ESOPHAGEAL DYSPLASIA IN HIGH RISK GROUP AND SIGNIFICANCE OF AgNOR IN ESOPHAGEAL DYSPLASIA

Dissertation submitted for

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CERTIFICATE

This is to certify that this Dissertation entitled **"ESOPHAGEAL DYSPLASIA IN HIGH RISK GROUP AND SIGNIFICANCE OF AgNOR IN ESOPHAGEAL DYSPLASIA"** is the bonafide original work of **Dr. S. GOMATHI**, in partial fulfilment of the requirement for M.D., (Branch III) in Pathology examination of the Tamilnadu Dr. M.G.R Medical University to be held in March 2007.

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DECLARATION

I, **Dr. S. GOMATHI**, solemnly declare that the dissertation titled "**ESOPHAGEAL DYSPLASIA IN HIGH RISK GROUP AND SIGNIFICANCE OF AgNOR IN ESOPHAGEAL DYSPLASIA"** is the bonafide work done by me at Govt. Stanley Medical College and Hospital during the period June 2004 to June 2006 under the expert guidance and supervision of **Dr. A. SUNDARAM,** M.D., Professor and Head of Department, Department of Pathology, Stanley Medical College. This Dissertation is submitted to the Tamilnadu Dr. M.G.R Medical University towards partial fulfilment of the requirement for the award of M.D. Degree (Branch III) in Pathology.

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CONTENTS

INTRODUCTION

Esophageal cancer is the sixth most common fatal cancer in the world. The five year survival rate is among the lowest for all cases (14%)[1] , the main reason being cancer of the esophagus becomes symptomatic at an advanced phase with a late diagnosis, when the tumor is already incurable. Most of these cases come to clinical attention after they have spread beyond the esophagus and become inoperable.

The only way to prevent this is by early detection of esophageal malignancies. For this ,the susceptible population should be subjected to some type of screening programme to identify any precancerous conditions. Squamous dysplasia and squamous cell carcinoma *in situ* are the two major categories which are thought to be precursor lesions of frank esophageal malignancy. All grades of squamous dysplasia and carcinoma *in situ* are associated with a significantly increased risk of developing esophageal squamous cell carcinoma. Increasing grades of dysplasia are associated with dramatically increased risk. If these dysplastic lesions can be identified in the high risk population, this dreadful condition can be dealt with more effectively.

There is well documented epidemiologic studies linking tobacco and alcohol use with the development of esophageal cancer. Alcohol was estimated to be causally associated with approximately 80% of esophageal cancers and the relative risk increases with the amount of alcohol consumed. Likewise, the risk associated with tobacco use appears to increase with the number of cigarettes smoked per day, duration of smoking and tar content. Ex-smokers have reduced risk compared to current smokers. A

synergistic effect for the combined habit of alcohol use and smoking or tobacco chewing has been reported. It is likely that these agents may also have a causal role in dysplasia of esophagus.

By studying this high risk group for the development of dysplasia, a causal association can be established, which can be used as a basis for devising effective screening protocols to pick up cases at an early stage. Once dysplasia is documented, it can be classified as mild, moderate or severe based on the extent to which the immature cells replace the normal epithelium. Mild dysplasia might be reevaluated periodically or treated by chemoprevention. Moderate dysplasia may be followed more closely or treated by focal endoscopic therapy. Severe dysplasia and carcinoma *in situ* should be treated by endoscopic mucosal resection which is a therapeutic method based on the principle of strip biopsy for resection of flat lesions of gastro intestinal tract which helps to prevent these lesions from progressing to inoperable stage.

With changing social habits, more and more people are becoming habituated to the use of alcohol and tobacco, which makes this kind of screening programme all the more mandatory. Hence an attempt has been made here to establish a causal association between these risk factors and development of dysplasia. The high risk group has been defined as individuals with habits like alcohol consumption, smoking, tobacco chewing and intake of hot beverages like coffee and tea either alone or in combination who are more prone to develop dysplasia.

Esophageal biopsies of asymptomatic individuals who belong to high risk group

are examined for the presence of dysplasia, comparing the findings with age matched subjects without the risk factors. Correlation of duration of risk factors and the occurrence of dysplasia is also evaluated.

Further the significance of AgNOR study in dysplasia of the esophagus is also evaluated. Various studies in different organs have proved that AgNOR numbers are increased in malignant cells compared to corresponding benign or normal cells. Moreover interphase AgNOR numbers are closely related to the proliferative activity of cells. The AgNOR numbers in quiescent cells is generally low while in proliferating cells such as cancer cells, a high AgNOR number is present.

AgNOR staining and counting of AgNOR numbers is done to determine any increase in AgNOR numbers in dysplastic esophagus compared to normal esophagus, thereby evaluating the degree of proliferative activity in dysplastic cells. Dysplastic cases are further subdivided into mild, moderate and severe and the variation in AgNOR numbers is also analysed, which could help in determining the prognostic significance of these lesions.

It is an attempt to find out the presence of dysplasia in asymptomatic individuals belonging to the high risk group and evaluate the significance of the role of risk factors in causation, which can be used for prevention research and also for early detection of cases by specific protocols in susceptible population thereby reducing the morbidity and mortality associated with this dreadful disease.

AIMS AND OBJECTIVES

1. Study of prevalence of dysplasia in the middle third of esophagus in asymptomatic individuals with high risk factors like alcohol consumption, smoking and tobacco chewing and comparing it with individuals without these risk factors.

2. Grading of esophageal dysplasia into mild, moderate and severe dysplasia on the basis of the thickness of the epithelium showing dysplastic changes.

3. AgNOR staining of the biopsy material and evaluation of AgNOR score in different grades of dysplasia and its comparison with the normal esophagus and malignancy.

4. To assess the usefulness of AgNOR study as a proliferation marker and prognostic indicator.

REVIEW OF LITERATURE

Squamous cell carcinoma is the most common malignant tumor of the esophagus and it is one of the most common fatal cancers worldwide. Especially high risk areas have been identified in Northern Iran, Central Asian Republics, Northern China and South Africa. In some of these areas over 20% of the population die of esophageal cancer. It is the sixth most common fatal cancer in the world, the five year survival rate among the lowest for all cancers (14%)[1]. The main reason for this poor prognosis is that most cases are asymptomatic and go undetected until they have spread beyond esophagus and are unresectable.

In sharp contrast to the rest of the gastro intestinal tract, the esophagus is mostly devoid of a serosal coat. Only a small segment of the intra abdominal esophagus is covered by serosa. The thoracic esophagus is surrounded by fascia that condenses around the esophagus to form a sheath like structure. The intimate anatomic proximity to important thoracic viscera, the great vessels and the tracheo bronchial tree are significant factors in facilitating the early and widespread dissemination of esophageal malignancy into the posterior mediastinum. The rich network of mucosal and submucosal lymphatics that run longitudinally along the esophagus further facilitate the tumor spread [2].

