REVIEW ARTICLE

SILVER STAINED NUCLEAR ORGANIZER REGIONS (AgNORs) – PREDICTORS OF INCIPIENT CELLULAR ALTERATIONS – A REVIEW

Thippeswamy SH[1], Bastian TS[2], Vikas Prasad[3], T. Suresh[4]

[1]Professor & Head, Department of Oral Pathology, DIRDS, Faridkot, Punjab, [2]Professor & Head, Department of Oral Pathology, Mahe Institute of Dental Sciences, Mahe, Pondicherry, [3]Professor & Head, Department of Oral Pathology, Geetanjali Dental College, Udaipur, [4]Reader, Department of Oral Pathology, Govt. Dental College, Bangalore

ABSTRACT:
Nucleolar Organizing Regions (NORs) are the loops of ribosomal DNA, which occur in the nuclei of cells possessing the genes for synthesizing rRNA. These regions (NORs) have been assigned the name 'fibrillar centers' (fcs) at the ultrastructural level and these should be regarded as the true interphase counterpart of the NORs present on the five human acrocentric chromosomes i.e. chromosome number 13, 14, 15, 21 and 22. Silver staining Nucleolar Organizer Regions (AgNORs) have been attracting much attention because of claims that their frequency within the nucleus has been significantly higher in malignant cells than in normal, and that they produce a very useful means for examination of nucleolar structures and variations in nucleolar activity.
Keywords: Chromosomes, Nucleolar Organizing Regions, Silver staining.

Corresponding author: Dr. Thippeswamy SH, Professor & Head, Department of Oral Pathology, DIRDS, Faridkot, Punjab

This article may be cited as: SH Thippeswamy, TS Bastian, Prasad V, Suresh T. Silver stained nuclear organizer regions (AgNORs) – Predictors Of Incipient Cellular Alterations – A Review. Int J Res Health Allied Sci 2015;1(1):16-19.

INTRODUCTION

Nucleolar organizer regions are the structure of central importance in the transcription of DNA to ribosomal RNA. This latter then forms ribosomes and these ultimately assemble proteins. The NORs consists of strips or loops of ribosomal DNA (rDNA; ribosomal cistrons) and may be regarded in simple terms as 'ribosomal factories'. Their molecular nature can be demonstrated by the binding of radiolabelled ribosomal RNA, which localizes on the NOR sites. The rDNA transcribes to rRNA under the catalytic influence of RNA polymerase I (RP I).[1]

STRUCTURE

Using specialized ultrastructral technique, in spreads of chromosomes the nascent RNA molecule can be seen to form the DNA cores; when this method is used the RNA appears in the form of lengthening branches of "Christmas tree" – like structures, representing the NOR complex. Indeed, the NORs lie in tandem, with non-transcribing spacer regions lying between them. The first RNA moiety to appear at the NOR is 45S unit, which is then converted into 18S and 28S subunits, about half of the 45 S material being lost or degraded in the processes. Once these components of ribosome are constructed, they pass through nucleolar pore and assembled in cytoplasm.[1]

NORs are usually demonstrated by binding of their associated proteins to silver ions (Ag^+). This silver binding reaction is relatively simple, which enables the Ag^+ ions to bind the nucleolar organizer region protein. This reaction traditionally runs at specific temperature and can be applied to paraffin tissue sections. For convenience, this is called AgNOR reaction and reaction sites, AgNORs (silver stained nucleolar organizer regions). [2]

NORS AND CHROMOSOMES:

NORs are the chromosomal segments in which ribosomal RNA (rRNA) is encoded and they are thus responsible for the development of the RNA containing nucleolus or nucleoli into which the NORs project on large loops of DNA. They are

situated in the nucleolus and are thought to reflect the proliferative activity of the cell. Their relationship to the DNA, the cell cycle and protein transcription makes them ideal as markers to detect cellular activity. The discovery that the protein associated with NORs are equally indicative of NORs activity as the NORs themselves, have led to reports in literature regarding their role in various disease and non-disease status.[3]

AgNOR DEMONSTRABILITY:

Transcriptionally active NORs associate to certain specific acidic non histone proteins, which are an essential component of the pre-RNA synthesis machinery and are selectively stained by a silver colloid technique and can be visualized as black dots; the structures thus demonstrated are called AgNORs. The silver staining technique identifies neither rRNA nor rDNA but the acidic protein

