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Prognostic Potential of Argyrophillic Nucleolar Organizer Regions (Agnors) in Oral Lesions: A Systematic Review

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Early detection of oral squamous cell carcinoma (OSCC) and oral potentially malignant disorders (OPMD) is important for dental professionals to improve patient survival rates. More than half of patients with oral squamous cell cancer had advanced disease at the time of diagnosis, indicating a lack of early detection and risk assessment biomarkers. The development of new protein biomarkers will help in early diagnosis and treatment.The argyrophilic nucleolar organiser regions (AgNORs) staining technique is simple and cost-effective. These are replicatory markers that identify epithelial dysplasia. And it also plays a very important role in differentiating the benign, pre-malignant, and malignant lesions of the oral cavity. The number of AgNORs per cell has been considered an indicator of cellular proliferative activity. Microscopically, NORs can be identified as well-defined black dots located throughout the cell nucleus. The agNOR quantity is strictly proportional to the proliferative activity of the cell. AgNOR quantification helps in the determination of the degree of epithelial dysplasia and, consequently, in the analysis of its potential for malignant transformation. AgNOR qualitative characteristics help in differentiating hyperplastic, premalignant, and malignant oral lesions. The sliver staining technique is useful for studying the structure of the nucleolus as well as the variations in its activity. AgNORs are a valuable parameter in tumour pathology.

Keywords: Border's Grading; Epithelial dysplasia; immunohistochemistry; Nucleolar Organizer Regions; Oral Squamous Cell Carcinoma; proliferating cell nuclear antigen; Tumor Markers.

Nucleolar organizer regions (NORs) are the loops of DNA present in the nucleoli of cells possessing the genes that transcribe for the synthesis of ribosomal RNA (rRNA).[1] These nucleolar organizer regions (NORs) correlate to secondary constrictions of metaphase chromosomes in eukaryotic cells. These NORs are believed to be the centre for proliferative activity in the cell, and they are located on the short arm of acrocentric chromosomes 13, 14, 15, 21, and 22.[2] NORs are associated with certain acidic, argyrophilic, non-histonic proteins, which are known as NOR associated proteins (NORAPs).[3]

Various cycle stages result in different patterns of nucleolar organiser regions as seen by sliver staining (AgNOR). These are

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replicatory markers that play a very important role in the diagnosis of epithelial dysplasia.[4] The transformation of a normal cell into a neoplastic cell is characterised by an increase in protein synthesis activity and a strong basophilia of the cytoplasm. Additionally, an increase in size as well as in the number of nucleoli is noticed, which implies amplified synthesis of the ribosomal RNA in order to meet the demand for proteins in tumors.[5] Microscopically, NORs can be identified as well-defined black dots located throughout the cell nucleus.[6] The number of AgNORs visualised depends on the number of NOR protein bearing chromosomes in the karyotype, the level of RNA transcriptional activity , and the stages of the cell cycle, as the nucleolus disperses before mitosis and reorganises afterwards.[7]

The World Health Organization (WHO) favours the term "Oral potentially malignant disorders" (OPMD) for clinically recognised diseases in which oral cancer may arise. This classification includes: oral leukoplakia, oral erythroplakia, oral lichen planus, nicotine stomatitis and oral submucous fibrosis.[8] Leukoplakia is one of the most common premalignant lesions affecting the oral mucosa. It has a potentially malignant transformation rate ranging from 3 - 33% over 10 years. Leukoplakia as a clinical entity may have a varied histopathological presentation ranging from mildly hyperkeratotic lesions to those exhibiting severe dysplastic features.[4]

Oral epithelial dysplasia (OED) is the diagnostic term used to describe the histopathologic changes seen in chronic, progressive, and premalignant disorders of the oral mucosa. Epithelial dysplasia is the histologic marker of pre-malignancy, and as such, it is predictive of an increased rate of development of oral squamous cell carcinoma (OSCC).[8] OSCC is the most common malignant neoplasm arising in the mucosa of the oral cavity and includes subsites like the buccal mucosa, tongue, palate and lip.[9]

There are basically two approaches to the early detection of oral dysplasia and cancer:

Oral cancer screening programs that identify asymptomatic patients with suspicious lesions.