Early diagnosis has been shown to improve the survival rate. Therefore successful strategies for primary prevention and early detection of curable lesions are critically required. If precursor stage of this malignancy can be identified by screening the susceptible population, it will avoid a great deal of morbidity and mortality associated with this dreadful disease.[3]

Esophagitis, atrophy and dysplasia may be precursor lesions of esophageal cancer in high risk population. Increasing grades of dysplasia are associated with increased risk. Severe dysplasia and carcinoma *in situ* have similar degrees of risk.[4]

Esophageal squamous dysplasia can be graded histologically depending on the thickness of epithelial involvement. Risk of developing invasive squamous cell carcinoma is closely correlated to the severity of esophageal dysplasia encountered. Correct recognition of esophageal dysplasia is thus important.[5]

Histological categories that can be encountered in a esophageal biopsy specimen include the following:[1]

Normal

A stratified squamous epithelium which shows no features diagnostic of other histological categories. Mature squamous cells with abundant clear cytoplasm, scattered lymphocytes and compressed nuclear fragments are occasionally seen in the epithelium. The lamina propria contains few scattered mononuclear inflammatory cells.

Acanthosis

An otherwise normal epithelium which is more than 0.5 mm thick.

Esophagitis

One or more of the following three criteria:

- Elongation of lamina propria papillae into upper third of epithelium with basal cell hyperplasia more than 15% of total epithelial thickness.
- Epithelial infiltration by neutrophils or eosinophils.

- A dense non follicular infiltrate of mononuclear inflammatory cells or neutrophils in the lamina propria.

Basal cell hyperplasia

An otherwise normal epithelium has a basal zone thickness of more than 15% total epithelial thickness, without elongation of lamina propria papillae or other abnormality.

Squamous dysplasia

Nuclear atypia (enlargement, pleomorphism and hyperchromasia), loss of normal cell polarity and abnormal tissue maturation present in

the lower one third of the epithelium - mild dysplasia

lower two thirds of the epithelium - moderate dysplasia

In full thickness of the epithelium - severe dysplasia.

Squamous dysplasia (NOS)

Dysplastic biopsies which can not be graded because of biopsy size or orientation are graded as squamous dysplasia (NOS) – Not otherwise specified.

Squamous cell carcinoma *in situ* (CIS)

Dysplasia of squamous cells involving the full thickness of the epithelium without invasion and with intact basement membrane.

Squamous cell carcinoma

Neoplastic squamous cells are present which have invaded through the basement membrane.

Grading of increased expansion in the basal layer, as the proliferating cells progress from normal to hyperplasia to dysplasia and to carcinoma can be done. The hyperplastic and dysplastic cells are fundamental phase of carcinomatous change in the esophageal mucosa. It shows a wide spectrum of cellular alterations in the course of malignant change and the close relationship between the morphological alterations and cell biology.[6] With quantitative morphometry it is possible to differentiate normal squamous epithelium from dysplastic carcinoma *in situ* in esophagus. The epithelium is divided into 3 zones.Nuclear density, nuclear area, nuclear irregularity and nuclear ovality are the morphological variables. Nuclear area in the superficial zone, nuclear perpendicularity in the intermediate zone, nuclear density in the total epithelial thickness and interzonal variation of these variables are helpful in this differentiation.[7]

Continuity of dysplastic lesions into the areas of carcinoma is more often encountered in severe dysplasia, which suggests some relationship between the severity of dysplasia and carcinoma.[8]

Dysplasia is considered to be the earliest malignancy of the esophagus based on such biologic features as the histopathological features, proliferative activity and altered expression of cancer associated genes. It therefore becomes essential to detect and treat these lesions endoscopically .[9]

Usha *et al* in their clinicopathologic study of carcinoma of the esophagus with reference to changes in the surrounding epithelium have found that dysplasia was present in all cases of carcinoma esophagus. 70% of the tumors were squamous cell carcinomas.[10]

Takiyama *et al* have observed that squamous dysplastic lesion is commonly associated with carcinoma of the esophagus. Squamous cell carcinoma of the esophagus develops from severe dysplasia in a similar manner to carcinoma of cervix. [11]

Ohta *et al* have studied the distribution of esophageal dysplasia in cancer of esophagus and a possible relationship of epithelial dysplasia to the origin of esophageal cancer which was shown by at least 2/3 of esophageal cancers.[12]

The wide geographic and cultural variations in the incidence of squamous cell carcinoma of the esophagus suggest that environmental exposure is causally important**.** Numerous epidemiologic studies have linked alcohol and tobacco use with the development of esophageal cancer. Ethanol was estimated to be causally associated with approximately 80% of the neoplasms and the relative risk increases with the amount of alcohol consumed. The association with esophageal cancer is strongest with liquor, intermediate with wine and weakest with beer. Since there is an inverse relationship between the caloric intake of alcohol and a wholesome diet, alcohol may increase the risk of esophageal cancer by reducing nutrient intake. The risks associated with tobacco use appear to increase with number of cigarettes smoked per day, duration of smoking and tar content. [13]

Ex smokers have a reduced risk compared to current smokers. A synergistic effect for the combined habit of alcohol drinking and tobacco smoking or chewing has been reported .

Squamous cell carcinoma of the esophagus becomes symptomatic at a late stage when the disease is already advanced,and this contributes to its poor prognosis.

Endoscopy of asymptomatic individuals exposed to known risk factors associated with the development of this cancer may facilitate the diagnosis of early cancers or precancerous lesions.

Esophageal squamous cell carcinoma has been reported to be epidemiologically associated with tobacco and alcohol consumption.[14] Individuals who abuse alcohol and smoke have a high prevalence of dysplastic lesions.[15] Gao *et al* in their study for risk factors for esophageal cancer have found that risk of esophageal cancer was increased among tobacco smokers and alcohol drinkers. Risk is increased with number of cigarettes smoked per day, duration of smoking, number of pack years and decreasing age at start of smoking. The combined effect of heavy smoking and drinking among men is more pronounced. Cigarette smoking and alcohol drinking combined account for almost 50% of esophageal cancers.[16]

Vaugen *et al* have come to the conclusion that an extraordinary high proportion of the excessive risk for esophageal cancer can be attributed to alcohol drinking.[17] Use of alcohol and tobacco are significant risk factors for squamous cell carcinoma of esophagus. Lu and Lion in their study of risk factors of esophageal cancer have come to the conclusion that cigarette smoking and alcohol drinking are risk factors for esophageal cancer.[18]

Takiyama *et al* studied the relationship between dysplasia and the consumption of alcohol and tobacco and have come to the conclusion that in some manner alcohol and tobacco consumption appear to increase the susceptibility of the squamous mucosa to malignant transformation.[19]

Takiyama *et al* in their clinicopathological study of dysplasia in human esophagus have observed that the incidence of dysplasia was frequent in men than in women. Carcinoma *in situ* and severe dysplasia were more frequent where there was a history of habitual drinking and smoking. Predisposing factors for high grade dysplasia of esophagus are gender, smoking and drinking.[20]

Saeki *et al* studied the biologic and clinical significance of squamous epithelial dysplasia of esophagus and consider dysplasia to be the earliest malignancy of the esophagus. The relation of alcohol consumption and cigarette smoking to the multiple occurrence of esophageal dysplasia and squamous cell carcinoma[21] was observed by them.