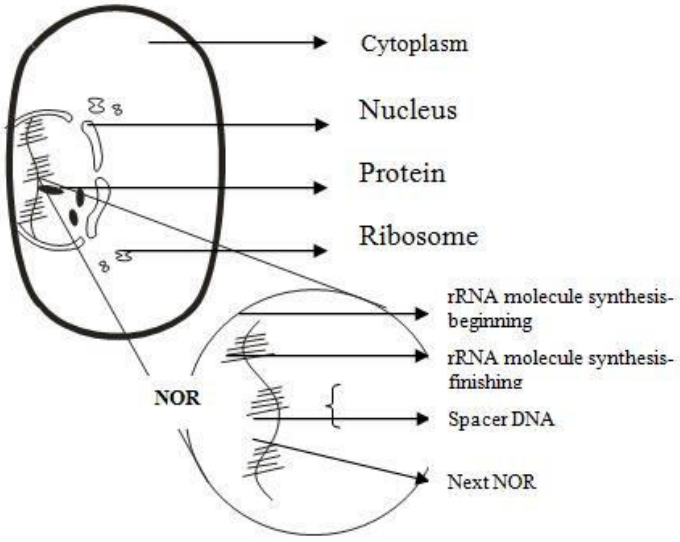

Figure 1: Diagrammatic illustration of NORs

NORs can be seen in the chromosomes as so called "secondary constriction". These are not true breaks in chromosomal integrity and areas can be shown to progress in an unusual ultrastructural configuration. In humans these regions occur on the short arm of the five acrocentric chromosomes No.13, 14, 15, 21 and 22. Usually these NORs are seen in close approximation to primary constriction.[1]

NOR-ASSOCIATED PROTEINS (NORAPS):

The nature of NOR associated proteins (NOAPs) is rather uncertain at present, but they probably act as regulators of rDNA transcription and maintain the extended configuration of DNA. NOR staining thus represents actively transcribing NORs (thus rDNA) and the frequency of NORs per nucleus may prove useful as a replicatory markers.[2,4] Major NORAPs are C_{23} (Nucleolin) and B_{23} protein. These proteins can be demonstrated by means of Ag^{+} binding or immunohistochemical technique using monoclonal antibodies to nucleolin. Other NORAPs include AgNOR protein, RNA polymerase I, 100 KDa, 80 KDa and phosphoprotein pp105 and pp135.[1]

associated with these sites of rRNA transcription.[3] Properly stained paraffin sections demonstrate, a pale yellow background with nuclei appearing as yellow ovals within black /brown AgNOR dots are visible.[5, 6]

The argyrophil method is directed against the NORAPs and is the most commonly used. Due to their high electron charge density, NORAPs, especially nucleoli, show affinity to silver stains. Affinity may also be expressed due to the presence of specific bonds and biochemical configuration of the NORAPs, for example, due to carboxyl and phosphate moieties.[6] NORs can be visualized directly by such specific methods as electron microscopy, in situ hybridization, and immunolabelling or indirectly by identifying the proteins associated (NORAPs). [3]Theoretically, 20 AgNOR sites should be present in the human diploid nucleus. However, AgNORs are found typically aggregated within one or two nucleoli during interphase in normal cells (Figure 2 and 3). The number of AgNORs visualized depend upon; [7]

1) Number of AgNOR bearing chromosome in the karyotype.
2) RNA transcriptional activity

3) Stage of the cell cycle as the nucleolus disperses before mitosis and recognizes afterwards. These properties of NORs have led to their use as diagnostic tools in various malignancies for evaluation of their prognosis and aggressiveness.