Employing specific diagnostic tools to identify dysplasia and early oral cancer in asymptomatic patients with an oral abnormality.[10]

The diagnosis of oral potentially malignant disorders (OPMD) and OSCC is of paramount importance given the mortality rate of late stage disease. A summary that synthesises the techniques for early diagnosis of oral cancer (Table 1).[8]

Tumor markers are substances that are produced either by the tumour itself or by the body in response to the existence of cancer or certain benign or noncancerous conditions that can help in the diagnosis of cancer and in the assessment of tumour burden.[11] In tissue sections, proliferating cells have been recognised using a variety of tumour markers. .[8] Identification of appropriate biomarkers can aid in the early diagnosis of oral cancer. However, a tumour biomarker must be characterised by accuracy, reproducibility, and reliability to be clinically useful and guide management. In oral cancer, several biomarkers have emerged, showing promising results in the diagnosis, early detection, and prognosis of the disease. [12]

Apart from many staining techniques which that were used for assessing the tumour tissue based on nuclear studies, the staining of AgNORs by silver compounds has become popular due to the following characteristic features:

- Simple technique
- Ease of use and to carrying out
- Low cost of staining
- Good proliferation with other proliferative markers.[13]

Hence, quantitative and qualitative assessment of argyrophilic nucleolar organizer regions (AgNORs) is a valuable parameter in tumour pathology.[8]The amount of AgNOR is proportional to the proliferative activity of neoplastic cells in the cell cycle, which progressively increases from the G_0 to the S phase.[14]

Staining Technique of AgNOR

According to Ploton et al., formalin fixed paraffin embedded tissues were submitted to 3ìm thick sections and extended in glass slides previously prepared with 3 – aminopropyltriethoxysilane (Sigma Chemical Co., St Louis, MO, USA).The sections were dewaxed in xylene and hydrated through decreasing grades of ethanol .The silver staining was applied according to the method of *Ploton et al., modified* by Rivero et al.[15]

AgNOR staining procedure, according to Crocker and Nar (1987):

4 µm thick sections were made

↓

Sections are then deparaffinized in xylene

↓

Hydrated through decreasing grades of ethanol to double distilled deionized water

↓

Sections were reacted with freshly prepared silver colloidal solution
(In a closed coplin jar for 35 minutes at room temperature)

* Ensuring a dark environment was maintained throughout the reaction time.

The silver colloidal solution was washed with double distilled ionised water. The sections were then treated with 5% sodium thiosulphate for 5 minutes, washed in double distilled deionized water, dehydrated through increasing grades of alcohol, cleared in xylene and then mounted.[1]

Quantitative Assessment of AgNOR

Giri et al (1989) proposed an AgNOR counting method. According to them, in all specimens, only 100 cells were selected randomly, and the AgNORs are visualised as black dots (100X magnification). A number of individually discernible separate black dots in each nucleus is recorded, and the average for each case is computed. A precaution is taken during counting: both intranucleolar and extranucleolar dots should be included.[1] When two or more dots are so closely aggregated or clumped within a nucleus that the precise number within the aggregate cannot be counted, the aggregate is considered as one.[16]

Qualitative Assessment of AgNOR

Pleomorphism is a term used in histology and cytopathology to describe variability in the size and shape of the cell or nucleus. Parameters such as cellular and nuclear areas, nuclear-cytoplasmic, and nucleolar – nuclear ratios have been utilized to characterize epithelial dysplasia.[17]

Qualitative assessment of AgNOR consists of morphological characteristics of AgNOR dots. Morphological variations of AgNORs depend on the size and shape of the individual AgNOR dots and their pattern of distribution.[13]

According to SA Khan et al. (2006), the grading of size variation and scores of distribution were given as the following: [14]

0 - More or less uniform in size;
1+ - Two different sizes;
2+ - More than two different sizes (but not those of 3+);
3+ - Including all grades and sizes.