Rotthawns *et al* in their article about prevention of esophageal cancer have said that smoking and alcohol are the major risk factors for squamous cell carcinoma. The prognosis is poor with 5 yr survival of less than 10%. Only early stages have a good prognosis. Prevention of squamous cell carcinoma can be done by avoiding drinking and smoking.[22]

Mascres and colleagues studied the morphological changes of esophageal mucosa in rats. 15 litter matched pairs of rats were divided into 2 groups. One was fed on alcoholic diet and the other was fed on isocaloric diet .Basal cell pleomorphism and tendency towards epithelial dysplasia were observed after long time alcohol consumption in the first group.[23]

AgNOR STUDY

Nucleolar organiser regions (NORs) are defined as nucleolar components containing a set of argyrophilic proteins, which are selectively stained by silver methods. After silver-staining, the NORs can be easily identified as black dots exclusively localised throughout the nucleolar area, and are called “AgNORs”. The NORs’ argyrophilia is due to a group of nucleolar proteins, which have a high affinity for silver (AgNOR proteins).

AgNOR proteins accumulate in highly proliferating cells whereas their expression is very low in non-proliferating cells. Some of these proteins remain associated with the nucleolar organizer regions (NORs) during mitosis. *In situ*, the expression of AgNOR proteins is measured globally by quantification of the level of silver staining using morphometry and image analysis. To go deeper into the understanding of the relationship between the cell cycle and quantity of AgNOR proteins, it is necessary to determine the phases of cell cycle during which expression of AgNOR varies and what are the most variable proteins in each phase. The amount of AgNOR proteins can be a marker of proliferation, because this amount is related to cell cycle phases, schematically low for G1 phase and high for S-G2 phase. Thus, it is a measure of the relative proportion of cells in each phase, and consequently of the timing of each phase. The higher value indicates that the major part of the cells are in the S-G2 phase and correlatively few are in the G1 phase, and this characterizes a rapid cell cycle.[24]

Interphase AgNORs are structural-functional units of the nucleolus in which all

the components necessary for ribosomal RNA synthesis are located. Two argyrophilic proteins involved in rRNA transcription and processing, nucleolin and nucleophosmin, are associated with interphase AgNORs and are responsible for their stainability with silver methods, thus allowing interphase AgNORs to be visulaized at light microscopic level, also in routine cyto-histopathological preparations. The number of interphase AgNORs is strictly related to rRNA transcriptional activity and, in continuously proliferating cells, to the rapidity of cell proliferation.

The quantity of interphase AgNORs, evaluated by morphometric analysis, increases in cycling cells from early G1-phase to the late S-phase. In cancer tissues, the AgNOR value is also closely related to both the percentage of cycling cells (measured by Ki67 or PCNA immunolabelling) and S-phase cells. Data from cell lines cultured in vitro have clearly shown that interphase AgNOR quantity is related to cell doubling time, the faster the rapidity of cell proliferation, the greater the interphase AgNOR quantity.[25]

SepLeek RD, has observed variations in the occurrence of silver-staining nucleolar organizer regions (AgNORs) in non-proliferating and proliferating cells. An increase in the size of AgNOR clusters rather than their number correlated positively with elevated labelling, particularly with the emergence of silver-staining regions of 2-3 microns visible diameter.[26]

AgNORs in normal cells are usually aggregated tightly within one or two nucleoli evident in histological or cytological preparations and individual AgNORs are often not discernible.The number of detectable AgNORs depends on several factors.[27]

1. The level of transcriptional activity.
2. The number of NOR bearing chromosomes in the karyotype.
3. The stage of the cell cycle.

Thus there is a striking discrepancy between AgNOR counts in chromosome spreads and those reportedly observed in histological sections of similar cell preparations.Histopathologists rarely record more than one or two AgNOR dots per nucleus in benign cells.This is due to the difficulty in perceiving the individual AgNORs when they are seen within a relatively small nucleus.

In malignancy,the AgNORs become dispersed through the nucleus to a varying extent,enabling the histopathologist to count them easily. The quantification of AgNOR interphase nuclei is related to their dispersion through the nucleoplasm than to the actual numbers present. Hence, the histologic AgNOR count in benign and malignant lesions denotes the numerical index of AgNOR dispersion rather than the absolute number of AgNORs.

Quantitative analysis of AgNOR proteins is a reliable marker of the rapidity of cell duplication and a significant prognostic parameter in tumour pathology. The rate at which a tumour proliferates represents an important parameter for predicting the clinical outcome of patients with neoplastic diseases. Rapidly growing tumours are expected to have a worse behaviour than slowly proliferating ones, and there is increasing evidence that high growth-rate cancers require aggressive treatment for better control of the disease. Cell kinetics data are, therefore, useful for pathologists and clinical oncologists in order to obtain valuable prognostic information and to define the most appropriate

therapeutic strategy in single cases. Over the past 20 years, several methods of cell proliferation assessment have been developed and employed in routine pathology. Among these, the quantitative analysis of AgNOR proteins has recently been proposed as a reliable indicator of the rapidity of cell duplication.[28]

Over the past 12 years, the "AgNOR method" has been applied in tumour pathology for both diagnostic and prognostic purposes. In order to achieve definitive standardisation of the AgNOR method and produce comparable data in all laboratories, the "International Committee on AgNOR Quantitation" was founded, and during the first Workshop "AgNORs in Oncology" held in Berlin in 1993 guidelines for AgNOR protein evaluation were first defined.[29]

Techniques for demonstration of NORs

The most popular method among all in locating the NORs is the silver staining technique. The structures demonstrated by this method are called AgNORs (Argyrophilic Nucleolar organizing Regions). The silver staining technique does not identify rRNA or rDNA, but the acidic NORAPs associated with the site of RNA transcription. The silver staining technique is based on the principle that, the acidic NORAPs have a high affinity for silver salts as a result of their high electron density and by virtue of their phosphate ,carboxyl and sulphur moieties.

The Argyrophil technique

The principle advantages of the AgNOR technique are the relative simplicity of the staining method and the ease of application to archival tissues. The one step method

consists of mixing silver nitrate and formic acid with gelatin acting as a colloid stabilizer. Paraffin embedded or frozen sections or cytological smears are incubated in this mixture for variable periods of time and then washed and mounted.

The silver reaction product is seen as discrete black dots at the light microscopic level which can be measured using oil immersion objective.Counting of AgNOR dots is done in a minimum of 100 cells and the results expressed as the mean number of AgNORs per nucleus. With minor modifications, this technique can be used in semiautomatic and fully automated image analysers[30] where the total amount of AgNOR material per nucleus rather than the number of dots counted.

Tissue fixation and processing

The intensity of AgNOR staining is dependant on the fixative used, and the results vary accordingly. Alcohol based fixatives, especially Carnoy's fixative gives optimal results. Mercurial and Dichromate fixatives are highly detrimental to AgNOR staining.[31] 10% neutral buffered formalin gives optimal staining comparable to alcohol fixatives.

An important factor to be standardized in this technique is the thickness of the sections. The ideal standard is a moderately thick section which contains all NOR profiles and at the same time, thin enough where NORs can be readily separated.[32]

AgNOR staining reaction

Usually the AgNOR staining reaction is run for 60 minutes irrespective of the type of tissue.However some minor alterations are recommended according to internal controls to allow subsidiary of AgNOR dots.

Non specific background silver grain deposits are a usual problem which can be minimized by the use of scrupulously clean glassware and double distilled water.

