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Value of Silver Binding Nucleolar Organiser Regions (AgNOR) in Squamous Cell Carcinomas of Upper Aero-digestive Tract

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Abstract: *Background: Study of Silver-binding nucleolar organizing regions (AgNORs) in 100 cases of squamous cell carcinomas (SCC) of upper Aero-digestive tract was carried out with an aim of correlating AgNOR positivity with histological grade of tumour and evaluating value of AgNOR staining in predicting the progress of disease.*
Method: Tissue sections of the cases were stained with 50 percent silver nitrate solution for AgNOR evaluation and the data analysed. All the sections were also stained with haematoxylin and eosin stain for tumour typing and grading.
Result: In this study, the mean AgNOR (mAgNOR) score of normal squamous epithelium (100 cases) was 1.56 (range1.00-2.80), that of well differentiated squamous carcinoma (54 cases) was 3.29 (range 2.4-4.6), moderately differentiated squamous carcinoma (42 cases) was 4.29 (range 2.7-5.6) and of poorly differentiated squamous cell carcinoma (4 cases) was 5.21.The mAgNOR scores were statistically significant. Comparison of the percentage AgNOR (pAgNOR) scores between well differentiated and moderately differentiated carcinomas showed that all the pAgNOR values (1-9) were significant. However, pAgNOR 3 and pAgNOR 4 were most significant. Cut off values for pAgNOR score to differentiate between the various grades of squamous cell carcinomas could not be calculated due to lack of data on survival rates.
Conclusion: AgNOR technique can definitely be used as a supportive tool to routinely performed Hemotoxylin and Eosin staining and may help in prognosis as well as therapeutic decision-making in squamous cell carcinomas. Studies of larger number of cases in a prospective study are needed to arrive at more substantial conclusions.
Keywords- *AgNOR, Theraputic, Squamous Cell Carcinoma.*

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I. Introduction

Accurate histopathological typing, grading and staging of tumours are of proven value in the clinical management of cancer. In many cases, however, histopathological assessment does not correlate accurately with clinical outcome and may not reveal all possible markers of prognostic importance. These problems have motivated the development of new techniques to augment routine methods and to improve the accuracy and reproducibility of prognostication [1,2]. Of the various newer techniques used for assessing tumour tissue based on nuclear studies, staining of the nucleolar organising regions by silver compound (AgNOR) has become popular for its

simplicity, ease of use, low cost and its good correlation with other proliferative markers, as their frequency within nucleus are significantly higher in malignant cells than in normal, reactive or benign neoplastic cell [3-5]. Nucleolar organiser regions [NORs] are loops of ribosomal DNA (rDNA) occurring in the nucleoli of cells. In the human genome NORs are located on short arm of five acrocentric chromosomes (13, 14, 15, 21 & 22). The nucleolar organiser regions can be visualised by staining with silver nitrate solution under prescribed conditions. The number of discernible and therefore countable dots (AgNOR) depends upon several factors.

The correlation between AgNOR numbers and cell proliferation has been widely investigated in tumours by comparing AgNOR values with kinetic data obtained by applying a panel of proliferation markers [2]. Therefore the AgNOR parameter can reflect the neoplastic nature of cells and represents a promising

prognostic indicator in tumour pathology [1-3,5-7]. The mean AgNOR (number per nucleus) and pAgNOR score (percentage of nuclei with more than one, more than two, more than three and more than four AgNOR) have been found to be significantly higher in malignant cells as compared to benign cells. They also correlate positively with tumour aggressiveness in several body sites and are higher in tumours with poor prognosis compared to those with good prognosis. Squamous cell carcinoma of the upper aero digestive tract is one of the commonest types of carcinomas affecting Indians [8]. The behavior of this carcinoma in

a patient is extremely variable. The TNM staging system of squamous cell carcinomas of the upper aerodigestive tract is well established and has stood the test of time as the most important prognostic indicator. It is however only of modest value in guiding the clinician in determining optimal therapy. There exists a sub group of cancers that even in early stages explicably behave in an aggressive manner with concomitant poorer prognosis. It is most essential to identify this sub-group at the earliest and initiate aggressive therapy [9]. Many studies have clearly brought out the usefulness of AgNOR in squamous cell carcinomas in this regard. [2-4] Thus studies have shown that AgNOR values can serve as a useful prognostic parameter and a marker for tumour progression in squamous cell carcinomas [3,4,10,11].

