Detection of Nucleolar Organizer Regions (NORs) as an Independent Proliferative Tumor Index

1Ibrahim El-Dosoky and 2Khaled Shahba

1Department of Pathology, Faculty of Medicine, Mansoura University, Egypt.
2Department of Zoology, Faculty of Science, Mousrata University, Libya.

Abstract: Argyrophilic nucleolar organizer regions (AgNORs) have been recently identified as a marker of proliferative index in various tumors. These were evaluated in 62 patients with breast carcinoma. AgNORs were correlated with histologic grading of infiltrating duct carcinoma. Six patients with benign breast tumors served as controls in the study. Methods: AgNORs were stained in paraffin sections of the tissues using silver stain technique. For each specimen, the number of AgNORs within the nuclei of 100 tumor cells were calculated. The average number of AgNORs per nucleus was calculated and the results expressed as mean ± S.D. Results: AgNOR count was significantly higher in breast carcinoma (3.8 ± 0.218) than in benign breast tumors (1.25 ± 0.088). Further, the AgNOR count in breast carcinoma showed a statistically significant increase as the stage of the cancer increase. Conclusions: These results indicate that breast tumors with a higher AgNOR count, even at the initial stage, have a poor prognosis and require aggressive treatment for better control of the disease. Further, it is suggested that the patients with a benign tumor and more than three AgNORs per nucleus need careful surveillance.

Key words: Breast carcinoma, Nucleolar organizer region, silver stain, AgNORs, proliferative index.

INTRODUCTION

The mitotic rate is an important characteristic of malignant tumors. A high mitotic rate is common in malignant neoplasm, whereas it is unusual for benign tumors to have a high mitotic index. In addition to the ability to separate benign from malignant tumors based on their mitotic activity, the number of mitosis within a given tumor provides a rough indication on the rate of tumor proliferation and its diagnosis (Cecilia and Fenoglio-Preiser, 1992).

NORs are loops or specific portions of DNA, called rDNA which occur in the nucleoli of cells and which are transcribed into ribosomal RNA (r-RNA) genes. These latter are transcribed by RNA polymerase, and are of vital significance in the ultimate synthesis of ribosomes and protein. Protein synthesis is a necessary step in the process of cell proliferation. Therefore a relation between NORs and cell proliferation is suggested (Bukhari et al., 2007).

The number of AgNORs and their configurations within the cells may be used as indicator of the activities of the cells (Eissa and Shoman, 1995) and (Kumar et al., 1997).

Location of the NORs is easily demonstrated by silver staining methods, showing the argyrophilic proteins (AgNORs) associated with NORs (Cromie et al., 1988).

Deregulated cell proliferation is a key factor in malignancy and therefore may be of prognostic significance silver binding argyrophilic nucleolar organizer AgNORs has been established as a valuable reflection of the tissue proliferative compartment and hence could be of value in studying the biologic behavior of malignant cells (Khanna et al., 2001).

The purpose of the present study was carried out to evaluate, if AgNORs count can act as a proliferative marker and can aid in the diagnosis and prognosis of invasive ductal breast carcinoma.

MATERIAL AND METHODS

1-Sample Collection:
A total of 62 female breast cases (excisional biopsy, quadrantectomy& mastectomy) were collected from Pathology Department Mansoura University, Egypt. Specimens were kept on ice after surgery for not longer than 2h. before delivery to the laboratory for storing in 10% formalin as a fixative till processed. Patient’s age ranging between 13 and 86 years (mean 47.54 ± 15.62).

2-Patients and Pathological Groups:
Sixty two cases of breast cancer lesions were classified into the pathological grading I, II and III according to WHO classification (WHO, 1982) and the standard methods (Bloom and Richardson, 1957).
A- Six cases were benign ductal hyperplasia (fibro-adenomas) obtained by excision biopsy.

B- Ten cases of grade (I) were infiltrating ductal carcinoma (IDC).

C- Nineteen cases of grade (II) IDC.

D- Twenty seven cases of grade (III) IDC.

3- The Methodology in the Present Study Comprised the Following:

3.1- Breast Tissue Specimens Processing:

The specimens were processed using automated tissue processor (Leica TP1020). Then specimens embedded in paraffin wax and sectioned by microtome (Olympus, USA). Sections were stained by Routine staining (Hematoxylin and Eosin). Hematoxylin, a natural dye which was first used about 1863 as a powerful nuclear stain. Counter stains for Hematoxylin with Eosin was probably the most commonly used (Luna, 1968).

