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Research Article

INVASIVE TUMOUR FRONT, AgNOR'S CHARACTERS IN PROGNOSTICATION OF OSCC: a SUGGESTED STANDARD CUTOFF AMONG INDIAN PATIENTS

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A B S T R A C T

The Nucleolar organizer regions (NOR) are unique elements of chromosomes that play a vital role in cellular proliferation at invasive tumour front of Oral squamous cell carcinoma (OSCC).

Aims and objective: The objective of this study is to determine cut-off value for invasive tumour front grade score, mean number and area of AgNORs that aids in prognostication of OSCC.

Materials and method: Two sections of formalin fixed paraffin embedded tissuesof OSCC were retrieved in 30 cases from archives. Tumorgrading, mean AgNOR number and area at the invasive frontwere elicited with H&E and silver staining respectively.

Results and observation: Tumor with least mean AgNOR number, area and invasive tumour front grade score had good prognosis significantly. We analyzedcut-off value of 12, 6, 2 μm2 for invasive tumour front grade score, mean number and area of AgNORs respectively which showed statistically good delineation prognosis. Thus cut-off value can serve as a guide for treatment planning of OSCC.

INTRODUCTION

Squamous cell carcinoma of the oral cavity (OSCC) is one of the ten most common cancers in the world and accounts for approximately 90% of oral cancers. (Piffko *et al*., 1997) Patients with primary oral carcinoma show varying prognosis for the same TNM staging, histopathological grading and treatment. (Anneroth *et al*., 1987, Bryne *et al*., 1989) Invasive front of the OSCC differs from the superficial areas of tumour on a molecular basis. It is this area where crucial molecular interactions takes place and also hosts most aggressive tumor cells. Malignancy grading of the deep invasive margins in oral carcinoma has been proved to yield highly significant prognostic information. (Bryne *et al*., 1989, 1992, 1998)

Various proliferative biological markers have been used to determine prognosis of OSCC, among them silver binding Nucleolar organizer regions (AgNORs) have been widely used. Nucleolar organizer regions (NORs) are loops of DNA coding for ribosomal formation and in turn protein synthesis. Silver binding Nucleolar organizer regions – associated protein (AgNORP) are acidic, non-histone in nature. AgNORP are best visualized on routine histopathological and cytological samples using silver solution. (Trerè *et al*., 1993, Fonseca *et al*., 2000, Schliphake *et al*., 2003)

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Many studies have indicated that AgNORs may be the only marker of cell proliferation that shows significant correlation with prognosis. The invasive front of a tumour is considered to be the aggressive advancing edge of the tumour that may display an increased cell proliferation that can be visualised by AgNORs. (Trerè *et al*., 1993, Fonseca *et al*., 2000, Schliphake *et al*., 2003)

The aim of the present study is to assess the prognostic value and possible relationship between the number of AgNORs, the mean area of AgNORs and the histopathological malignancy grade of the invasive tumour front. An attempt is also made to obtain a cut-off value for AgNOR's (number and area) and invasive front grading that could potentially serve as a prognostic marker in oral squamous cell carcinoma.

MATERIALS AND METHOD

The study group comprised of 30 histopathologically diagnosed cases of oral squamous cell carcinoma. The clinical details and follow up data up to 5 years were retrieved from the patient's medical records. Patients who had undergone surgery as the only mode of treatment were selected. Patients who had undergone post-operative radiotherapy/chemotherapy and cases which had tumour islands at the surgical excision margin were excluded from the study. The patients were categorized based on their follow-up progress [Table 1].The formalin fixed paraffin embedded (FFPE) tissue blocks of all the cases were retrieved from the archives of the department. Two sections of 5μm thickness were cut from these FFPE

blocks. One section was stained with routine Hematoxylin and Eosin (H&E) and malignancy grading of the deep invasive front of the tumour was scored using criteria given by Bryne *et al* [Fig 1]. (Bryne *et al*., 1992, 1998) The other tissue section was stained with silver solution for the visualisation of AgNORs as per the standard protocol. (Kamath and Sastry., 2004)

Table 1 case selection for the study

Category	Criteria	Number of cases
Group I	Patients with no recurrence of the tumour. In a follow-up of 5 years (considered to have a good prognosis)	13
Group II	Patients who presented with recurrence of tumour in a follow-up of 5 yrs. (poor prognosis – with recurrence)	11
Group III	Patients who presented with recurrence of tumour in a follow-up of 5 yrs. (poor prognosis – expired due to tumor)	6