The minor modifications suggested are:

1. Pre incubation in glycine prior to AgNOR staining which reduces background staining by blocking the free or reversibly bound aldehyde residues left by formalin fixation.
2. Use of polyethylene glycol as a protective colloidal developer in place of gelatin.[33]
3. Use of 10% nitric acid on the slides after completion of the staining procedure.
4. Use of inverted incubation technique, wherein the slides are inverted in the staining solution, which maintains a high degree of contrast between the background and the AgNORs.[34]

Enumeration of AgNORs

Three main types of AgNOR configuration can be described in normal or neoplastic cells. The NORs can aggregate to form a solitary rounded argyrophilic structure called as AgNORs corresponding to the nucleolus per se. Individual NORs cannot be resolved within the nucleus.

The second configuration is called the 'Nucleolar pattern', which is often seen in proliferating cells. The NORs can be seen within the nucleolus with careful use of AgNOR technique. Finally the true AgNORs may be dispersed through the nucleoplasm. This pattern is frequently observed in malignant cells. These features are demonstrated especially well in cytological preparations, but can be seen in carefully prepared paraffin sections.

There are two methods of counting AgNORs:

1. All silver stained structures could be counted, but when seen in groups, each cluster is counted as one structure.
2. When AgNORs can be seen separately within a nucleolus, each AgNOR can be counted as a single unit, together with small AgNORs seen outside the nucleolus.

If the AgNOR count represents nucleolar disaggregation, which in turn reflects cellular activity, it is important to assess if AgNORs can be resolved within nucleoli. Meticulous attention should be paid in such cases to technique and reaction timing.

AgNOR count (mAgNOR**)** – Mean AgNORs/ nucleus.

AgNOR proliferative index (p AgNOR) – Percentage of cells exhibiting five or more AgNORs / nucleus.

The mAgNOR represents the mean DNA content of the cells or ploidy whereas pAgNOR represents the S- phase fraction.[35]

AgNOR score

Each AgNOR dot is classified as small, medium and large according to its size. A small dot is defined as a just visible but distinct one, when seen under the oil immersion objective. Dots about three times the size of a small one are classified as medium and those about five times or more the size of a small dots are classified as large.

The AgNOR score is calculated by multiplying the number of small dots by a factor of one, medium dots by a factor of three, and the number of large dots by a factor of five and adding up the three.

Improvements in the AgNOR technique

Ploton first described the Argyrophilic staining technique in 1986. Several modifications of the technique have been designed since then, aimed at improving the overall staining reaction as well as to enhance the information that is obtained. Some of the modifications include the following:

1. Sequential demonstration of Nucleolar Organizer regions and various antigens in both paraffin and frozen sections: This method is of great value in the evaluation of AgNOR numbers in neoplasms where cell populations are heterogenous. Immunostaining for proliferative markers demarcate the cell populations accurately prior to AgNOR counting. Combining immunocytochemistry and AgNOR staining on a single preparation gives positive results on fixed tissue with immuno alkaline phophatase methods providing better contrast. Trypsinisation of the sections prior to

immunostaining does not alter the count of silver stained granules.

2. Combination of a modified AgNOR staining technique with Feulgen reaction: This technique not only enables the counting of active NORs but also the evaluation of the amount of DNA in the nucleus.

3. Combination of cyto fluorometric analysis with conventional silver staining of NORs on cell suspensions: This AgNOR – FCM technique can be applied to analyze clinicopathological samples including neoplastic tissues more quickly and quantitatively than regular staining with AgNORs.

4. Use of AgNOR technique with minor modifications in semi-automatic and automatic image analysis hardware: This method enables the measurement of the total amount of Argyrophilic material per nucleus instead of counting the number of AgNOR sites.[36] Moreover, the incorporation of automated image analysis hardware in the technique makes it less subjective than the traditional methods.

5. Pretreatment with 7% nitric acid: This method gives very distinct dark brown images of AgNORs on a yellow background. The gradient of background colors allow easy discrimination of nucleolar, nuclear and cytoplasmic structures. Many morphometric parameters related to number, size and shape of AgNORs can be evaluated quantitatively by image analysis on sections pretreated with nitric acid and on adjacent sections treated with citrate buffer in a wet autoclave according to the most widely accepted method for image analysis of AgNOR. Both methods give similar results. A second improvement

is achieved by coating the slides with 7% celloidin solution in ethyl alcohol-ether prior to AgNOR staining and acid pretreatment. This coating prevents nonspecific silver deposition on argyrophilic bacteria and other tissue debris in human vaginal smears that could make visualizing AgNOR sites difficult.

6. Finally, placing sections face down on the staining solution prevents the formation of nonspecific silver precipitates. These procedures can be applied together or separately according to the requirements of the material to be evaluated.[37]

7. Quantitation of AgNORs by flow versus image cytometry: AgNORs are nucleolar proteins that interact specifically with silver salts. The size of silver precipitates measured by image analysis (ICM) in cycling cells proved to be inversely proportional to the cell cycle time and provided a significant correlation with prognosis for a large spectrum of cancers. Because ICM is time-consuming and poorly reproducible among laboratories using different imaging settings, a new approach to AgNOR quantitation is based on flow cytometry (FCM). Silver precipitates caused a great decrease in the forward scattered light and that this effect was correlated with the AgNOR's relative area as measured by ICM. These results were confirmed by measuring cell lines having different cell cycle durations. Moreover, double staining using APase-Fast red fluorescence to reveal the Ki-67/MIB 1 antigen of cycling cells and silver nitrate to stain the AgNORs was successfully analyzed by FCM. This procedure makes it possible, for the first time, to validly and rapidly compare the growth fraction and cycling speed of partially proliferating cell

populations, such as tumors.[38]

A revised version of the AgNOR staining technique involves the use of microwave radiation in order to shorten the processing time, the use of gelatin as a protective colloid, and a Farmer's solution to optimize the specificity of the technique giving highly contrasting AgNORs with minimal unspecific silver precipitation, thus facilitating both manual and computerized counting.[39]

Yekeler H has described a sensitive staining method for NORs instead of silver staining. As NORs are loops of DNA which are transcribed into ribosomal RNA, they can be demonstrated by staining with silver nitrate, since NOR-associated proteins are argyrophilic, producing structures termed AgNORs. The number and resolution of NORs increased 2-3 times by blue toning (30mmol/l FeCl3, 11 mmol/l potassium hexacyanoferrate(III), and 33 mmol/l oxalic acid) compared with silver staining.

A significant difference in the number of NORs was noticed between silver-stained and blue-toned cells ($P<0.001$). The blue toning technique thus appears to be more sensitive in detecting NORs than the AgNOR method and may prove a useful alternative for applications in histopathology.[40]

Applications of the AgNOR technique

The AgNOR technique was first applied in histopatholgy in prostatic tissue. Since then numerous studies have been performed in a variety of tissues for both malignant and non-malignant conditions.