3.2- Pathological Evaluation (Diagnosis) of the Specimens:

Pathologic evaluation of the specimens included both gross and microscopic examination.

Histopathologic Examination:

The tissue specimens were fixed in 10% neutral buffered (pH = 7) formalin at RT. and processed for routine paraffin embedding. Five μm sections were stained by Hx & E to be examined microscopically and to confirm the diagnosis and grading of mammary carcinoma.

Breast Cancer Grading:

1- Histological Grading:

Several systems of histologic grading have described, the most frequently used are based on those of Bloom and Richardson (1957) and Fisher and Redmond (1980), these both take into account histologic architecture (tubule formation) as well as nuclear features.

2- Nuclear Grading:

This is based on nuclear pleomorphism and mitotic activity, because no account taken of growth pattern, the system can be applied to all types of mammary carcinoma. The former method as modified by Elston and Ellis (1991) is described in Table (1).

### Table 1:

<table>
<thead>
<tr>
<th>Feature</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Tubule formation (extent within tumor)</td>
<td></td>
</tr>
<tr>
<td>• 75%</td>
<td>1</td>
</tr>
<tr>
<td>• 10% - 75%</td>
<td>2</td>
</tr>
<tr>
<td>• &lt; 10%</td>
<td>3</td>
</tr>
<tr>
<td>- Nuclear polymorphism</td>
<td></td>
</tr>
<tr>
<td>• Small regular uniform</td>
<td>1</td>
</tr>
<tr>
<td>• Moderate variation in shape and size</td>
<td>2</td>
</tr>
<tr>
<td>• Marked variation in shape and size</td>
<td>3</td>
</tr>
<tr>
<td>- Mitotic count per 10 HPF (dependent on microscopic field area)</td>
<td></td>
</tr>
<tr>
<td>Field diameter 0.59 diameter 10.274 mm² area</td>
<td></td>
</tr>
<tr>
<td>• 0 – 9</td>
<td>1</td>
</tr>
<tr>
<td>• 10 – 19</td>
<td>2</td>
</tr>
<tr>
<td>• &gt; 20</td>
<td>3</td>
</tr>
<tr>
<td>Field diameter 0.44 mm diameter 10.152 mm² area</td>
<td></td>
</tr>
<tr>
<td>• 0 – 5</td>
<td>1</td>
</tr>
<tr>
<td>• 6 – 10</td>
<td>2</td>
</tr>
<tr>
<td>• &gt;11</td>
<td>3</td>
</tr>
</tbody>
</table>

Total score: 3–5 = grade (I), well differentiated, 6 – 7 grade (II), moderately differentiated, 8 – 9 grade (III), poorly differentiated.

3.3- Staining of NORs as a Proliferative Tumor Marker in Breast Cancer:

A sensitive staining method of NORs was described by Croker et al. (1989). By using the silver nucleolar organizer region (AgNOR) impregnation technique the number, size and shape of NORs can be studied in a fast and simple way, not only in fresh frozen tissue specimens but also in formalin fixed paraffin embedded material. The amount of silver deposit in a cell, reflecting the amount of NORs that are involved in protein-synthesis, is thought to be related to the proliferative capacity of that cell. The exact relationship between proliferation, protein-synthesis and expression of AgNORs is, however, not yet well understood. But the expression of AgNOR is either causally or indirectly coupled to DNA-synthesis and thus AgNOR can be considered as a cell proliferation marker (Bukhari et al., 2007).
**Counting Pattern and Procedure:**

NORs represent the distribution of small true AgNORs throughout the nucleoplasm as a frequently observed in high malignant cells. The brown AgNORs dots were counted at a magnification of x1000 (oil emersion), using a color filter (such as green) since this reduces chromatic aberration and increases the clarity of AgNOR perimeters (Croker and Egan, 1988). One hundred cells were selected randomly from each specimen for NORs counting. All visible AgNORs dots were counted individually by careful focusing (Croker et al., 1989). AgNORs are seen as dark dots within the cell nuclei. It is counted by enumerating these dots and the mean number is calculated. The number of individual and separable black dots are only counted.

