Predictive Value of Argyrophilic Nucleolar Organizer Regions at Invasive Tumor Front in Oral Squamous Cell Carcinoma

Bhagyashri R Latti, Jitendra V Kalburge

Abstract

Purpose: While grading the tumor, histopathological evaluation of invasive front of the tumor is important as the morphological features registered reflect the important biological events within the tumor cells. This study was undertaken to determine the prognostic value of malignancy grading and Argyrophilic Nucleolar Organizer Region (AgNOR) method in assessment of proliferative activity at the invasive tumor front of OSCC. Material and Methods: Excised tumor tissue and normal tissue were fixed, processed and stained with Hematoxylin and Eosin stain and diagnosed, then successive section was stained with AgNOR and its counting was done at invasive tumor front. Correlation between conventionally used grading systems of Broder’s (1927) with that of Invasive Tumor Front grading of Bryne et al (1998) was done. Results: Increased proliferative activity and AgNOR count was found in tumor tissue than in normal tissue. A high statistical significance was found for mean AgNOR count and average of mean AgNOR / nucleus (p<0.01), average AgNOR count/nucleus according to clinical stage (p<0.05). Conclusions: Invasive front grading has much better predictive potential than any single tumor marker at the invasive front.

Keywords: Argyrophilic Nucleolar Organizer Region; Broder’s grading; Invasive tumor front grading; Oral Squamous cell Carcinoma; TNM staging.

Introduction

In India, oral cancer is one of the five leading sites of cancer in either gender. According to the cancer registry data, it is estimated that annually 75,000-80,000 new oral cancer cases develop in India. Oral squamous cell carcinoma (OSCC) is the most common malignant neoplasm of the oral cavity. Its incidence in the world is increasing and the survival index continues to be small (50%), despite the progress in diagnosis and treatment. Thus, there is a need for improvement in early detection of oral carcinomas because treatment is more effective and the morbidity is minimal. Tobacco and alcohol are the two most important known risk factors for the development of oral cancer. Most invasive oral carcinomas are preceded by a preinvasive stage that may last for years. Given the relatively poor survival rates of oral cancer patients, cessation of tobacco remains the key element in oral cancer prevention and control.

Dr. Barbara McClintock, an American, first described the “nucleolar-organizing body” in Zea mays in 1934. The argyrophilic nucleolar organizer regions (AgNOR’s) are nucleic acid-argyrophilic non-histone protein complexes in the nucleoli, and the silver staining allows their identification and enumeration during interphase at the light microscopic level. AgNOR’s study provides understanding of the tumoral behavior since their frequency, size and cell arrangement have been reported as a discriminatory factor between certain benign and malignant lesions, reflecting the cell proliferative activity, cell cycle phase, cell ploidy and DNA content. The histopathological features of oral squamous cell carcinoma may differ widely from area to area within the same tumor, and it is believed that the most useful prognostic information can be deduced from the invasive front of the tumor, where the deepest and presumably most aggressive cells reside. The aim of our study was to determine the prognostic value of malignancy grading and AgNOR method in assessment of proliferative activity at the invasive tumor front of oral squamous cell carcinoma.

Materials & Methods

The study was conducted in the Department of Oral Pathology and Microbiology. Patients selected for the study include those with clinical
and histopathological diagnosis of oral squamous cell carcinoma who was treated in the form of complete surgical resection and radical neck dissection. A total of 60 cases, 30 of oral squamous cell carcinoma and 30 normal subjects as control were studied. Patients with other systemic diseases and other malignancies were excluded from the study.

The excised tumor tissue was fixed using 10% formalin, grossed for the exact identification of tumor area and margins. The tumor was dissected as per the margins and kept for fixation using 10% formalin. After the fixation of tissues routine processing was done by using alcohol as dehydrating agent and xylene as clearing agent. After processing, embedding of the tissues was done in molten paraffin wax and blocks were prepared. From the blocks, 5-6µm thick sections were cut using semi-automatic microtome (Microm, Germany) and mounted on the glass slide. The normal tissue was also excised after taking patient's consent and was processed in the similar manner as that of the tumor tissue. Slides were stained separately for Hematoxylin and Eosin (H & E) and silver nitrate (AgNOR) for both normal and tumor tissue. AgNOR slides were stained by method given by Ploton et al (1986). The slides were then mounted using Distrene dibutyl Phthalate Xylene (DPX). The stained sections of H and E were observed under 4X, 10X and 40X magnification and AgNOR stained sections were observed under oil immersion using research microscope (Carl Zeiss, Germany) and AgNOR counting was done. For counting we followed the method used by Xie et al,11 who also evaluated each section under 5 fields using a 100X oil immersion lens. For normal epithelia and dysplasia’s, five fields covering the basal and adjacent parabasal layers were evaluated. By careful focusing, all clearly distinguishable black dots within the nuclei were identified and counted. Black dots within nucleoli or aggregated clusters were treated as one AgNOR (Figure 1 & 2). Statistical analysis was done by applying Student’s unpaired ‘t’ test and Kruskal-Wallis H test (Non-parametric analysis of variance (ANOVA) test).