Nucleolar organizer region staining patterns in paraffin-embedded tissue,

increased number of nucleoli (nucleolar organizer regions, NORs) with abnormal shapes and sizes, including small dots, has been used as prognostic tools to evaluate tumor proliferation levels and troublesome borderline lesions. All types of cancer cells show variable numbers of abnormally shaped nucleoli and dot-like structures. Only tumor cells presented four or more nucleoli, with or without dots, while 85% of the normal cells have one single NOR without dots. Changes in the number and shape of nucleoli present in malignant cells could be attributed to increased levels of rDNA transcription on cancer cells, besides abnormal remodeling of chromatin, which could disrupt proper nucleoli association.[41]

Prognostic significance of argyrophilic nucleolar organizer regions (AgNOR) in oesophageal cancer: AgNOR has been shown in recent times, to have value in knowing the prognosis of carcinoma oesophagus. Since the AgNOR counting is a simple method and can be applied to paraffin embedded sections, estimation of the AgNOR number may help in determination of prognosis in patients with oesophageal carcinoma. Preoperative radiotherapy seems to decrease AgNOR count with improved survival.[42]

Also the number of argyrophilic nucleolar organizer regions is a good indicator of lymph node metastasis in patients with esophageal carcinoma. The number of argyrophilic nucleolar organizer regions (AgNORs) were evaluated as a predictor of lymph node metastasis in patients who underwent resection of advanced squamous cell carcinoma of the esophagus. Results indicated that the AgNOR score is a good indicator of lymph node metastasis and suggest that it might also be a useful prognostic marker in patients with esophageal cancer.[43]

AgNORs can also be used as predictors of post operative distant recurrences in esophageal carcinoma. Pathologic factors and the number of nucleolar organizer region proteins (AgNORs) were evaluated as predictors of post operative recurrence in distant organs in patients with curatively resected esophageal carcinoma. Findings indicated that the combination of venous invasion and a high AgNOR score predicts distant post operative recurrences in patients with curatively resected esophageal carcinoma.[44]

Prognostic significance of argyrophilic nucleolar organizer regions in esophageal carcinoma was studied by Morita M, and the AgNOR number was found to be one of the independent and significant variables ($P<0.01$). Because the AgNOR method is simple and can be applied to paraffin-embedded sections, the AgNOR number may provide potential benefit in the pretherapeutic assessment of malignant potentiality in esophageal carcinoma.[45]

Remmerbach, in his study of diagnostic value of nucleolar organizer regions (AgNORs) found that the best cut-off value of the mean number of AgNOR dots per nucleus, distinguishing benign from malignant cells was 4.8. The percentage of nuclei with more than three AgNORs had a cut-off level of 70%. Applying these methods to doubtful or suspicious cytological diagnoses, he was able to correctly establish the diagnosis of malignancy in histologically proven cases. AgNOR-analysis may be a useful adjunct to other methods in routine cytological diagnosis of cancer that can help to solve cytologically suspicious or doubtful cases.[46]

Prathiba studied the value of AgNORs in premalignant and malignant lesions of the cervix and observed that statistically significant difference in AgNOR counts was

noted in different sets of lesions and state that this technique can serve as a useful adjunct to routine histopathology. Study of the argyrophilic nucleolar organizer regions (AgNORs), which was used extensively in cytogenetics, has been identified as a reliable indicator of cell proliferation and in turn, the malignant potential of a lesion.[47]

Xie evaluated the diagnostic and prognostic value of nucleolar organizer regions in normal epithelium, dysplasia, and squamous cell carcinoma of the oral cavity and concluded that AgNOR enumeration, in particular pAgNOR > 1, appears to be a useful tool in distinguishing between normal epithelium, dysplasia, and squamous cell carcinoma of the oral cavity. In this study, AgNOR counts were strong prognostic markers for patients with squamous cell carcinoma.[48] Pelusi in his study observed that AgNOR protein quantity of cervical smears correlates with that of histological sections in cervical intraepithelial neoplasia. AgNOR protein quantity of cervical smears actually reflects that of the cervical epithelium *in situ* and may therefore be useful for the cytological diagnosis of cervical lesions.[49]

Xu in his study on morphological typing of argyrophilic nucleolar organizer regions has observed that the diffuse type (78%) was the most frequently seen, and in benign lesions, the nucleolar type (92.85%); The difference was thus highly significant ($P<0.001$). The intranucleolar and aggregate types were not observed in benign lesions. There was no obvious difference in the proportion of the mixed type in benign and malignant lesions ($P>0.05$).[50]

Progressive increase in AgNOR counts was observed by Misra JS, when the severity of pathologic lesions increased. Statistical analysis revealed a significant

difference in AgNOR counts between normal and inflammatory smears, but it was highly significant between inflammatory and LSIL cases, between Low grade squamous intraepithelial lesions (LSIL) and High grade squamous intraepithelial lesions (HSIL), and between severe dysplasia and frank malignancy. This underscores the diagnostic importance of AgNOR counts, especially in discriminating between LSIL and HSIL of the cervix.[51]

A morphometric study of nucleolar organiser regions in cervical intraepithelial neoplasia by Terlikowski showed that the number increases with CIN level when the cells contain 4 and more AgNORs. The number of granules per 100 cells also increases with the degree of CIN. It indicates that the number of cells with 4 and more AgNOR granules can serve as a CIN differentiation exponent.[52]

Pahuja in his study of proliferative activity in squamous intraepithelial and invasive lesions aimed at assessing the proliferation in preinvasive and invasive squamous epithelial lesions of cervix using a simple and inexpensive proliferation marker observed that highest mean of AgNORs per nucleus was observed in poorly differentiated squamous cell carcinoma of cervix. He concluded that AgNORs can prove to be a simple, inexpensive and reliable proliferation marker in lesions of cervix.[53]

Kaushik studied AgNOR counts in cervical lesions. The mean AgNOR counts in cervical epithelium showed a progressive and statistically significant increase from normal to chronic cervicitis to CIN I, II and III ($P<0.001$). Scores in carcinoma also exceeded that of CIN ($P<0.05$). This can be a useful adjunct to routine histopathology to evaluate cervical lesions.[54]

PCNA immunostaining combined with AgNOR staining in esophageal squamous cell carcinoma to identify patients with a poor prognosis was done. In this analysis PCNA staining was combined with an analysis of argyrophilic nucleolar organizer region (AgNOR) staining, and a correlation with prognosis was found. The results indicated that this combined evaluation may be useful for the identification of patients with a poor prognosis among those undergoing surgery for esophageal squamous cell carcinoma.[55]

Pahuja in his study of proliferation in squamous cell carcinoma of cervix did a comparative assessment by two markers. Study was aimed at assessing the cell proliferation in various grades of squamous cell carcinoma of cervix using two proliferation markers, AgNORs and Ki-67. Highest mean number of AgNORs was found in poorly differentiated squamous cell carcinoma and statistically significant difference was observed between well and poorly differentiated SCC and between moderately and poorly differentiated SCC. Highest mean Ki-67 was seen in poorly differentiated SCC. He observed correlation between histological grade and Ki-67 and AgNORs separately but no statistically significant correlation was found between Ki-67 and AgNOR counts. AgNORs and Ki-67 are both simple and easily performed techniques. They may prove to be a useful adjunct in estimating tumor proliferation and hence in determining the management strategy of the patients.[53]

AgNOR COUNTS IN VARIOUS ORGAN DISORDERS

AgNOR studies have been done extensively in various organs disorders .Some of

the studies have documented the following results.[56-60]

I LIVER

Diagnosis	**Mean**	**± Std. Dev.**
Normal hepatocytes	1.53	0.21
Alcoholic cirrhosis	1.57	0.06
Post hepatitis cirrhosis	3.65	0.53
Hepatocellular carcinoma	7.94	1.18