**IV-Results:**

The present study was conducted on 62 female patient suffering from breast disease. Breast tissue specimens were processed for diagnostic purposes by Hematoxyline and Eosin (Hx&E) staining. Breast carcinoma was classified into two major divisions are carcinoma in situ and infiltrating carcinoma each of which can be of either lobular or ductal type. Characteristic of all subjected cases were listed in Table (1).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Number of patients</td>
<td>62</td>
</tr>
<tr>
<td>- Age (mean)</td>
<td>47.5±13.6</td>
</tr>
<tr>
<td>- Histopathology (classification &amp; grading):</td>
<td></td>
</tr>
<tr>
<td>• Fibroadenoma</td>
<td></td>
</tr>
<tr>
<td>• IDC*</td>
<td></td>
</tr>
<tr>
<td>Grade (I)</td>
<td></td>
</tr>
<tr>
<td>Grade (II)</td>
<td></td>
</tr>
<tr>
<td>Grade (III)</td>
<td></td>
</tr>
<tr>
<td>• ILC**</td>
<td></td>
</tr>
<tr>
<td>Grade (I)</td>
<td></td>
</tr>
</tbody>
</table>

* = Infiltrating Duct Carcinoma. ** = Infiltrating lobular Carcinoma.

Plate 1: Revealed (ILC) grade (I): (A) Showing tumor cells arranged in cords with Indian file arrangement. They are separated by demo- plastic stroma (Hx & E, x 100), (B) Showing (ILC) grade (I) with tumor tissue arranged in cords of and rows of malignant small darkly stained cells. The tumor cells are condensed by demoplastic stroma forming Indian file arrangement (Hx & E, x200).

The higher AgNORs counts reported in malignancy are attributed to:

- Absolute or real increase in AgNORs due to increase cell ploidy.
- Increased transcription activity and nucleolar dispersion resulting in easier counting of individual AgNORs (Fakan and Hernandez-Verdum, 1986).

In this work, AgNORs were applied in paraffin section of female breast tissue specimens using Croker and Egan (1988) assay giving brown or/dark brown color in the nuclei as shown in the Plates (4A,B,C & D).

Silver binding nucleolar organizer regions (AgNORs) were counted in tissue sections and were shown to assist in the distinction between benign and malignant breast lesions (Rzymowska, 1997).

For each specimens, the number of AgNORs within the nuclei of one hundred tumor cells were calculated. The over number of AgNORs pre-nucleus was calculated and the results expressed as mean ± standard deviation (S.D.).
In this study we found that AgNORs count was significantly higher in breast carcinomas (3.83±0.393) than in benign breast cases (1.25±0.088). Further, the AgNORs count in breast carcinoma showed a significant increase in correlation with the increase in the grade of cancer, as shown in the Table (2).

Plate 2: Revealed IDC grade (II):(A) Showing variable sized sheets of malignant rounded cells with occasional acinar forms separated by marked degree desmoplasia with good lymphocytic infiltration. There is moderate nuclear pleomorphism and scarce mitosis (Hx & E, x100), (B) Showing IDC grade (II), with marked inflammatory cell in the stroma (Medullary carcinoma), (Hx & E, x200).

Plate 3: Revealed (A) IDC grade (III), with tumor tissue formed of malignant spheroidal cells exhibiting high anaplasia. No tendency at acinar formation (Hx & E, x400). (B) IDC grade (III): Showing tumor cells with highly anaplastic features cells arranged in irregular sheets and cords (Hx & E, x400).

Table 2: Illustrates the AgNORs counts in both benign and malignant grades of breast carcinoma.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of cases (n = 62)</th>
<th>Mean ±SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibro-adenoma</td>
<td>6</td>
<td>1.25±0.088</td>
</tr>
<tr>
<td>Grade (I)</td>
<td>10</td>
<td>3.21±0.073</td>
</tr>
<tr>
<td>Grade (II)</td>
<td>19</td>
<td>3.42±0.186</td>
</tr>
<tr>
<td>Grade (III)</td>
<td>27</td>
<td>4.20±0.393</td>
</tr>
</tbody>
</table>

*SD = Standard deviation.

- This table represents the mean ± SD of AgNORs count in both benign and malignant tumor cases of a total 62 female breast specimens.
- Table (2), showing that the average of AgNORs count of both benign and malignant tumor cases are (1.25±0.088) and (3.8±0.218) respectively (p < 0.0001) extremely significant.
- Also we can note that the direct proportional relationship between the mean count of AgNORs and histological grading.

The direct proportional relationship between the histopathology of different groups (benign & grades I, II, III) and their AgNORs average count can be observed also in the figure (1).
Also we were observed the morphological changes in the AgNORs as well as reduction of the AgNORs count and AgNORs aggregation and forming a single large spherical AgNORs. We found about 37% of AgNORs in the low grade cancer cases were aggregated to one large spherical figure while about 26% of AgNORs in higher grade cases were aggregated.