The dots dispersion grading and scores of AgNOR dots were given as the following:

0 - Limited to nucleoli;

Table 1. Techniques that contribute to the diagnosis of oral cancer

1.Vital Staining (Conventional Method)	• Toluidine Blue • Methylene Blue • Lugol's Iodine • Rose Bengal
2. Light Based Detection Systems	• Chemiluminscence • Tissue fluorescence imaging • Tissue fluorescence spectrometry
3.Histological Techniques	• Incisional biopsy • Excisional biopsy
4. Cytological techniques	• Oral Brush biopsy • Liquid based cytology • Laser Microdisection
5.Molecular Analysis	• Gene alteration • Viral genome studies • AgNORs assessment • Immunohistochemical identification of tumor markers
6.Imaging techniques	• Magnetic resonance imaging (MRI) • Computed tomography (CT) • Positron emission tomography (PET)

1+ - Occasional dispersion outside nucleoli;
2+ - Moderate dispersion outside nucleoli;
3+ - Widely dispersed throughout the nucleus.

According to Elangovan T et al in 2008, qualitative assessment depends on the size and shape of the individual AgNOR dots and their pattern of distribution, as defined by Warnakulasuriya and Johnson, who identified three patterns of AgNOR distribution (Fig.1).[18]
Type I: single /few large dots within nucleolus, representing nucleolus;
Type II: discrete small dots within the nucleolus;
Type III: fine black dots dispersed throughout the nucleoplasm.

DISCUSSION

Oral squamous cell carcinoma is the most common malignant neoplasm arising in the mucosa of the oral cavity.[19] In India, oral cancer represents a major health problem accounting for upto 40% of all cancers, and is the most common cancer in males and third most common cancer in females.[20] It often arises from Oral potential malignant disorder (OPMDs) such as leukoplakia, erythroplakia and oral lichen planus. Leukoplakia is the most common OPMD.[21]Risk factors for oral cancers are well established and include tobacco and alcohol consumption.[22]

Early diagnosis seems to significantly decrease the morbidity rate in oral cancer.[23] The current method for the early diagnosis, visual examination of the oral cavity, relies on clinical expertise in recognizing early neoplastic changes.[24] Many techniques till date have been reviewed so far e.g. vital stating procedure, brush biopsy, micronuclei analysis, DNA ploidy but have certain limitations.[21]

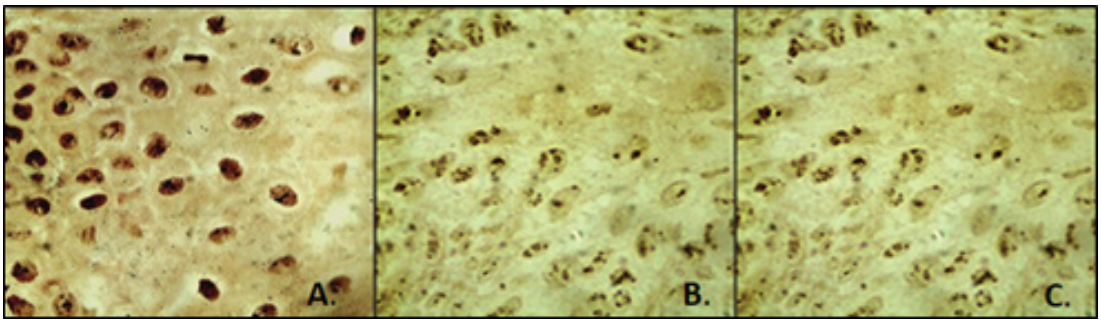

Fig. 1. A silver-stained section : A. shows a single or few spherical dots in the nucleolus; B. shows small dots in the nucleolus; C. shows numerous fine dots in the nucleoplasm
Source: KP Khot et al.2015.Pleomorphism of argyrophilic nucleolar organizer regions in oral submucous fibrosis and oral squamous cell carcinoma. J Nat Sci Biol Med.

