The role of determining the nucleolar organizers in assessing the evolution of neoplasms

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Abstract

Nucleolar organizer regions are parts of chromosomes that code ribosomal RNA and correlate to cellular proliferation. They have been identified by means of an argyrophilic technique that reveals NORs as black dots in the nuclei. The method has been recently applied in some malignancies, as lymphoma or melanoma. Only few investigations on hormonal-dependent tumours have been published. Our aim was to investigate on the total number of AgNORs and cell type of endometrial tumors.

We studied 21 cases of formalin fixed paraffin embedded endometrial tumors including hyperplasia. The number of AgNORs was counted in both epithelial and stromal components. Results: A total of 13 cases were benign and 8 malignant lesions. Total number of AgNORs was found to correlate with mitotic counts both in benign lesions and malignancies. The mean number of AgNORs increased from 2.79 in simple endometrial hyperplasia to 10.0 in adenocarcinoma.

Our data indicate that the AgNORs visualization can assist to differentiate between different malignancy grades of endometrial proliferations, especially between complex endometrial hyperplasia and atypical hyperplasia as well as endometrial adenocarcinomas.

Keywords: Endometrial Hyperplasia, Endometrial Adenocarcinoma, AgNOR, Mitotic count.

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Introduction

The nucleolar organizer regions have been introduced as an appreciation method on the evolution and prognosis of proliferative lesions. The method was mentioned by Ploton in 1986 [1] and Crocker in 1988 [2]. Several studies have been reported in literature regarding the utility of AgNOR count as a prognostic marker in different tumors, as astrocytomas [3], gliomas, meningiomas [4], laryngeal cancers [5], gastric endocrine tumors [6] or lymphomas [1].

In hormonal-depending tumors the AgNOR method has been used in diagnosis and as a predictive factor for patient outcome in uterine sarcoma [7], cervical adenocarcinoma[8,9] or in prostate adenocarcinoma [10]. Trere et al. consider that “the quantity of AgNOR protein represents an independent marker with highly significant predictive value in numerous human tumors [10].

Winzer finds a strong correlation between the overall survival in 244 patients with breast cancer and proliferation markers, as DNA content, proliferation index and selected AgNOR parameters; that could replace the conventional tumoral grading [11].

The nucleolar organizer regions are parts of chromosomes that code ribosomal RNA and are arranged on specific DNA loops. They are the result of an argyrophilic reaction of the nucleolar region [12]. There are few data on nucleolar organizers (AgNORs) in endometrial proliferations and the individual studies on the AgNOR count and distribution are very controversial [12, 13].

The principle of the method

On the usual chromosomal preparations one could notice secondary restrictions or gaps. They appear in the electronic microscopy under the form of some pale regions, poorly delimited within some electron-dense areas, and in the optical microscopy, the Giemsa stain, they look like some clear areas.

These areas hybridized with ribosomal RNA are located on the short arms of five human acrocentric chromosomes: 13, 14, 15, 21, 22 and present DNA under an extended form. These regions, also known as NORs (nucleolar organizer regions) and NOR antigens (AgNORs), can be distinguished by coupling the proteins associated with the silver ions. The usuriousness of these proteins (NOR-associated proteins) is due to the uptake of Ag+ ions by the carbonyl groups, disulphide, and sulfhydryl groups. If the histone proteins are removed from the chromosomes or from the nucleuses, the argyrophilic sites remain, proving that the argentie reaction is independent of the histone [14].
Material and methods

The argentinc method of distinguishing the NORs is simple, reproductive and remarkably specified. It included three sequential stages:

- Silver nitrate solution – 50g
- ammoniacal silver solution – 40g (silver nitrate in aqueous ammonium hydroxide with pH=12-13)
- The development by using a formaldehyde solution 3%, adjusted to a pH=7 with sodium acetate, then adjusted at pH=5-6 with formic acid.