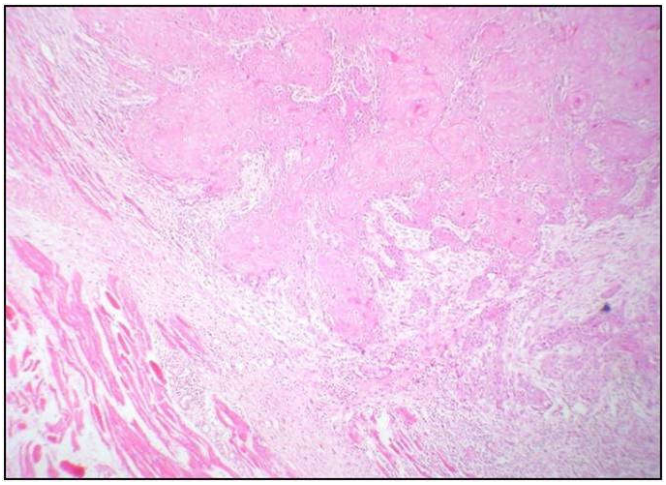

Fig 1 Photomicrograph showing the invasive tumour front

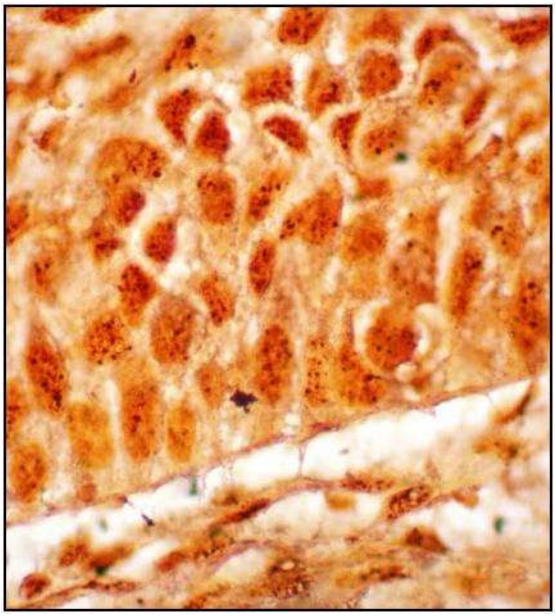

Fig 2 Photomicrograph showing AgNORs at the invasive front of oral squamous cell carcinoma

Counting the number of Ag NORs per nucleus

The number of AgNORs per nucleus was counted in 200 nuclei using x1000 magnification among the malignant cells at the invasive front of the tumor per case [Fig 2]. Only those areas in the nucleus that appeared as separate black dots were recorded; 2 or more dots closely aggregated were counted as one. (Crocker *et al*., 1989) The mean number of AgNORs per cell (N) was calculated using the formula given by Ruschoff J *et al*. (Ruschoff J *et al*., 1990)

Mean AgNORs per cell (N) = Σ (AgNORs) / No. of cells (200)

Evaluation of AgNORs area

Image j was used to calculate the mean AgNORs area, 200 cells were visualized in 100X objective under oil immersion. The invasive front area of the tumor was selected and was digitally captured in jpeg format and Computer-aided Image Analysis was performed. AgNORs were clearly delineated easily discriminated from the surrounding background by selectively thresholdingusing the image analysis software [Fig 3a, 3b]. To minimize nucleolus-biased sampling, we followed the protocol proposed by Ruschoff J *et al* and the field of the image was reduced to central portion with only 2-5 cells in the field. (Ruschoff J *et al*., 1990) Mean AgNORs area per nucleus was calculated using the following the proposed formula. (Ruschoff *et al*., 1990, Derenzini and Trere, 1991)

Mean AgNORs area per nucleus (A) = Σ (Area of AgNORs) / No. of cells (200)

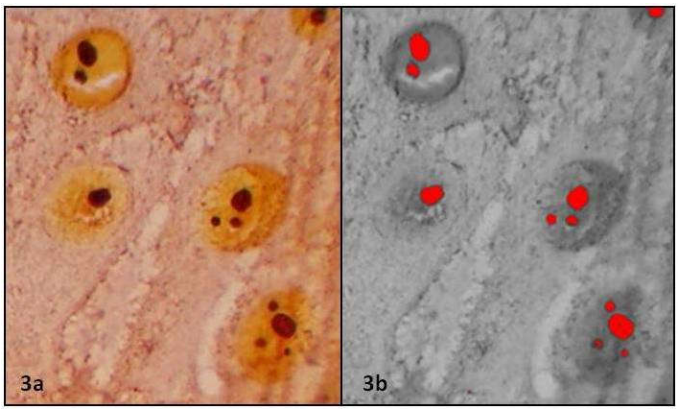

Fig 3 Photographs showing AgNORs in the nucleus (3a)and selection of AgNORs using threshold tool bar of the image analysis software (3b)

Statistical analysis

Mann Whitney test was used to compare prognosis and invasive front grading score, mean AgNORs number and the mean AgNORs area. Log Rank test performed for survival analysis and to obtain a cut-off value for the above mentioned criteria for the same group.