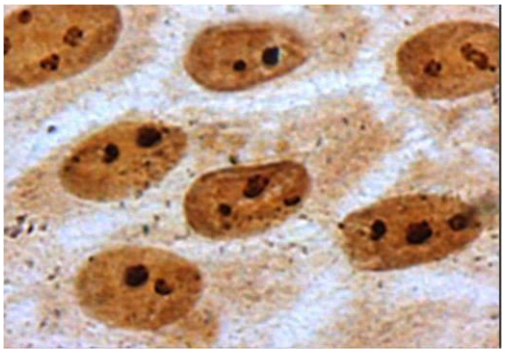

Figure 2: AgNORs in normal cells

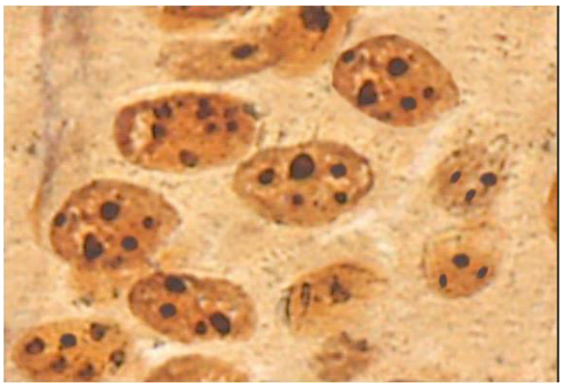

Figure 3: AgNORs in dysplastic cells

The AgNOR method:

The acidic AgNOR proteins were first localized at the electron microscope level by Hernandez-Verdun et al using the usual three step method of Good Posture and Bloom (1975). They were found within the fibrillar centers and the dense fibrillar apparatus of interphasic nucleoli of cultured cells, fixed with Conroy's solution[8]. However, some modifications have been proposed for this technique, since pathologists often experience one or more of the following problems.[9]

i) Silver precipitate over the slide caused by too rapid development.
ii) Development time cannot be standardized because the ammonical-silver formalin developer solutions are unstable, causing over or under developed AgNORs
iii) Uneven staining of the AgNORs occurs across the slide, making reliable AgNORs counts difficult.
iv) Incubation in aqueous silver nitrate alone is time consuming from 3-24 hours. Moreover ammonical silver is expensive and has a short shelf life.

Subsequently, Howell and Black [9]suggested a one step technique to reduce time. This reaction primarily uses gelatin as a protective colloid to control silver staining and consists of mixing silver nitrate and formic acid in optimal proportions. This method required only two minutes to perform.

Various modifications of this technique have been proposed and utilized; preincubation with glycine to reduce incubation time, substitution of gelatin with polyethylene glycol as a protective colloidal developer, primarily used to reduce background staining. Celloidin film has also been used to reduce non specific deposits [3]. Internal controls, period of incubation, control of staining time and reduction of background deposits are integral to the process.[10]

Modified one step silver technique can be performed at 20^{o}C.Here it utilizes localization of the argyrophilic proteins of the nucleolar organizer regions, to various materials including cells in smears, chromosomes semi-thin sections of plastic embedded cells and sections of paraffin embedded human pathologic tissues. Use of reflected light based on the ability of silver to reflect incident light specifically, and give images with an improved resolution. This method at room temperature improves the specificity of the staining and optimizes the conditions of observation.[11]

Degree of staining is also dependent on the fixation regime employed and results may vary greatly from one fixative to another. In general alcohol-based fixatives give optimal results, Carnoy's fluid being especially recommended. Mercurial and dichromate-containing fixatives were found to have highly detrimental effects on NOR staining. Routine 10 per cent formol saline fixation gave adequate results whereas 10 percent neutral buffered formalin gave optimal staining, similar to alcohol-based fixation.[12]

Use of celloidin films have been recommended to reduce the non specific background deposits[10]. Further simple modifications are added to the silver staining nucleolar organizer regions, which includes, performing background staining. The method being straight forward and fast, maintained a high degree of contrast between the background and the AgNORs[12]. Further improvements in silver staining include, pre-reduction of sections, selection of an optimal gelatin, and post treatment of sections to produce a permanent preparation. These methods were compatible with many fixatives, other stains used before or after the silver stain.[3]

Visualization of proteins associated with nucleolar organizer regions proteins (AgNORs) on formalin fixed and paraffin embedded archival tissues is substantially improved after application of wet autoclave pretreatment. Microwave irradiation can also be advised in order to shorten the processing time.[13,14] To achieve reliable and reproducible results, the committee for AgNOR quantification in 1999 recommended the standard AgNOR technique to assess AgNOR quantity in the paraffin embedded sections, which includes, a wet autoclave pretreatment required in getting consistently high staining quality of single interphase AgNORs independent of duration of formalin fixation and archival storage. [15]It has also been suggested the AgNOR staining has to be done at 37^{o}C in a thermo statistically controlled environment[16].