II CERVIX

Diagnosis	**Mean**	**± Std. Dev.**
Normal cervix	1.36	0.23
Chronic cervicitis	2.31	0.53
C I N – I	2.56	0.69
C I N – II	4.28	1.08
C I N – III	5.16	1.64
Invasive squamous cell carcinoma	6.40	0.81

III PROSTATE

Diagnosis	**Mean**	**± Std. Dev.**
Benign Prostatic Hyperplasia	2.93	0.40
BPH with Chronic Prostatitis	3.84	0.44
Prostatic adenocarcinoma		
Grade I	4.98	0.72
Grade II	7.26	0.76
Grade III	10.98	1.64

IV GALL BLADDER

Diagnosis	**Mean**	**± Std. Dev.**

Chronic cholecystitis	1.89	0.96
Epithelial hyperplasia	3.99	1.03
Adenocarcinoma	7.65	1.32

V SOFT TISSUE TUMORS

Category	Mean	± Std. Dev.
Benign tumors group	**1.51**	**0.21**
Fibroma	1.7	0.11
Benign fibrous histicytoma	1.80	0.13
Lipoma	1.21	0.06
Leiomyoma	1.94	0.31
Haemangioma	1.79	0.12
Neurofibroma / Neurilemmoma	1.47	0.03
Malignant tumors group	**4.96**	**1.33**
Fibrosarcoma	1.70	0.11
Malignant fibrous histiocytoma	1.80	0.13
Rhabdomyosarcoma	1.21	0.06
Liposarcoma	3.12	0.58
Haemangiopericytoma	6.3	0.87
Metaplastic malignant lesions	6.46	0.92
Round cell tumor(sarcomas)	6.83	1.0
Alveolar soft part sarcoma	2.44	0.73

MATERIALS AND METHODS

121 Consecutive male patients above 25 years of age who attended the Department of Medical Gastro Enterology, Govt. Stanley Medical College and Hospital for upper GI endoscopy with complaints of dyspepsia without alarm between July 2004 and May 2006 constituted the study group. The study was undertaken after getting clearance from Ethical committee. Adequate material for study was obtained only in 79 cases as the rest were found to be either too tiny for processing or inadequate for study.

Information obtained included demographic data, occupation, literacy, socio economic status and habits like smoking, alcohol use, tobacco chewing, use of nasal snuff and use of beverages like hot tea or coffee and were noted in a pre-structured proforma and recorded by a single person. Endoscopic biopsy specimens were obtained from the anterior and posterior wall of the middle third of the esophagus. The biopsy material was fixed in formalin and the sections of 3-5 microns were obtained and were stained with routine Heamatoxylin and Eosin staining method. Necessary micro photographs were taken.

Stained sections were studied for the presence or absence of dysplasia and the dysplastic cases were further graded into mild, moderate, and severe dysplasia, based on the thickness of the dysplastic epithelium and the results were recorded. Any bias in reporting was avoided by blinding procedure.

Based on the histopathological report, cases and controls were segregated.

A case was defined as one with dysplasia on histopathology

irrespective of risk factors.

A control was defined as one with no dysplasia on histopathology irrespective of the risk factors.

After studying the H&E stained sections, a further study was done to assess the proliferative activity of the dysplastic cells by staining the sections with silver staining procedure. The silver staining for Nucleolar organizing regions was done using silver impregnation method and the AgNOR count was done by manual counting using oil immersion objective and the results were tabulated

AgNOR STAINING METHOD

1. Paraffin sections of 3-5 micron thickness were cut.
2. Sections were dewaxed in xylene.
3. Sections were immersed in distilled water.
4. Sections were then layered with 50% silver nitrate in 2% gelatin-formic acid solution and incubated in a dark room for 40 minutes at room temperature.
5. Sections were washed in distilled water, dehydrated, cleared and mounted with DPX.

Slides were studied under oil immersion objective and intranuclear silver stained dots were manually counted. Total of 100 cells were studied in each section and AgNOR score (mean number of AgNOR dots per nucleus) was calculated and the results were tabulated.

The results of H&E study and AgNOR study were tabulated and analysed statistically. Odds Ratio which indicates the strength of association between risk factors and outcome was calculated for each risk factor using standard statistical formulas and the results were analysed.

RESULTS

Endoscopic biopsies obtained from the middle third of esophagus from the study group were stained with Haematoxylin and Eosin staining and examined for the presence of dysplasia

Out of 79 biopsies studied, dysplastic changes of varying degrees were found in 40 biopsies and 39 were reported as normal. Dysplastic cases were further subtyped into mild, moderate and severe dysplasia depending on the thickness of the epithelium showing dysplastic changes.

Total number of biopsies studied	:	79
Biopsies showing dysplasia	:	40
Biopsies reported as normal	:	39
Mild dysplasia	:	21
Moderate dysplasia	:	12
Severe dysplasia	:	7

The study group was divided into cases and controls based on the results of the histopathological examination.

Cases were defined as the group of individuals exhibiting dysplastic changes in esophageal biopsy with or without exposure to the risk factors under study namely alcohol, smoking, other tobacco products and beverages.

Controls were defined as the group of individuals who do not have dysplasia of

the esophagus irrespective of the presence or absence of exposure to risk factors.

All cases and controls were analysed statistically for different variables and the following results were observed.

Number of cases exposed to risk factors:

Alcohol – 28

Smoking – 28

Alcohol and Smoking – 23

Other tobacco products – 9

Beverages – 28

Number of controls exposed to risk factors:

Alcohol – 4

Smoking – 8

Alcohol and Smoking – 3

Other tobacco products – 9

Beverages – 34

Mean age of Cases :44.64 ± 9.65

Mean age of Controls : 43.35 ± 9.70

In each category of exposure, mean age is as follows:

MEAN AGE IN CASES

Risk factor	Mean Age
Alcohol	44.10 ± 9.63
Smoking	46.35 ± 10.13

Other tobacco products	44.00 ± 10.6
Beverages	44.20 ± 9.73

MEAN AGE IN CONTROLS

Risk factor	Mean Age
Alcohol	41.20 ± 7.45
Smoking	38.75 ± 8.27
Other tobacco products	39.80 ± 9.50
Beverages	42.40 ± 9.32

Odds ratio (Cross-Product ratio) which is defined as the measure of the strength of association between risk factors and outcome was calculated in each category with the following formula:

Odds Ratio = ad / bc

a - Number of subjects with exposure and disease

b- Number of subjects with exposure and without disease

c- Number of subjects with out exposure and with disease

d- Number of subjects with out exposure and without disease

ODDS RATIO IN DIFFERENT RISK FACTORS

Risk factor	Odds Ratio
Alcohol	22.90
Smoking	10.10
Alcohol and smoking	17. 7
Other tobacco products	1.03
Beverages	0.45

From this data it is very clear that the strength of association is very significant for alcohol and smoking as individual factors and also in combination. The association with beverages and other tobacco products was not significant as the Odds Ratio was found to be less than 2.

Further, the risk factors were analysed for the duration of exposure. In each category the number of patients showing dysplasia and the duration of exposure to each risk factor was analysed and an attempt was made to ascertain if prolonged exposure has any significant increase in the occurrence of more number of dysplasias. Sub categories in each risk factor were also analysed.