RESULTS AND OBSERVATIONS

In this study invasive front grading score, mean AgNORs number per nucleus and mean AgNORs area per nucleus were obtained for all the three groups [table 2, figure 4]. Mann Whitney analysis showed a significant (p= 0.001, 0.002, 0.001) correlation between increase in grading score, mean AgNORs number and area per nucleus and low chance of patient survival respectively [table 3].

Survival analysis (Log rank test) was performed to compare the cumulative survival and the invasive front grading score,

mean AgNORs number andarea per nucleus among these patients [Figure 5]. We obtained a cut-off value of 12, 6 and 2 μm^2 for invasive front grading score, mean AgNORs number andarea per nucleus respectively, below which patients had a good prognosis.We also found that patient survival rate decreases when the mean AgNOR number is more than 7.9. We were unable to obtain the similar cut-off value for invasive front grading and mean AgNORs area per nucleus.

Table 2 Range and Average values of all the three parameters in the present study

	Invasive front grading	Mean AgNOR's number per nucleus	Mean AgNOR's Area per nucleus
Group I	8-12 (mean 9.4615)	3.65-6.89 (mean 5.1031)	0.65 µm2 - 2.92 µm² (mean 1.4330 µm²)
Group II	13-18 (mean 14.636)	4.53-9.75 (mean 6.7682)	1.58 µm²- 2.91 µm² (mean 2.3291 µm²)
Group III	14-16 (mean 14.833)	6.225-8.89 (mean 7.6967)	1.64 µm2-2.95 µm² (mean 2.5250 µm²)

Table 3 Comparison of mean invasive front grade score, AgNOR number and AgNOR area among the three groups

Parameter	Group	N	Mean	SD	H	P-value
Invasive front grading	Group I	13	9.4615	6.3296	21.825	**0.001** (sig)
	Group II	11	14.636	11.385		
	Group III	6	14.833	3.0110		
AgNOR number	Group I	13	5.1031	0.9818	12.902	**0.002** (sig)
	Group II	11	6.7682	1.6477		
	Group III	6	7.6967	0.9820		
AgNOR area	Group I	13	1.4330	0.6044	14.079	**0.001** (sig)
	Group II	11	2.3291	0.3758		
	Group III	6	2.5250	0.4712		

H- Mann Whitney test

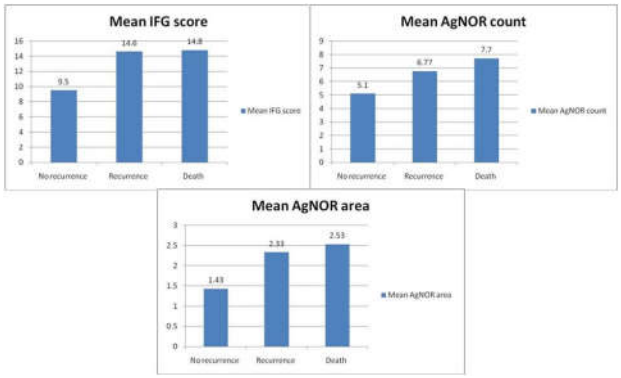

Fig 4 The mean invasive front grading score, Mean AgNOR's number and Mean AgNOR's area in the nucleus of tumor cells of group I, II and III

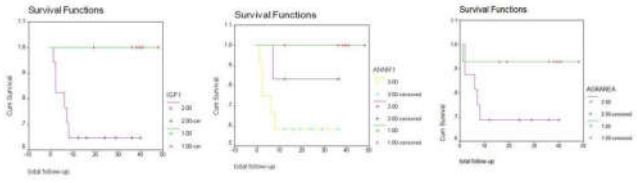

Fig 5 Survival analysis (Log Rank test) for mean invasive front grading score, Mean AgNOR's number and Mean AgNOR's area

DISCUSSION

It is known that greater prognostic information can be obtained from the invasive tumor front since it resides the most aggressive tumor cells. (Kurokawa *et al*., 2005 a,b) Silver binding nucleolar organizer regions (AgNORs) have been widely used in tumour pathology mainly for assessing the prognosis of malignant tumours. (Trerè *et al*., 1993, Fonseca *et al*., 2000, Schliphake *et al*., 2003, Underwood and Giri, 1991)

In the present study the prognostic value of the histological grade of malignancy in the deep invasive tumour front of oral squamous cell carcinoma was analysed. It was found that patients with a invasive front grading of less than 12 had a good prognosis. These values correlate with values obtained by Bryne M *et al* in oral squamous cell carcinoma of floor of the mouth (score of 9-12 indicative of poor prognosis).