**Plate 4:** (A) Revealed NORs staining of IDC grade (I) (x1000). (B): Revealed NORs staining of IDC grade (II) Note. The aggregated AgNORs (x1000). (C): Revealed NORs staining of IDC grade (III) (x1000). (D): Revealed the nuclear organizer regions (NORs) staining of fibroadenoma case (x1000).

**Fig. 1:** Show the relationship between histopathology of different groups and their mean count of AgNORs.

The statistical test (Turkey-Kramer multiple comparisons test) results were illustrated in Table (3), for evaluating the significance of the AgNORs count in both benign and malignant breast cancer. The comparison was carried out between each histological group and the other one as follows.
a) Fibroadenoma as an example of commonest benign of the breast disease versus grade (I) malignant tumor breast cancer we found that (q) value is 15.24 and (p value is < 0.0001). Then the relationship between fibroadenoma group and breast cancer grade(II) is extremely significant.
b) Fibroadenoma versus grade (II) malignant tumor breast cancer we found that (q) value is 17.849 and (p value is < 0.0001). Then the relationship between fibroadenoma AgNORs count and breast cancer grade (II) AgNORs count is extremely significant.
c) Fibroadenoma versus grade (III) malignant tumor breast cancer we found that (q) value is 27.66 and (p value is < 0.0001). Then the relationship between AgNORs mean count in both fibroadenoma group and breast cancer grade (III) is extremely significant.
d) Grade (I) versus grade (II), we found that (q) value is 1.198 and (p) value is 0.1246 (p > 0.05). Then the relationship between AgNORs average count in both breast cancer grade (I) and grade (II) is not significant.
e) Grade (I) versus grade (III), we found that (q) value is 7.542 and (p value is 0.0002). Then the relationship between AgNORs mean count in both breast cancer grade (I) and grade (III) is extremely significant.
f) Grade (II) versus grade (III), we found that (q) value is 6.985 and (p value is 0.0003). Then the relationship between AgNORs mean count in both breast cancer grade (II) and grade (III) is extremely significant.

Table 3: Illustrates the comparison between AgNORs of different histological groups and the other one.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Mean difference</th>
<th>t value</th>
<th>q value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign vs. GI</td>
<td>-1.963</td>
<td>39.628</td>
<td>15.241</td>
<td>*** p &lt; 0.001</td>
</tr>
<tr>
<td>Benign vs. GII</td>
<td>-2.112</td>
<td>25.279</td>
<td>17.849</td>
<td>*** p&lt; 0.001</td>
</tr>
<tr>
<td>Benign vs. GIII</td>
<td>-2.774</td>
<td>16.908</td>
<td>27.665</td>
<td>*** p &lt; 0.001</td>
</tr>
<tr>
<td>GI vs. GII</td>
<td>-0.149</td>
<td>1.677</td>
<td>1.198</td>
<td>ns p &gt; 0.05</td>
</tr>
<tr>
<td>GI vs. GIII</td>
<td>-0.810</td>
<td>4.518</td>
<td>7.542</td>
<td>*** p &lt; 0.001</td>
</tr>
<tr>
<td>GII vs. GIII</td>
<td>-0.661</td>
<td>4.231</td>
<td>6.985</td>
<td>*** p &lt; 0.001</td>
</tr>
</tbody>
</table>

If the value of (q) is greater than 3.834 then (p) value is less than 0.05 (p value is significant).
- *** = p value considered extremely significant.
- ns = p value considered extremely not significant.
- G = grade.

V- Discussion:
Breast cancer is the leading cause of death among solid tumours in women, and its incidence is increasing all over the world. Adjuvant chemotherapy and hormonal treatment improve survival but have potentially serious side effects, and are costly. Because adjuvant treatment should be given to high risk patients only, and traditional prognostic factors (lymph node status, tumour size) are insufficiently accurate, better predictors of high risk and treatment response are needed. Many breast cancer prognosticators are directly or indirectly related to proliferation. Although studies evaluating the role of individual proliferation markers like as AgNORs which have greatly increased our knowledge of this complex process, the functional end result cells dividing has remained the most important prognostic factor (van Diest et al., 2004).

In the present study we detected the proliferation activity in the female breast cancer specimens by investigation activity of AgNORs which was expressed as a brown dots after staining by silver stain technique, these dots can be counted and then the proliferation activity can be evaluated.