II. Materials and Methods

The study was conducted on 100 biopsies and resected specimens (formalin fixed paraffin embedded blocks) of squamous cell carcinoma of the upper aerodigestive tract received by the Pathology department. Relevant clinical data for the cases were collected from records. For each case, a minimum of two sections was made from the blocks. One section was stained with Hemotoxylin and Eosin to give a morphological diagnosis and to verify the grade of tumour. The other section was stained for AgNOR by using the method of Plotton et al., [12].

Based on the grade, the 100 cases were divided into three groups namely well differentiated, moderately differentiated and poorly differentiated. Suitable specimens of squamous epithelium, cervical tissue and skin were studied to standardize the staining technique. Similarly 100 controls of normal squamous epithelium were also evaluated for AgNOR values.

Method of Ag NOR Staining: Formalin fixed 5 mm paraffin sections were stained with a solution made up of 50 percent Silver nitrate solution (prepared fresh.) and two percent Gelatin solution (stored at room temperature). Working solution was freshly prepared using two parts of Silver nitrate solution and one part of Gelatin solution. To carry out AgNOR staining the cut sections were deparaffinized and rehydrated using distilled deionized water. They were then placed in a plastic jar (slide container) filled with the staining solution and incubated at 37^0 C in dark for 40 min. Slides were washed thoroughly with distilled deionized water and dehydrated. After drying, the slides were cleared in xylene and mounted in DPX.

AgNOR counting: In each section 50 cells were counted using high power (40X) and oil immersion lens (100X). Areas with necrosis, pronounced inflammation, artificial damage and
marked keratinization were avoided. By careful focusing, all clear distinguishable black dots within the nucleus were identified and counted. Black dots (both smaller and larger) corresponding to 'clusters' were treated as one AgNOR. Two methods were used. The overall mean AgNOR (mAgNOR) per nucleus was obtained by counting the AgNOR dots in 50 nuclei and the average was calculated. The number of nuclei with one, two and so on upto10 and greater than10 AgNOR dots in the 50 nuclei were counted and the percentage of nuclei with more than one (pAgNOR >1), more than two (pAgNOR >2), more than three (pAgNOR >3) and so on up to pAgNOR greater than 10 were calculated. These values of pAgNOR and mAgNOR were correlated with histological grade of tumour and statistically analysed for their significance. In the 50 controls only the mAgNOR scores were calculated and statistically analysed.

III. Results

In this study, 100 cases of squamous cell carcinoma of the upper aerodigestive tract were studied. The distribution of these tumours as per site is summarized in Table 1. The age range was from 25-75years with the average age of 54.22years. Maximum cases were in stage IV (56%). All poorly differentiated carcinomas were in stage IV at the time of diagnosis. Only one case of laryngeal carcinoma was at stage I and was well differentiated. Mean AgNOR scores were calculated for normal squamous tissue and for the three grades of squamous cell carcinomas (well differentiated, moderately differentiated and poorly differentiated). Similarly the pAgNOR (1-10) values were calculated for the three grades of squamous cell carcinomas. Since there were only 4 cases of poorly differentiated squamous cell carcinoma, this grade was not included for statistical analysis as the sample size was inadequate. Comparison of mAgNOR scores were done between normal squamous tissue and well differentiated carcinoma, moderately differentiated carcinoma and in between well differentiated and moderately differentiated carcinoma

IV. Discussion

The one stage argyrophilic method for demonstration of nucleolar organizer regions (AgNOR) is proving to be a useful technique in the field of tumour histopathology as a prognostic indicator in morphology. The present study was undertaken to standardize this technique and to study AgNOR counts in squamous cell carcinomas of upper aerodigestive tract and to correlate the AgNOR scores with the grade of tumour.

All efforts were made to standardize the tissue processing and staining technique. Enumeration of AgNOR dots was done using the method suggested by Crocker et al [15]. Moreover lymphocytes that showed a single well stained AgNOR dot was used as internal control. Fine focusing was used to eliminate dust particles and deposit debris that interfered with the counting of AgNOR dots. In most tissues, AgNOR could be counted accurately using high power (40X objective). Oil lens (100X objective) was used in counting AgNOR dots especially in moderately and poorly differentiated tumours that had numerous dots. It was observed that

AgNOR dots tended to be large, homogenously staining and regular in the nucleolus of normal epithelial tissue as well as in well differentiated carcinomas . As the grade increased, AgNOR dots became smaller and irregular and were more widely dispersed in the nucleus. (Fig 1) In some cells, large giant dots with satellite of small dots were also noticed especially in poorly differentiated carcinoma. Similar observations have been made by Crocker J et al [15], Derenzini [17], Giri et al [18], Khanna et al [19]. A study that carried out a cumulative mean technique to determine the number of nuclei that needs to be counted before the mean became stable , have recommended 50 nuclei (range 20-70) for invasive carcinomas [20].

