nuclear AgNOR dots (AgNOR X 100 oil immersion); C. Photomicrograph reveals follicular type of ameloblastoma with peripheral columnar cells exhibiting (H &E X 40);D. AgNOR staining showing nucleolar organizer regions as black dots in follicular type of ameloblastoma (AgNOR stain X 100)

Table 1: Mean age, sex, mAgNOR and pAgNOR counts in different variants of ameloblastoma

Histological variants	Mean age (Year)	Gender (n=50)		Mean Values	
		Male	Female	mAgNOR	pAgNOR
Follicular	39.7±15.8	30	10	1.39±0.82	2.64±1.21
Plexiform	39.8±25.0	3	1	1.62±0.72	2.60±0.69
Desmoplastic	37.3±16.6	2	1	3.05±0.05	5.73±0.32
Acanthomatous	39.3±10.3	2	1	3.15±0.30	8.43±0.80
Total		37	13	P < 0.001	P < 0.001

Table 2: Histological variants having mAgNOR and pAgNOR more than or less than or equal to normal

Histological variant	mAgNOR		pAgNOR		Total
	≤ 3.0	>3.0	≤ 5.0	> 5.0	
Follicular	40	0	39	1	40
Plexiform	4	0	4	0	4
Desmoplastic	1	2	0	3	3
Acanthomatous	2	1	0	3	3
Total	47	3	43	7	50

mAgNOR P-value = 0.003 and pAgNOR P-value < 0.001

Table 3: Comparison of AgNOR size in variants of ameloblastoma

Histological variant	Size			Total
	0	+1	+2	
Follicular	34	6	0	40
Plexiform	4	0	0	4
Desmoplastic	0	2	1	3
Acanthomatous	0	0	3	3
Total	38	8	4	50

P-value = 0.001, 0=More or less uniform, 1+=Two different sizes, 2+=More than two different sizes, 3+=All grades and sizes including too minute to be counted

Table 4: Comparison of AgNOR dispersion in different variants of ameloblastoma

Histological variant	Size			Total
	0	+1	+2	
Follicular	38	2	0	40
Plexiform	4	0	0	4
Desmoplastic	1	1	1	3
Acanthomatous	0	1	2	3
Total	43	4	3	50

P-value <0.001, 0=Limited to nucleoli, 1+=Occasional dispersion outside the nucleoli, 2+=Moderate dispersion outside the nucleoli, 3+=Widely dispersed throughout the nucleus

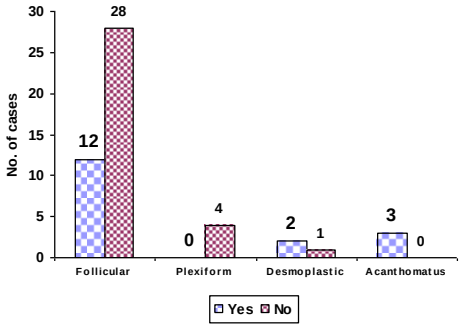

Fig. 1: Ameloblastic histological variants and their association with pain

Table 5: The most common site for histological different variants of ameloblastoma

Histological variant	Site	
	Mandible	Maxilla
Follicular	36	4
Plexiform	4	0
Desmoplastic	3	0
Acanthomatous	2	1
Total	**45 (90%)**	**5 (10%)**

Table-6: Criteria for Grading of Ameloblastoma

Grade	mAgNOR	pAgNOR	Size	dispersion
Low	<3	<5%	0	0
High	>3	>5%	Equal or > +1	Equal or > +1

Table 7: Grading of different variants of Ameloblastoma

Grade	Variants of ameloblastoma
Low grade	Follicular
	Plexiform
High grade	Desmoplastic
	Acanthomatous

DISCUSSION

The present study was conducted to determine the possible correlation between clinical behaviour and proliferation activity of different variants of ameloblastoma and to histologically grade malignant potential of different variants of ameloblastoma with the help of AgNOR staining technique. It included 50 cases of ameloblastoma. In our study, most of the patients were between third to fourth decade of life which shows strong similarity with previous studies[11,12,13]. Male predominance was seen as out of 50 cases 33 were males. Most of the studies on ameloblastomas give male preponderance[14,15], however, some studies differ from this statement[16].