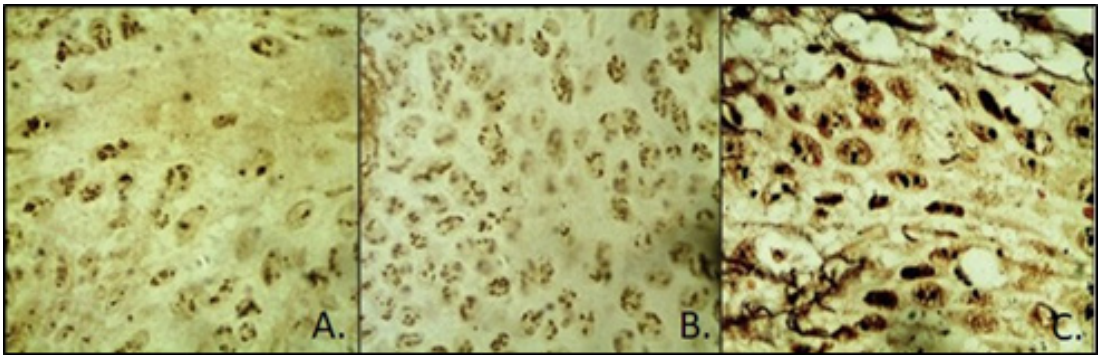

Fig. 2. A silver-stained section shows: A. small dots in the nucleolus in advanced OSMF; B. in advanced OSMF, fine dots are present throughout the nucleoplasm, which gives a granular appearance; and C. large kidney shaped dots seen in moderately and poorly developed OSCC.
Source: Khot KP et al.2015. Pleomorphism of argyrophilic nucleolar organizer regions in oral submucous fibrosis and oral squamous cell carcinoma. J Nat Sci Biol Med.

Of the various newer techniques, NORs are useful in the determination of cellular activity and application in neoplastic lesions. Proliferation rate can be assessed by AgNORs on cytologic or histologic preparations.[13]

The first observation of the nucleolus is attributed to Fontana in 1781 and silver staining was first described by Good Pasture and Bloom in 1975. Hubbel et al. (1977) carried out a study to identify NORs in normal and neoplastic cells by the silver staining method.[25]Nucleolar organizer regions are focal aggregates of intranuclear non-histone proteins that are associated with potential sites of ribosomal DNA transcription. These proteins are easily localized by virtue of their argyrophilia; hence, they are known as AgNOR (argyrophilic nucleolar organizing region).[16] AgNOR dots are seen as dark brown to black dots inside a brownish nucleus within a yellow cytoplasm.[15] The mean number of AgNORs per nucleus accurately correlates with the mitotic rate in tumour cell lines.[16]

Most of the studies on AgNOR have been done focusing on its application in many oral lesions. NORs have received a great deal of attention recently because of the observations that their frequency within the nucleus is significantly higher in dysplastic cells than in normal cells. Consequently, they are of diagnostic value in the characterization of the invasiveness of carcinomas. They also play a very important role in the estimation of the cellular activity that is applied to a variety of neoplastic as well as hyperplastic lesions.[26]

Variations in the size and/or number of the AgNOR dots may depend on the stage of the cell cycle, the transcriptional and metabolic activity of the cell, or the number of NOR bearing chromosomes in the karyotypes. The AgNOR count is used as a marker of cellular proliferation.[2]

Xie X et al. stated in 1997 that the number of AgNOR at any given stage in the cell cycle appears to be inversely proportional to the cell cycle time, i.e., the higher the amount of

Table 2. List of Tumor Markers

Epithelial Markers	• Cytokeratins (CK)
	• Epithelial membrane antigen (EMA)
	• Oncofetal antigens
	• Alpha fetoprotein (AFP)
	• Carcinoembryonic antigen (CEA)
Mesenchymal Markers	Muscle antigens
	• Desmin
	• Actin
	• Myoglobin
	• Myosin
	Vascular antigen
	• CD 34
	• CD 31
	Neural antigens
	• S 100
	• Glial fibrillary acidic protein (GFAP)
	• Synaptophysin
	• Nerve growth factor receptor
Prognostic Markers	Cell adhesion molecules
	• Cadherins
	• Integrins
	• Selectins
	Proliferation markers
	• PCNA
	• Ki67
	• AgNORs

AgNOR, the shorter the cell cycle time. AgNORs are basically considered to reflect the biosynthetic and nucleolar functional activity of a cell cycle and serve as an indicator of the rapidity of the cell cycle. The AgNOR count increases with increased cell ploidy and with increased transcriptional activity during active cell proliferation.[27] Ray J G et al. (2003) suggested that premalignant lesions show higher mean AgNOR counts in leukoplakia as compared to normal epithelium. Their study shows a 1.47 mean AgNOR count of normal epithelium and 2.37 of leukoplakia.[30]