Results
The normal tissue findings were compared with that of the tumor tissue and found that there was high proliferative activity and AgNOR count in tumor tissue as compared to that of control tissue. Distribution of mean and standard deviation (SD) values of AgNOR in Control group showed that by applying Student’s unpaired ‘t’ test there is a highly significant difference between mean AgNOR count and average of mean AgNOR/nucleus (i.e. p<0.01), with value of t = 13.33, p<0.01, highly significant (Table 1).

Distribution of mean values of AgNOR according to the margins showed that of the margins examined at the invasive tumor front, deeper and anterior margins had higher mean AgNOR count with standard deviation i.e., 0.57±0.09 and 0.56±0.39 respectively, whereas central and posterior margins had lower mean AgNOR count with standard deviation i.e., 0.50±0.06 and 0.49±0.06 respectively (Table 2).

### Table 1: Distribution of mean and SD values of AgNOR in Control group

<table>
<thead>
<tr>
<th>Margins</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior</td>
<td>0.56 ± 0.39</td>
</tr>
<tr>
<td>Posterior</td>
<td>0.49 ± 0.06</td>
</tr>
<tr>
<td>Superior/medial</td>
<td>0.54 ± 0.31</td>
</tr>
<tr>
<td>Deeper</td>
<td>0.57 ± 0.09</td>
</tr>
<tr>
<td>Central</td>
<td>0.50 ± 0.06</td>
</tr>
<tr>
<td>Total mean</td>
<td>0.50 ± 0.03</td>
</tr>
</tbody>
</table>

### Table 2: Distribution of mean values of AgNOR

The margins examined at the invasive tumor front, deeper and anterior margins had higher mean AgNOR count with standard deviation i.e., 0.57±0.09 and 0.56±0.39 respectively, whereas central and posterior margins had lower mean AgNOR count with standard deviation i.e., 0.50±0.06 and 0.49±0.06 respectively.

Distribution of mean and SD values of Average/Nucleus according to clinical stage
showed that by applying Kruskal-Wallis H test (Non-parametric analysis of variance (ANOVA) test), p value <0.05 was considered to be statistically significant (Graph 1). Mean AgNOR count in study and control group showed that of 30 patients each, a mean with standard deviation in the control group was 1.8966±0.1937 as compared to the study group 2.75±0.31 (Graph 2).

**Graph 1:** Distribution of mean and SD(Standard Deviation) values of Average/Nucleus according to clinical stage showed that by applying Kruskal-Wallis H test (Non-parametric analysis of variance (ANOVA) test), p value <0.05 was considered to be statistically significant.

**Graph 2:** Mean AgNOR count in study and control group of 30 patients each showed that, a mean with standard deviation in the control group was 1.8966±0.1937 as compared to the study group 2.75±0.31.

**Discussion**

Different histopathological grading systems employed in diagnosing OSCC are conventional Broder’s grading, invasive tumor front grading proposed by Jakobsson et al (1973), Fisher et al (1975), Lund et al (1975), Willen et al (1975), Crissman et al (1980), Anneroth and Hansen et al (1984), Yamamoto et al (1984) and Anneroth et al (1987). Bryne et al developed a malignancy grading system focusing only on the invasive front of the tumor. In this invasive front grading (IFG) system, five different histological features (components) are graded and assigned scores from 1 to 4, the score for each variable is summed to provide a total malignancy score for each tumor. The grading suggested by Bryne et al, is preferred as compared to that of Jakobsson et al and Anneroth et al, because higher reproducibility has been obtained by,

a. Omitting the mitotic count (Bryne et al, 1992);
b. Calibration and training of pathologists (Bryne and Kristensen et al, 1997); and
c. Simplification of diagnostic criteria (Woolgar et al, 1995).

**Figure 1:** Photomicrograph of moderately differentiated squamous cell carcinoma showing moderate AgNOR count (AgNOR stain 100X Oil Immersion)

**Figure 2:** Photomicrograph of poorly differentiated squamous cell carcinoma showing high AgNOR count (AgNOR stain 100X Oil Immersion)

Recent seminal studies by M Bryne et al (1998) suggested that the invasive tumor front is the most prognostic region of tumor. These findings led us to explore the
relationship between invasive tumor front (ITF) grading and conventional Broder’s grading method in assessing the tumor biological modalities and clinical behavior by correlating both the grading systems to each other and also to the initial clinical staging based on TNM, and in established pathologic metastases status of the lymph nodes in operated specimens. 

Nucleolar organizer regions (NOR’s) are loops of DNA that contain ribosomal RNA genes. These genes are transcribed by RNA polymerase I and ultimately direct ribosome formation and protein synthesis. One of the proliferative markers which are focused is Argyrophilic Nucleolar organizer region staining in histopathological diagnosis. The amount of silver deposited in the nucleus in this cytochemical reaction is a reflection of the transcriptional activity of ribosomal genes. With advancements in histopathologic grading systems and need for the assessment of invasive tumor front, this study is designed to evaluate the AgNOR count at ITF and to assess the utility of this proliferative marker as prognostic marker.