According to our study, the most common site for development of ameloblastoma was mandible which is in favour of those studies reported earlier.[17,18] The number and morphological features of AgNOR are thought to reflect the cellular proliferative activity and grade of malignancy. Follicular was the commonest and the most benign type of ameloblastoma (mAgNOR = 1.39, pAgNOR = 2.64, size = 0, dispersion = 0) .Because mAgNOR and pAgNOR were insignificant.Similarly, plexiform pattern (mAgNOR = 1.62, pAgNOR = 2.60, size =0, dispersion = 0) also showed insignificant variation. However, when acanthomatous (mAgNOR =3.15, pAgNOR = 8.43, size =+2, dispersion = +2) and desmoplastic (mAgNOR =3.05 ,pAgNOR =5.73, size = +2, dispersion = +1) varieties were compared with each other and also with other two variants, the difference was quite obvious. Both the varieties showed significant values at proliferative index. AgNOR dots per hundred cells and percentage of the dots per hundred fields were raised as leisions showed malignant potential. Similarly, size and dispersion were also significantly in these cases.

A positive correlation was seen between DNA index and mean number of nucleolar organizing regions. It was also found that average maximum nucleolar organizer region count exhibited a slight tendency to increase for each increasing rate of dysplasia.[19]

In our study, it was observed that mean AgNOR and proliferative AgNOR were directly proportional to each other. Some studies reported a positive relationship that the AgNOR numbers correlate well with the number of cells in the S-phase by means of DNA flow cytometry[20] while another reported a positive relationship between the mean number of AgNOR per nucleus and tumour grade fraction. Hence, the AgNOR numbers could be probably related to cellular activity.[21] The mean AgNOR count increased gradually with increasing grades and stages of tumours.[22] Above study had similarity with our research, where mAgNOR is observed directly proportional to malignant potential of the tumour.

Size and dispersion has also a very important impact on character of a tumour. Small sizes, large numbers and scattered distribution of AgNORs are characteristic of malignant tumour cells, while large size, small number and clustered distribution of AgNORs are hallmark of benign tumour cells.[23] The above mentioned studies have thrown light on the importance of AgNOR size and dispersion in malignancies. Previously, some authors have tried to grade AgNOR size and dispersion for benign and malignant leisions.[24-26]

We, in the current study, followed this method of grading AgNOR size and dispersion. It was observed that AgNOR size and dispersion were significantly high in acanthomatous and desmoplastic variants of ameloblastoma than that of follicular and plexiform varieties. As far as malignant potential in desmoplastic variety was concerned, it was in the favour of the statement that this variant had more aggressive behavior.[11] However, according to our work there is another reality that acanthomatous had also such potential. The correlation between AgNOR numbers and cellular activity has been a subject of interest for years.[27] Its further well understood that the mean nuclear profile reflects the cell proliferation. There was an emerging evidence to comment that the number of the intranuclear visible NORs were indicative of proliferative activity of the tissue being examined, mAgNOR could represent mean DNA content of cells and percentage of the cells showing five or more nucleus would represent S-phase fraction. In this study, pAgNOR of acanthomatous and desmoplastic variants was significantly higher than that of follicular and plexiform patterns.

Contrast to the literature, three cases of follicular variety showed slight malignant potential as mAgNOR and pAgNOR were on the higher side. Another very important thing was observed that all the three cases of follicular variety showing slight malignant potential were of females between sixth to seventh decade of life. With the help of this study, we can comment that old age has strong association with malignant behaviour of the tumour. In the light of our study, we can comment that AgNOR staining is a

simple, inexpensive, rapid and easily reproducible technique and it can be performed very easily on small biopsies even on cytological preparations. By relying on AgNOR typing and proliferative index (pAgNOR) it has become possible to grade histological variants of ameloblastoma that will undoubtedly, help in improving and modifying treatment modalities and thus enhancing prognosis of the tumour.

CONCLUSION

AgNOR staining technique can be useful in histopathological grading of ameloblastoma. Quantitative and qualitative evaluation of silver nitrate staining reflect more malignant potential in acanthomatous and desmoplastic variants than in follicular and plexiform varieties. AnGOR staining technique is undoubtedly effective, easily reproducible and inexpensive. It use in laboratories should be encouraged in routine. However, still to prove its efficacy, we need multicentred, large scale studies using big sample size. However in future, more clinical follow-up studies should be performed ideally.

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