Immunohistochemistry (IHC) markers have lately acquired popularity as a reliable diagnostic tool.[31] IHC markers such as Ki-67, p53, CK17, CK13, laminin -52, and type IV collagen are used for detection of cancer. [32]

Table 3. Summarizes the qualitative and quantitative assessment of AgNORs in oral lesions

Author (year)	Sample	Intervention	Summary of findings
Xie X et al. 1997 (27)	OSCC	AgNORs' relevance in malignant oral lesions as a screening tool and prognostic marker	Discrimination between normal epithelium, dysplasia, and OSCC is possible using both mAgNOR and pAgNOR counts. For SCC patients, AgNOR counts were reliable prognostic indicators.
Sarbjeet Singh et al. 2006 (28)	Malignant oral lesion, premalignant lesion, and condition	Evaluation of AgNORs counts in the pre-therapeutic assessment of the disease's	AgNOR value of more than 1.7 was suggestive of precancer and more than 6.0 was suggestive of cancer.
Elangovan T et al. 2008 (3)	Hyperplastic, premalignat and malignant oral lesions	AgNOR was evaluated on both a quantitative and qualitative level.	The amount of AgNOR is inversely correlated with the rate of cell proliferation. It is possible to discriminate between hyperplastic, premalignant, and malignant lesions based on the qualitative properties of AGNOR.
Sandhya Panjet Gulia et al. 2011 (2)	Premailgnant & malignant oral lesions	An analysis of the role of AgNORs in identifying benign from pre-malignant and malignant lesions in the oral cavity	OSCC showed a significantly high mean AgNOR count. In benign lesions, the AgNOR dots are typically small, uniformly stained, and of regular shape; however, when the tumor's grade increased, the AgNOR dots tended to become irregular, large dots, or bizarre clusters.
Aman Chowdhry et al.2013 (26)	Premalignant & malignant oral lesions	Quantitative evaluation of large and small AgNORs in oral normal mucosa, precancerous mucosal lesions, and infiltrating squamous cell carcinomas	Indicated that AgNORs in OSCC become smaller as they proliferate. Additionally, as a cell's potential for malignancy develops, AgNOR levels rise. This may aid in the early diagnosis, prognosis, and malignant transformation of dysplastic mucosal lesions.
Fahad Mansoor Samadi et al.2014(4)	Leukoplakia & OSCC	Quantitative analysis was done.	AgNOR counts to be much higher in OSCC, when compared to oral leukoplakia.The continuous application of the AgNOR stain to dysplastic lesions and early OSCC aids in the accurate identification of these lesions. Komal
P Khot et al.2015 (17)	OSMF & OSCC	To ascertain whether the AgNOR qualitative analysis may be employed as a marker for disease severity and lesion progression	AgNOR pleomorphism appears to be an accurate depiction of the underlying tissue alterations, and as such, it can be helpful in the diagnosis and prognosis of tumours.
Ritu Sharma et al.2016(13)	OSCC	The qualitative assessment of AgNORs was done.	This study has been suggested that AgNOR staining can be used to differentiate different grades of OSCC.
Hindangmayyum Denish Sharma et al.2021 (29)	OSCC	The quantitative assessment of AgNORs was conducted.	Summarized that both standardized AgNOR analysis and TATE count of the invasive front of OSCC provide outstanding information about the clinical course of the tumor.

Abbreviations: OSCC: oral squamous cell carcinoma; OSMF: oral sub mucous fibrosis; TATE: tumor associated tissue eosinophilia.

Table 4. Summarizes the comparison among AgNOR and some other immunohistochemical markers used for oral cancer prognosis

Reference	Aim	Result
Nakamura M et al.(33)	The value of localized fluorodeoxyglucose (FDG) uptake in conjunction with the histochemical expression of AgNORs was examined.	An useful index for figuring out how best to treat each patient after neoadjuvant chemoradiotherapy is the combination of FDG-PET and AgNORs score.
Madan M et al. (34)	Proliferating cell nuclear antigen (PCNA) expression and AgNOR counts are used to quantify the rate of cell proliferation.	According to the results, the mean AgNOR count by itself cannot be a helpful metric to distinguish between the normal epithelium and the DL epithelium and OSCC. On the other hand, PCNA can be a useful biomarker for making this distinction.
Jagtap M M et al. (35)	Examined the association between the immunohistochemical (p53) and histochemical (AgNOR) profiles of OSCCs	The mean AgNOR count and p53 expression both showed an upward trend as the OSCC's histological grade progressed. In addition to the gold standard, Anneroth's histomorphological grading system of OSCC, the nuclear proliferative indices (AgNORs and p53) are trustworthy prediction markers.