In order to obtain a correct prognostic evaluation, we decided to follow the cell proliferative activity in different types of endometrial hyperplasia, as well as in the endometrial adenocarcinoma, using both the 1994 WHO classification of endometrial hyperplasia and the new 2015 WHO classification [15](Table 1).

<table>
<thead>
<tr>
<th>Types of lesions</th>
<th>Number of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WHO classification 2013</strong></td>
<td></td>
</tr>
<tr>
<td>Hyperplasia without atypia</td>
<td></td>
</tr>
<tr>
<td>Simple endometrial hyperplasia</td>
<td>4</td>
</tr>
<tr>
<td>Complex endometrial hyperplasia without cytological atypia</td>
<td>4</td>
</tr>
<tr>
<td><strong>WHO classification 1994</strong></td>
<td></td>
</tr>
<tr>
<td>Complex endometrial hyperplasia without cytological atypia</td>
<td>4</td>
</tr>
<tr>
<td>Atypical hyperplasia/endometrial intraepithelial neoplasia EIN</td>
<td>8</td>
</tr>
<tr>
<td>Complex endometrial hyperplasia with cytological atypia</td>
<td>6</td>
</tr>
<tr>
<td>Atypical endometrial hyperplasia</td>
<td>5</td>
</tr>
<tr>
<td>Endometrial invasive neoplasia</td>
<td></td>
</tr>
<tr>
<td>Well differentiated endometrial adenocarcinoma</td>
<td>4</td>
</tr>
<tr>
<td>Moderately differentiated endometrial adenocarcinoma</td>
<td>2</td>
</tr>
<tr>
<td>Poorly differentiated endometrial adenocarcinoma</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 1. Case distribution according to the histopathological type.
Using Ploton’s method (the argyrophilic technique) we obtained positive reactions in all the cases characterized by the appearance of some intranuclear black dots [16].

The Issues encountered were grouped into 4 classes:

- high and round solitary structures, suggesting a nucleolus
- numerous small dots, within a big and round structure
- a cluster of small dots, not delimited by an argyrophilic structure
- small, black and extranuclear dots.

100 cells have been examined for each case, using x100 (with immersion). The evaluation of the AgNORs was made using the formula:

\[ N_{\text{AgNORs}} = \text{sum of AgNORs/cell number}. \]

A total of 19 cases of endometrial hyperplasia have been studied and 8 cases of endometrial adenocarcinoma.

Results

The 19 cases of endometrial hyperplasia included (following WHO classification 1994 [15]): 4 cases of simple endometrial hyperplasia, in which the AgNORs were intraepithelial distributed in 2 cases (Fig. 1), whereas in the other two cases of simple hyperplasia, the AgNORs distribution was both intraepithelial as well as in the stroma (Fig.2, Fig. 3).

Figure 1. Simple endometrial hyperplasia with an intraepithelial and stromal distribution of the AgNORs.

Figure 2. Simple endometrial hyperplasia with intraepithelial AgNORs.
Only 2 cases out of 4 investigated cases of complex endometrial hyperplasia and without detectable cytological atypias presented with intraepithelial AgNORs (Fig. 4), herein one case with pseudostratification and of intraluminal papillary proliferation (Fig. 6); the 2 additional case presented with both intraepithelial and stromal distribution (Fig. 5).

The third investigated group includes 6 cases of complex endometrial hyperplasia, with cytological atypia. In 2/6 cases, the AgNORs were distributed in both the epithelium and the stroma (Fig. 7) whereas the remaining 4 cases displayed with a stromal AgNORs distribution (Fig. 8).

The forth investigated group comprises of 5 cases of atypical endometrial hyperplasia. Herein, the histopathological aspect was a “back-to-back” appearance. Consecutively, AgNORs could be noted in the atypical epithelial cells (Fig. 9).

The fifth investigated group consists of 8 cases of endometrial carcinomas. They were graded into 4 well differentiated cases. The AgNORs were unequally distributed in the tumor cells and in the stroma (Fig. 10).
Two additional endometrial carcinomas were moderately differentiated. Their distribution of AgNORs was intraepithelial (Fig. 11).