We also obtained mean AgNORs number per nucleus of patients in group I ranged from 3.65-6.89, while patients in group II ranged from AgNORs number per nucleus that ranged from 4.53-9.75 (mean 6.7682) and group III ranged from 6.225-8.89 (mean 7.6967). From the above observations it is clear that the risk of recurrence of tumour or death due to tumour is high with the increase in the number of AgNORs. The present study supports the results of studies by Sano K *et al*, Piffko J *et al*, Pillai K.R. *et al* and De Rosa I *et al*. (Piffko *et al*., 1997, Pillai *et al*., 2005, De Rosa *et al*., 1999, Teixeria *et al*., 1996) Sano K *et al* concluded that the pooled mean AgNOR count of patients with poor prognosis was higher than that in patients with good prognosis. Patients with a 5-year survival had a low AgNOR count (less than 6.5) compared with patients in the group with poor prognosis (greater than 6.5). (Sano *et al*., 1991) Piffko J *et al* concluded that carcinoma patients with favourable prognosis had fewer mean AgNORs per nucleus (cut-off point of 3) than patients with poor clinical outcome. (Piffko *et al*., 1997) De Rosa I *et al* obtained results that showed that the size and number of AgNORs are sensitive parameters for discriminating potentially malignant lesions (3.21 AgNORs per nucleus) from oral squamous cell carcinoma (3.73 AgNORs per nucleus). AgNORs can also help in determining the prognosis of patients with oral squamous cell carcinoma as the mean AgNORs number per nucleus was lesser than 3.48 (SD = 0.62) for patients who had a good prognosis. (De Rosa *et al*., 1999)

Pillai K.R. *et al* examined the AgNOR counts in normal, premalignant and malignant oral mucosa to evaluate their potential as a biological marker for tumour progression and a prognostic predictor for treatment outcome in oral carcinomas. Analyzing the prognostic significance of AgNORs in oral carcinomas, a mean count of greater than 2.8 was seen in patients with poor prognosis. It was concluded that patients presenting with T3 and T4 tumours, and with mean AgNOR counts of more than 2.8 have tumours that are aggressive and that may exhibit resistance to current treatment. (Pillai *et al*., 2005)

In the present study we also obtained a cut-off value of less than 6 for mean AgNORs number in patients with good prognosis (without any recurrence or death). As the mean AgNORs number per nucleus increases to more than 7.9, patient survival rate decreases. These values are in concordance with the values obtained by Sano K *et al* [low AgNORs count (less than 6.5) compared with poor prognostic group (greater than 6.5)]. (Sano *et al*., 1991)

We obtained a cut-off value of less than 2 μm^2 for the mean AgNORs area per nucleus in patients with good prognosis. These findings are in agreement with the values obtained by Piffko J *et al* (mean AgNORs area per nucleus less than 1.9 μm^2 in patients with good prognosis). (Piffko *et al*., 1997) But the cut-off value for survival rate was not obtained because all the values of mean AgNORs area per nucleus in cases with

poor survival rate are well within the range of values for patients with recurrence.

In contrast, Teixeira *et al* in 1996 compared AgNORs area of the entire thickness of tumour epithelium with the prognosis of patients with oral squamous cell carcinoma of the tongue and floor of the mouth. Their result concluded that patients with good prognosis had lower AgNOR area (less than 7.77μm^2 in 17 of 27 patients), while patients with poor prognosis had higher AgNOR area (greater than 7.77 μm^2 in 12 of 16 patients). (Cabrini *et al*., 1992) These values are quite higher than the values obtained by Piffko J *et al* (1.9 μm^2), and the present study (2 μm^2). (Piffko *et al*., 1997) The high values obtained by Teixeira *et al* may be partly explained as the AgNORs area per nucleus is raised steadily towards the upper layers in normal mucosa. (Cabrini *et al*., 1992)

From the above observations it is clear that squamous cell carcinoma patients with higher mean AgNORs number, higher mean AgNOR area at invasive front and higher score for the invasive front grading will have a high risk of developing metastasis or dying from the disease. The importance of aggressive treatment and thorough follow-up has to be appreciated for improving the survival of these patients.

AgNORs count appears to be a useful tool in distinguishing between normal epithelium, dysplasia, and squamous cell carcinoma of the oral cavity. Numerous studies have even supported the above findings. (Warnakulasuriya and Johnson, 1993, Chattopadhyay and Ray, 2008, Eslami *et al*., 2006, Xie *et al*., 1998, Wang *et al*., 2004)

CONCLUSION

Since AgNORs can be identified at a light microscopic level, the evaluation of AgNORs (both number and area) especially along with invasive front grading can serve as important adjunct to clinical staging and grading of the neoplasm. This will enhance the ability to stratify patients into more realistic risk categories. However, the results of the present study need to be confirmed using a larger sample size with longer follow-up to validate the use of the cut-off values as indicators of tumor behaviour.

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