According to Gyanchandani(2022) assessement, CK17 expression can be used as an adjunct in the grading of OSCC in conjugation with Broder's system. They've concluded that CK 17 expression useful in early diagnosis or determining the aggressiveness of the tumour.[29] Wei et al. carried out a study on the overexpression of the CK17 protein in OSCC. The study revealed increased CK17 expression in cancerous tissues from OSCC patients compared with paired adjacent non-malignant epithelia. [36] Nobusawa et al. conducted a study on the immunohistochemical staining patterns of CK13, CK14, and CK17 in oral epithelial dysplasia, including orthokeratotic dysplasia. It was found from the study that CK14 expression can be used to detect early epithelial dysplasia and that CK13 and CK17 expression are useful for detecting neoplastic changes. [37] Patankar S et al. (2022) concluded that higher CD10 expression was observed with disease progression from oral epithelial dysplasia to increasing grades of OSCC, which may be associated with a poor prognosis. [38]

AgNORs are assessed qualitatively based on their size, shape, and distribution, which allows for the determination of the differentiation characteristics of the transformed cells. [27]

Warnakylsuriya K & Johnson NW in 1993 observed single, double, or few AgNORs per nucleus (Type I pattern) in most of the benign oral lesions. In epithelial dysplasia, predominantly multiple small AgNOR dots were observed within the nucleolus (Type II), whereas in OSCC, multiple small AgNOR dots were scattered throughout the nucleoplasm (Type III). As per them, on the other hand, the epithelial dysplasia group also shows an admixture of all the three types of sliver stained products.[7] According to Khot et al., as such, no difference was noted in AgNOR's shape between normal epithelium and early OSMF. In Mod-Adv and Adv OSMF, AgNORs predominantly varied from small dots present in the nucleolus to fine dots present throughout the nucleoplasm, giving a granular appearance (Fig.2 A & B).While in well differentiated OSCC, the dots were predominantly small and irregularly shaped, but some of them were slightly larger and kidney shaped. In moderately and poorly differentiated OSCC, irregular and bizarre shapes are found (Fig.2 C).[17]

According to Gulia S. P. et al. (2011), oral squamous cell carcinoma showed notable variations (irregular, giant, and bizarre clusters) from normal epithelial tissues in terms of the AgNOR dots,

which tended to be large, homogenously stained, and regular in the nucleus. [2]

AgNOR has essentially been shown to be helpful in diagnostic pathology, particularly to identify benign lesions from their malignant counterparts. AgNORs reflect the state of activation and the proliferation activity of the cell and the degree of malignant transformation of certain tissues.[38] Protein synthesis is faster in rapidly dividing cells as compared to slowly proliferating cells. Therefore, an increase should occur in the nucleolar structures (AgNORs) where rRNA synthesis takes place. [39] For the above reasons, AgNORs have found widespread application in tumour histopathology, in assessing the growth potential and malignant potential of tumours as well as in distinguishing between benign and malignant neoplasms, to assess the prognosis and to evaluate the risk of recurrence.[2] In oral pathology, the AgNORs technique is also a useful tool for the differentiation between odontogenic cysts and tumours and to distinguish recurrent and non-recurrent giant cell lesions of the jaws.[1]

CONCLUSION

AgNOR staining is rapid, efficient and inexpensive procedure which provides useful information about cellular proliferation. AgNOR method can be used as a therapeutic assessment of the biologic aggressiveness of the diseases. The AgNOR counts increase with increased cell ploidy and with increased transcriptional activity in the stages of active cell proliferation. The fact that AgNOR count increased from normal to precancer to cancer, can be used to detect the degree of malignant potential in premalignant lesion and condition.

According to many studies, the AgNORs count represents a valuable criteria to help the gradation of epithelial dysplasia and, therefore help in examining their potential for malignant transformation.

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