Our study comprised of total 30 patients of which 17 males and 13 females and the ratio was found to be 1.3:1, i.e. males were affected more commonly as compared to that of females. We analyzed the mean AgNOR count in study and control group and found that there was difference in the mean values with standard deviation which were 2.75±0.31 and 1.8966±0.1937 respectively. But we found that there was a highly significant difference between mean AgNOR count and average of mean AgNOR / nucleus with a p<0.01. Our results were in accordance to Manu et al., who also found that the mean AgNOR counts in study group were higher (3.29-5.21) as compared to that in the control group (1.00-2.80). Our results were in accordance with Xie et al. who found that mAgNOR count for carcinomas was 6.2 (4.7-11.3). Patients with counts below the overall mean had a significantly longer disease free period and longer survival time compared with those with higher mAgNOR counts.

In our study, we examined the mean values of histopathological grading at the invasive tumor front using different margins and found that the mean values with standard deviation were higher in deeper margins (9.83±1.84) as compared to that in the central margins (5.7±1.26). To our knowledge this is the first study performed using AgNOR at the margins of invasive tumor front in oral squamous cell carcinoma. Our results were in accordance with J Piffko et al., who found that the mean value of AgNOR in oral squamous cell carcinoma was significantly higher at the invasive tumor front than at the central parts of the tumor. The dynamism of the cell cycle, characterized by the AgNOR content, probably represents one of the biological functions that underlie the prognostic significance of the histomorphological features of the invasive zone of OSCC’s.

In this study, relationship between mean AgNOR values and histopathological grading was analyzed and it was found that grade I (n=5) had a mean with standard deviation was 2.86±0.11 as that compared to grade IV (n=1) which had a mean value with standard deviation of 2.32±0.0. According to Manu et al., among carcinomas they found that the mean AgNOR count increased with the stage of the disease (p<0.001) but they did not find any correlation between the AgNOR scores and grade of tumor. According to Oliveira et al., they found that the AgNOR mean value/nucleus increases as the histopathological grading increases and thus has a clear relationship with tumor differentiation.

According to Chattopadhyay and Sano et al., a higher count of AgNOR in seen in poor differentiated carcinoma. According to Okada et al., according to histological malignancy classification proposed by Anneroth et al., they found that there was a significant relationship between histological malignancy grading and metastasis in the cervical lymph nodes and concluded that when the sum of the degree of histological malignancy grading exceeds mean points of 2.5 or more, metastasis in the cervical lymph nodes should be considered.

We correlated clinical staging (TNM Staging) and histopathological grading and found that there was a significant correlation between them (i.e p<0.05). According to Dantas et al., there was no correlation between TNM classification and histological scores of malignancy. Tumors that are well-differentiated are likely to invade tissues in a pattern characterized by well-defined margins, while the more undifferentiated tumors infiltrate tissues in small cell groups.
or even as isolated cells, which may explain its correlation with the prognosis.

The ideal approach to assess the prognostic value of any grading or staging system would be to carry out a randomized controlled mortality study, however these are difficult to perform owing to the obvious drawbacks with respect to the period of the study and difficulties in collection of the data. Prolonged follow-up periods, with sufficiently large number of sample size involving comprehensive and homogenous data might clarify whether the grading of oral squamous cell carcinoma at the invasive front is of any greater significance in reflecting the growth capacity and malignancy of the tumor and in predicting the outcome of this dreadful disease at an early stage.

Conclusion
The morphological features registered at the tumor invasive front probably reflect both the important biological events within the tumor cells and the microenvironment. The main conclusion from previous studies was that invasive front grading has much better predictive potential than any single tumor marker at the invasive front. Thus, traditional histopathology is at present still very useful as a prognostic tool, despite the introduction of molecular biological techniques. From this study, we conclude that AgNOR at the tumor invasive front in oral squamous cell carcinoma is a useful proliferative marker, helps in treatment planning depending on the grade and stage of the tumor and also helps in predicting the prognosis.

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Author Affiliations
1. Dr. Bhagyashri R. Latti, Assistant Professor, Department of Oral and Maxillofacial Pathology, Yashwantrao Chavan Memorial Medical and Rural Development Foundation’s Dental College, Vadgaon, Ahmednagar, 2. Dr. Jitendra V. Kalburge, Professor & Head, Dept of Oral and Maxillofacial Pathology, Government Dental College & Hospital, Jamnagar, Gujarat-361008.

References


Corresponding Author
Dr. Bhagyashri R Latti, Assistant Professor, Dept of Oral and Maxillofacial Pathology, Yashwantrao Chavan Memorial Medical and Rural Development Foundation's Dental College, Vadgaon Gupta, Ahmednagar-414003. Ph: +91-9503994650 Email: bhagya_1229@rediffmail.com

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