One of the most recent methods to determine the proliferative activity as a marker in various tumors as well as the breast cancer is silver-staining nucleolar organizer region (AgNOR) staining (Saluia and Vandan, 2001). Khanna et al. (2001), reported that, AgNORs count was significantly higher in breast carcinoma (6.61±1.75) than in benign breast cancer (1.88±0.19). Furthermore AgNORs count in breast carcinoma showed a statistically significant increase in correlation with the increase in the size of tumor, stage of the cancer, tumor recurrence at various sites and negative expression of hormonal receptors.

In the present work, AgNORs were evaluated in 62 female breast cancer formalin-fixed paraffin-embedded tissues, we found that, AgNORs average count was significantly higher in invasive breast cancer cases (3.83±0.393) than in benign cases (1.25 ± 0.088) (p < 0.0001). Furthermore the AgNORs count in this study showed a statistically significant increase in correlation with the increase in the tumor stage, i.e. AgNORs count in tumors grades subjected in this study I, II and III were (3.21±0.073), (3.42±0.186) and (4.2±0.393) respectively (direct proportional relationship between AgNORs main count and the histological grades), this result was supported by various other studies. From the above obtained results in this work we reported that, AgNORs shown to assist in the distinction between benign and malignant breast cancer and AgNORs were correlated with various parameters as tumor grading.

Dasgupta et al. (1997) reported that, in fibrocystic disease the main AgNORs count was (1.60), in fibroadenoma it was (1.61). The main count in carcinoma was (12.0). The differences in AgNORs count in fibrocystic and fibroadenoma was not significant, but that between benign breast lesion and carcinoma was significant. Thus the simple AgNORs staining technique can be used as an additional criterion to differentiate the benign and malignant lesions of breast.
Also in the current study we observed that, the differences in AgNORs count in fibroadenoma versus malignant tumor of breast cancer (grades I, II and III) is extremely significant ($P<0.001$). Also, the relationship between AgNORs count in both breast cancer grade (I) versus grade (III) and grade (II) versus grade (III) are extremely significance ($p < 0.001$), whereas, the differences in AgNORs count in breast cancer grade (I) versus grade (II) is not significant ($p>0.05$). This result supported by other studies and confirmed the fact of the AgNORs as a proliferative index can be used for discrimination between the malignant and benign lesions of female breast (Mijović et al., 2006) and (Ceccarelli et al., 2000).

Rzymowska (1997) reported that the main AgNORs counts in fibroadenomas were $1.05\pm0.85$, in lobular carcinoma $3.55\pm0.56$ and in intraductal carcinoma $4.83\pm1.2$. AgNORs were shown to assist in the distinction between benign and malignant breast lesions.

Ceccarelli et al. (2000) reported that the quantitative distribution of AgNORs protein is a proliferation-related parameter that can be used as prognostic index in tumor pathology. In breast cancer some authors found a significant prognostic correlation of AgNORs protein quantity.

Treatment with RNA polymerase inhibitor (actinomycin D) reduced the size of AgNORs. Messenger RNA polymerase inhibitor (alpha-amanitin) also increased AgNORs. However translation-blocking agents closely related to ribosomal RNA (cycloheximide and anisomycin) caused a decrease in the number of AgNORs, which seemed to fuse to an aggregated around the nucleolus and formed single large spherical AgNORs in the final stage. These changes were observed typically when cells were treated with fluorouracil or 5-fluorouridine. This morphological changes in the AgNORs pattern, AgNORs aggregation, might reflect certain damage in ribosomal RNA (Ofner et al.,1996).

The current study observed the morphological changes in the AgNORs as well as reduction of the AgNORs count and AgNORs aggregation and forming a single large spherical AgNORs, as a response for chemotherapy. We found About 37% of AgNORs in primary cancer grade were aggregated to one large spherical figure while about 26% of AgNORs in higher grade cases were aggregated.

Furuya et al. (1997) reported that typical 5-fluorouracil induced AgNORs aggregation (FFA) was observed in cases with breast cancer tissue. In these cases, AgNORs were aggregated to one large spherical figure in more than 39% of tumor cells.

Conclusion:

AgNORs seem to reflect proliferation independent cellular and nuclear activity of tumor cells as a proliferative index in various tumors. AgNORs count even at the initial stage has a poor prognosis and require aggressive treatment for better control of the disease.

In this study we observed that AgNORs staining assay could discriminated significantly between low grades (I) & (II) and high-grade (III), also between benign and malignant cases. We recommend for using it as routine specific stain as Hx&E stains for using in breast cancer diagnosis.

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