ALCOHOL

Dysplasia (28) **No Dysplasia (4)**

Type of spirit	Dysplasia	No dysplasia
High spirit	26	4
Toddy / Beer	2	0
Both	0	0

Correlation of duration of exposure with dysplasia

Duration of exposure	Dysplasia	% of cases
< 5 years	5	17.5%
6-10 years	11	39.2%
>10 years	12	42.9%

Among the alcohol users when the duration was more than 5 years, risk was found to be 39.2% and with duration of more than 10 years the risk was found to be 42.9%.

SMOKING

Dysplasia (28) **No Dysplasia (8)**

Type of smoke	Dysplasia	No dysplasia
Cigarettes	10	2
Beedi	9	4
Both	9	2

Correlation of duration of exposure with dysplasia

Duration of exposure	Dysplasias	% of cases
< 5 years	5	17.8 %
6-10 years	10	35.7 %
>10 years	13	46.4 %

In smokers, when the duration was more than 5 years, risk was found to be 35.5 % and with duration of more than 10 years the risk was found to be 46.4 %.

OTHER TOBACCO PRODUCTS

Dysplasia (9) **No Dysplasia (9)**

Type of tobacco	Dysplasia	No dysplasia
Snuff	2	2
Beetel nut	7	6
Both	0	1

Correlation of duration of exposure with dysplasia

Duration of exposure	Dysplasia	% of cases
< 5 years	5	55.5 %
6-10 years	3	33.3 %
>10 years	1	11.1 %

BEVERAGES

Dysplasia (28) **No Dysplasia (34)**

Type of beverage	Dysplasia	No dysplasia
Coffee	10	8
Tea	18	24
Both	0	2

Correlation of duration of exposure with dysplasia

Duration of exposure	Dysplasia	% of cases
< 5 years	4	14.2 %
6-10 years	7	25 %
>10 years	17	60.7 %

RESULTS OF AgNOR STUDY

All the sections were stained with silver staining technique and number of AgNOR dots were counted using oil immersion objective .The results were analysed and compared with AgNOR counts of histopathologically proven esophageal squamous cell carcinomas.

The results of AgNOR study are as follows:

HPE report	Number of cases	Mean ± Std. Dev.
Normal	29	2.28 ± 0.76
Mild dysplasia	18	2.43 ± 0.39
Moderate dysplasia	15	3.0073 ± 0.63
Severe dysplasia	6	3.4885 ± 0.84
Malignancy	8	4.65 ± 0.47

AgNOR counts were found to increase with the level of dysplasia indicating increased proliferation in dysplasia and malignancy.

DISCUSSION

In India, Squamous cell carcinoma of the esophagus is the third most common malignancy in men and fourth most common malignancy in women. In men, majority of the cases occur in the 6th and 7th decades. It is commonly seen in lower socio economic group and is more prevalent in those who smoke, drink alcohol and use pan or tobacco. There is an increase in the incidence of malignancies of 2.8 and 2.5 fold, seen in smokers and tobacco chewers respectively. The risk with alcohol is found to be increased three fold, which is significantly higher than that was reported earlier from other centers in our country. A strong association is found between cancer of the esophagus and risk factors like alcohol, smoking and betel nut chewing among South Indian patients.

An attempt was made here to ascertain the association of these risk factors to the development of dysplasia in the middle third of the esophagus, the segment where carcinoma of the esophagus frequently occurs.[60] The study attempted to establish if there is an increased risk of dysplasia in individuals with these risk factors (High risk group) compared to individuals without these habits (Low risk group).

On analyzing the results it was found that the high risk group is more prone to develop dysplasia of the esophagus compared to the low risk group without these risk factors.

When the risk factors were analysed individually as well as in combination, smoking and alcohol were found to have a significant association with development of

dysplasia. This finding correlates with the previous studies implicating smoking and alcohol in the causation of squamous cell carcinoma of the esophagus. Thus the aim of trying to establish smoking and alcohol as high risk factors associated with the development of dysplasia is achieved with the results of this study, and can be a strong indicator of the susceptible population which should be subjected to vigorous screening programmes to detect early lesions.

When the dysplastic cases were studied for subtyping into mild, moderate and severe dysplasia it was observed that in the same case different areas showed different grades of dysplasia and this is a clear indication of the natural progression of increasing grades of dysplasia similar to other dysplasia - carcinoma sequences like carcinoma of the cervix. This is a very strong point which argues in favour of the need for routine screening protocols as being practiced for carcinoma of the cervix. This could lead to early intervention which may help to abort these lesions from progressing unchecked to inoperable advanced malignancy.

Some of the individuals showed dysplastic changes without history of exposure to any of the risk factors included in this study. It implies that there could be other agents or risk factors responsible for the development of dysplasia in such cases and it underscores the need for more such studies to unravel the unknown culprits.

The study of AgNORs has recently received increased attention because of claims that the frequency within the nuclei is significantly higher in malignant cells compared to normal, reactive or benign cells indicating highly increased proliferative activity in malignancies. Counting of AgNORs gives a score which is representative of

the level of proliferative activity of the sample studied. While evaluating AgNOR study, various parameters like mAgNOR, and AgNOR score are done.

Hence, AgNOR staining was done in dysplastic cases as well as normal epithelium in this study to evaluate the number of AgNOR dots and to document the degree of proliferative activity in dysplastic lesions and the results were compared with normal esophageal epithelium. It was also attempted to establish if there is any variation in the number of AgNOR dots in the different grades of dysplasia. Most of the cells showed well delineated NORs with irregular morphology dispersed within the nucleoplasm.

It was found that AgNOR counts are of definite value in grading of dysplasia and when considered along with other proliferation markers it could be of even more significance.The application of AgNOR as a potential prognostic indicator or predictor of optimal treatment protocol may require long term clinical follow up.

AgNOR can be combined with other prolifeative markers as PCNA, Ki67, or P53 which are other cell cycle associated proteins. Since increase in the AgNOR count implies increased proliferative activity which is a fertile soil for development of malignancy, those with increased AgNOR count should be considered as potential candidates for developing malignancy and these individuals should have frequent follow up.

The increase in the proliferative activity of the cells can be evaluated and the association between various grades of dysplasia and the development of overt malignancy can be documented by regular follow-up study of these individuals who can

be considered for endoscopic mucosal resection if there is any indication of progression.

On histopathological examination of the esophageal biopsies the dysplastic cases were sub typed into mild, moderate and severe and AgNOR count was done in all these categories and results were noted.

HPE report	Number of cases	Mean ± Std. Dev.
Normal	29	2.28 ± 0.76
Mild dysplasia	18	2.43 ± 0.39
Moderate dysplasia	15	3.0073 ± 0.63
Severe dysplasia	6	3.4885 ± 0.84
Malignancy	8	4.65 ± 0.47

It was observed that the difference in AgNOR values in normal epithelium and dysplasia is very significant and the AgNOR values in malignant epithelium was very much increased compared to normal epithelium. However when the values for mild moderate and severe dysplasias were compared as separate groups, the difference between normal epithelium and mild dysplasia was not marked. But moderate and severe dysplasia showed significant difference in values as compared to normal epithelium. Since mild dysplasia is not considered as a potential premalignant lesion compared to moderate and severe dysplasia this observation in mAgNOR is understandable.