The last subgroup comprised 2 cases of poorly differentiated endometrial carcinoma in which an intraepithelial and stromal AgNOR distribution noted (Fig. 12).
Discussion

Niwa (1991) used the AgNOR staining in different endometrial lesions and in normal endometrium [17]. He has found a significantly higher mean AgNOR count in well-differentiated endometrial carcinoma (5.5) than in hyperplastic lesions without atypia (3.6). Miller [18] considers AgNOR staining as a valuable method to identify the high-risk patients for recurrences in endometrial carcinoma.

The lack of a standardised silver-staining protocol has led to a lot of misinterpretations in the early ‘90. In 1993, in Berlin, the International Committee on AgNOR Quantitation defined the guidelines for AgNOR protein evaluation [19].

Further studies were performed on endometrial lesions using different AgNOR parameters, as mean AgNOR count or mean AgNOR area value. The results pointed out
a significantly increased value for AgNOR area from normal proliferative to hyperplastic and well differentiated carcinoma [20], but still controversial in respect to a significant difference between atypical hyperplasia and adenocarcinoma [21]. Brustmann suggest in his study that AgNOR counts are reliable markers of endometrial proliferation and allow a clear distinction between benign, premalignant and malignant epithelial changes [22]. Miller, in a study on AgNORs counts in 35 cases of uterine cervical adenocarcinomas do not find any significant correlation with tumoral stage and size, but with tumoral grading [8].

![Figure 13. Comparative values for AgNORs in different endometrial lesions.](image)

Our studies have revealed a significant increase in the values of the AgNORs from the simple to the complex lesions of the hyperplasia, with or without atypias, the highest values increasing within the endometrial adenocarcinomas, especially in the not differential ones. The AgNORs value distribution of the analysed groups is presented in Fig.13 and Table 2.

<table>
<thead>
<tr>
<th>Types of lesions</th>
<th>No. of cases</th>
<th>AgNORs Average</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple endometrial hyperplasia</td>
<td>4</td>
<td>2,79</td>
<td>0,38</td>
</tr>
<tr>
<td>Complex endometrial hyperplasia without cytological atypias</td>
<td>4</td>
<td>3,43</td>
<td>0,38</td>
</tr>
<tr>
<td>Complex endometrial hyperplasia with cytological atypias</td>
<td>6</td>
<td>4,17</td>
<td>0,40</td>
</tr>
</tbody>
</table>
Table 2. AgNORs values in different histopathological types of endometrial lesions ((WHO classification 1994).

<table>
<thead>
<tr>
<th>Lesion</th>
<th>AgNORs Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atypical endometrial hyperplasia</td>
<td>5</td>
</tr>
<tr>
<td>Endometrial adenocarcinoma</td>
<td>8</td>
</tr>
</tbody>
</table>

Although this technique is simple, fast and requires reagents that are accessible in everyday practice, people rarely use the information obtained through this method. If we could access the automated quantification, the use of this research technique would prove valuable.

At the same time, it outlines the prerequisites for a better understanding of the evolution of endometrial borderline lesions and their risk of malignization.

Due to the fact that in recurrent lesions there is an increased number of AgNORs in comparison with the primary lesions, it is possible to identify the risks of relapse. Our research on AgNORs highlights the fact that different degrees of hyperplasia can be differentiated on morphological features. The results are therefore in concordance with other similar studies in literature on larger groups of cases [23].

**In conclusion**, the AgNOR staining method is simple and reproducible and represents a significant marker of endometrial proliferation. The AgNOR count allows us to distinguish between different proliferative endometrial lesions and to identify lesions with high-risk of malignization. We consider it a useful tool for a much objective differentiation between the benign, premalignant and malignant endometrial changes.

**References**


11. Winzer JW, Bellach J, Hufnagl P, Long-term analysis to objectify the tumour grading by means of automated microscopic image analysis of the nucleolar organizer regions (AgNORs) in the case of breast carcinoma, Diagnostic Pathology, 2013, 8:56.