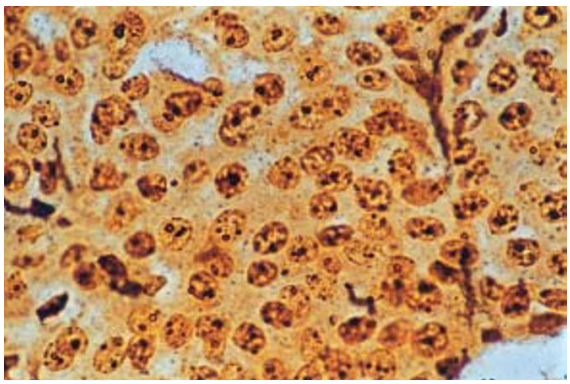

Fig. 1: Moderately differentiated squamous cell carcinoma showing 3 - 4 small AgNOR dots dispersed within the nucleus (AgNOR stain x 20)

Thus we have also counted 50 nuclei per section in this study. Hirsch SM et al in their study of 66 cases of squamous cell carcinoma of the head and neck found that carcinomas had a significantly higher AgNOR count than benign epithelia ($p<0.0001$). Among carcinomas, mean AgNOR count increased with stage of disease ($p<0.001$) but they did not find any correlation between the AgNOR scores and grade of tumour [3]. Xinxie et al in their study of 91 cases of squamous cell carcinomas of the oral cavity along with 20 cases of dysplasia and eight normal epithelia used both the mAgNOR and pAgNOR method of counting and found that both the mAgNOR and pAgNOR counts enabled significant discrimination between normal epithelium and dysplasia ($p< 0.0003$) and between dysplasia and squamous cell carcinoma ($p<0.0001$). They also found that overall means for mAgNOR and pAgNOR correlated with disease free period and survival time ($p< 0.004$) and that pAgNOR > 1 (at 88% cut off level) was the best discriminator regarding the disease free period and survival time [4]. Sano K et al in their study of AgNORs in 37 cases of oral squamous cell carcinomas found that mAgNOR count was higher in poor prognostic group than good prognostic group. Counts >= 6.5 showed lesser five year survival rates than those with low AgNOR score (< 6.5) [21]. In our study the mAgNOR score of normal squamous epithelium was 1.56 (range 1.00-2.80), well-differentiated squamous carcinoma was 3.29 (range2.4-4.6), moderately differentiated squamous carcinoma was 4.29 (range 2.7-5.6) and that of poorly differentiated squamous cell carcinoma was 5.21. (Table 2). When we consider AgNOR counts between normal, benign and malignant lesions and between various grades of squamous cell carcinoma, we found the comparison of the mAgNOR scores to be statistically significant. Comparison of the pAgNOR scores between well differentiated and moderately differentiated carcinomas showed all the pAgNOR values (1-9) were significant. However, pAgNOR 3 and pAgNOR 4 were most significant. Cut off values for pAgNOR score to differentiate between the various grades of squamous cell carcinomas could not be calculated due to lack of data on survival rates.

Site	Well differentiated	Moderately differentiated	Poorly differentiated	Total
Bucco alveolar complex	26	20	2	48
tongue	12	6	0	18
lip	4	0	0	4
layrnx	8	10	2	20
oesophagus	4	6	0	10
total	54	42	4	100

Table 1- Distribution of squamous cell carcinoma of the upper aero digestive tract according to the site and grade

Grade	Total count	mAgNOR value	Standard deviation	Variance
Normal	100	1.56	0.42	0.17
Well differentiated	54	3.29	0.63	0.39
Moderately differentiated	42	4.29	0.78	0.62
Poorly differentiated	4	5.21	0.16	0.02

Table 2- mAgNOR, standard deviation and variance value for normal and different grades of tumour

V. Conclusion

The findings of the present study are comparable with the previous studies performed on AgNOR by various authors, The AgNOR stain is of value in giving an idea about the tumour aggressiveness. Though the staining procedure is simple and cost effective it needs a lot of dedication, standardization and meticulous bench work to achieve good results. Thus we feel that AgNOR technique can definitely be used as a supportive tool to routinely performed H & E staining and will help in prognosis as well as all therapeutic decision making in squamous cell carcinomas of upper aerodigestive tract.

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