CONCLUSSION:

The patterns of Nucleolar Organizer Regions by size and distribution, as visualized by silver staining (AgNORs) are known to vary with different cell cycle stages. The number of individual dots may increase during the mitotic phase whereas in the G_1 phase there is a tendency for the dots to decrease in number and increase in AgNOR area. Thus, analysis of number of AgNORs, AgNOR area, size, and shape are the better agreed methods to obtain an accurate and reproducible marker of the proliferative potential and cell stability of the tissue analyzed.

REFERENCES

1. McGee JOD, Isaacson PG, Wright NA. Oxford Textbook of Pathology Vol 1, 1993.Oxford University press, Oxford: 586-90.
2. Kamath VV and Sastry KARH. Nucleolar Organizer regions (NORs) in oral lesions. Ind J Oral Pathol 1994; 1:1-11.
3. Lindner LE. Improvements in silver staining technique for Nucleolar Organizer Regions (AgNOR). J Histochem Cytochem. 1993; 41 (3): 439 - 45.
4. Chattopadhyay A. AgNORs in tumoral pathology. Review of literature and observations on the technique and reaction in normal oral epithelium. Indian J Dent Res. 1993; 4: 47-53.
5. Thippeswamy SH, Bastian TS, Einstein A, Mujib A. Morphometric evaluation of AgNORs can help in identifying incipient cellular alterations in clinically normal-appearing oral mucosa adjacent to oral squamous cell carcinoma. J Dent Res Sci Develop 2014;1:29-33.
6. Tekade SA, Chaudhary MS, Gawande MN, Bagri K. Correlation between mucoepidermoid carcinoma grade and AgNOR count. J Oral Sci 2010;52:275-9.
7. Griffiths HP. Silver stained structures (AgNORs) their dependence on tissue fixation and absence of prognostic relevance in rectal adenocarcinoma. Journal of Pathology. 1989; 159: 121-27.
8. Daniele Hernandez –Verdun, Hubert J, Bourgeois CA, Bouteille M. Ultra structural localization of AgNOR stained proteins in the nucleolus during the cell cycle and other structure. Chromosoma – (Berl) 1980; 79:349-62.
9. Howell WM and Black DA. Controlled silver staining of Nucleolus Organizer Regions with a protective colloidal developer: a 1-step method. Experimentia. 1980; 3: 1014-1015.
10. Chiu KY, Loke SL,Wong EK. Improved silver technique for showing Nuclear Organizer Regions in paraffin wax sections. J Clin Pathol 1989; 42: 992-994.
11. Platon D, Meneger M, Jeannesson P, Himber G, Pigeont, Adent JJ. Improvement in staining and in the visualization of the argyrophilic proteins of the Nucleolar Organizer Region at the optical level Histochem.1986; 5 (18):5-14
12. Coghill G, Grant A, Orrell JM, Jankowski J, Evans AT. Improved silver staining of nucleolar organizer regions in paraffin wax sections using an inverted incubation technique. J Clin Pathol 1990; 43: 1029-1031.
13. Massimo D and David T. Standardization of inter phase AgNOR measurement by means of an automated image analysis system using lymphocytes as an internal control. Journal of Pathology. 1991; 165: 337-342.
14. Ofner D, Bankfalvi A, Richemann K, Bocker W, Scmid KW. A standardized AgNOR stain method for formalin fixed and paraffin embedded material. J Pathol 1994; 15 (4): 226-230.
15. Tuccari G, Giuffre G, Ofner D, Ruschoff J. Standardized use of the AgNOR method. J Oral Pathol Med; 29:526-7.
16. Fahad Mansoor Samadi, Bastain Thattil Sebastian, Anil Singh, Shaleen Chandra, Shadab Mohammad, Arun Singh, Thippeswamy Halappa, Firoza Samadi. Silver Binding Nucleolar Organizer Regions Dots in Oral Leukoplakia with Epithelial Dysplasia and Oral Squamous Cell Carcinoma: An In Vivo Study. ISRN dentistry 2014:4

Source of support: Nil

Conflict of interest: None declared