The marked difference between mAgNOR of normal epithelium and malignant epithelium signifies the value of AgNORs as a proliferative marker and its utility in dysplasia as a screening tool. When it is used in combination with other proliferative markers it will definitely give very significant information about the malignant or premalignant potential of the sample studied. Specifically it can be applied to organs like cervix, esophagus, skin etc., where dysplasia has been proved as premalignant condition.

AgNOR study similar to the present study has been done in cases of cervical

dysplasia which showed the following values.

Category	Mean ± Std. Dev.
Normal Cervix	1.36 ± 0.23
Chronic cervicitis	2.31 ± 0.53
C I N - I	2.56 ± 0.69
C I N - II	4.28 ± 1.08
C I N - III	5.16 ± 1.64
Invasive squamous cell carcinoma	6.40 ± 0.81

In this AgNOR study the following values were observed:

Category	Mean ± Std. Dev
Normal esophagus	2.28 ± 0.76
Mild dysplasia	2.43 ± 0.39
Moderate dysplasia	3.0073 ± 0.63
Severe dysplasia	3.488 ± 0.84
Malignancy	4.65 ± 0.47

The AgNOR values observed in the study of esophageal dysplasia and the values observed in the study of cervical dysplasia are more or less similar,whereas the values in other organ system lesions were not comparable with this study, indicating that different organ systems may have different AgNOR counts in dysplasia as well as in malignant lesions. It could also underline the lack of standardization in AgNOR study and inter observer variations and the need for elimination of these shortcomings so as to make AgNOR study which is a simple and inexpensive procedure, a valuable proliferation marker and prognostic indicator.

SUMMARY

Study was contemplated to evaluate the risk factors for esophageal dysplasia and to assess the correlation between the duration of exposure to the risk factor and the grade of dysplasia in the high risk population.

Risk factors included in the study

Alcohol, Smoking, Other tobacco products and Beverages.

Total number of patients included in the study group : 79

Cases - Patients who showed dysplasia on H&E examinations: 40

Controls - Number of biopsies which showed no dysplasia : 39

Dysplastic biopsies were further graded into mild, moderate and severe dysplasia.

Mild dysplasia :21

Moderate dysplasia :12

Severe dysplasia:7

The results were analysed statistically and Odds Ratio was calculated for each risk factor. The results are summarised as follows:

Risk factors	Cases	Controls	Odds ratio
Alcohol	28	4	22.9
Smokers	28	8	10.1
Alcohol & smoking	23	3	17.7
Other tobacco users	9	9	1.03
Beverages	28	34	0.45

Alcohol consumption and risk of dysplasia

Duration of exposure > 5 years : 39.2 %

Duration of exposure > 10 years : 42.9 %

Smoking and risk of dysplasia

Duration of exposure > 5 years : 35.5%

Duration of exposure >10 years : 46.4%

Further the significance of AgNOR study in dysplasia of the esophagus was also evaluated.The results were analysed and compared with AgNOR counts of normal esophageal epithelium and esophageal squamous cell carcinoma.

The results of AgNOR study are as follows:

HPE report	Number of cases	Mean ± Std. Dev.
Normal	29	2.28 ± 0.76
Mild dysplasia	18	2.43 ± 0.39
Moderate dysplasia	15	3.0073 ± 0.63
Severe dysplasia	6	3.4885 ± 0.84
Malignancy	8	4.65 ± 0.47

CONCLUSION

- Study has attempted at correlation of risk factors and their duration with onset of dysplasia in the oesophagus. vailable literature does not show any similar studies.
- Alcohol and smoking (either alone or in combination), with a duration of more than 10 years is likely to predispose an individual to dysplasia.
- Conclusions on beverages needs further validation.
- Dysplasia is more prevalent in the high risk group as compared to low risk group.
- Dysplasia in persons in the low risk group may be due to other risk factors not included in the study (18%).
- AgNOR study is a simple,inexpensive and reliable proliferation marker in esophageal dysplasia.

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PROFORMA

Study of Dysplasia in Endoscopic Biopsy from Esophagus

Sl.No. Name: Age: Sex:

MGE No.

Indication for endoscopy:

Smoker: YES / NO Duration: ___years Type:Beedi / Cig/ Others
No.per day_____

Alcohol:YES / NO Duration: ___years Type:Whisky / Rum/
Beer / Arrack/Others
Quantity_____ ml/day

Tobacco: YES / NO Duration: ___ years No. of times/day:

Snuff: YES / NO Duration: ____ years No. of times/day:

Coffee: YES / NO Duration: ____years No. of cups/day:

Tea: YES / NO Duration: ____ years No. of cups/day:

Endoscopy findings:

HPE Report:

AgNOR STATISTICS				
Sl.	**Normal**		**Normal**	
No.	**Biopsy No.**	**mAgNOR**	**Biopsy No.**	**mAgNOR**
1	E-3	2.94	E-66	1.77
2	E-6	2.06	E-75	2.84
3	E-12	2.13	E-84	2.73
4	E-17	2.89	E-99	2.27
5	E-21	2.18	E-100	3.04
6	E-24	1.78	E-108	3.06
7	E-26	4.19	E-111	1.96
8	E-27	1.65	E-112	2.7
9	E-28	4.33	312	1.76
10	E-30	2.13	712	1.87
11	E-45	2.19	1576	1.65
12	E-52	1.58	1995	1.73
13	E-57	1.02	2093	1.55
14	E-58	2.67	2111	1.56
15	E-63	1.81		
	Average		2.27724	
	± Std. Dev.		0.76292	

AgNOR STATISTICS				
Sl. No.	**Mild Dysplasia**		**Moderate Dysplasia**	
	Biopsy No.	**mAgNOR**	**Biopsy No.**	**mAgNOR**
1	E-4	2.14	E-10	3.91
2	E-8	3.39	E-14	3.05
3	E-9	2.57	E-23	3.31
4	E-11	2.29	E-35	3.1
5	E-16	2.46	E-38	1.85
6	E-25	2.26	E-40	2.72
7	E-31	2.95	E-44	2.28
8	E-41	2.65	E-46	2.69
9	E-42	2.87	E-47	3.2
10	E-50	2.34	E-71	2.78
11	E-59	2.39	E-76	2.46
12	E-60	1.73	E-77	3.69
13	E-67	2.31	E-78	2.53
14	E-69	1.94	E-102	3.34
15	E-73	2.13	E-104	4.2
16	E-91	2.17		
17	E-101	2.8		
18	E-110	2.35		
	Average	2.434705882		3.0073333
	± Std. Dev.	0.40584969		0.631545346

AgNOR STATISTICS				
Sl. No.	**Severe Dysplasia**		**Malignancy**	
	Biopsy No.	**mAgNOR**	**Biopsy No.**	**mAgNOR**
1	E-51	2.48	4470	4.52
2	E-62	3.99	4480	4.24
3	E-74	2.52	350	5.09
4	E-88	2.12	4537	3.84
5	E-92	2.67	351	4.67
6	E-103	3.3	946	5.15
7	ES-1	3.93	183	5.12
8	ES-2	3.47	1099	4.55
9	ES-3	4.12		
10	ES-4	3.89		
11	ES-5	4.52		
12	ES-6	3.48		
13	ES-7	4.86		
	Average	3.488461538		4.6475
	± Std. Dev.	0.84089		0.46536468