The effect of delayed fixation on the demonstration of AgNORs adenocarcinoma

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ABSTRACT
This paper studies the significance of a modified nucleolar organizer region silver stain on neoplastic tissue using paraffin and cryostat sections in order to determine the effects of delayed fixation. **Settings**: The study was conducted in the Riyadh Al Kharj Armed Forces Hospital Pathology Department, Kingdom of Saudi Arabia. **Design**: The study involves the staining of paraffin and cryostat sections with a modified silver stain. **Results**: Tissue that had been left unfixed for more than thirty minutes was not suitable for AgNOR demonstration. Cryostat sections and tumor tissue that had been fixed immediately gave excellent and comparable results. A modification of the AgNOR technique appears to be an improvement on the original method. **Conclusion**: A delay in fixation of adenocarcinomas for more than thirty minutes results in unsatisfactory AgNOR demonstration.

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**Keywords**: Nucleolar organizer regions, AgNOR method, fixation, frozen sections.

Interest is increasing in the application of the AgNOR technique for the demonstration of AgNOR associated proteins in neoplastic tissues. Recent studies have shown that AgNOR counts are a diagnostic and prognostic discriminant in certain in certain tumors. Changes in AgNOR areas have also been shown to be important in the assessment of malignant lesions. AgNOR area may be measured using a Kontron Vidas automatic image analysis system. The instrument uses the segmentation and measurement of a digitized image. In order to provide this instrument with accurate information it is important to demonstrate AgNORs with a high density of staining and minimal background. The current methodology which is relatively new to paraffin embedded tissues has been reported to give results of inconsistent quality. Documentation of the effect of any delay in fixation is therefore essential in order to standardize the technique. It is also important to compare qualitatively, frozen and paraffin sections. Two modifications to the original staining method are described which can improve the quality of the results with frozen and paraffin sections.

**Materials and methods.** Samples, 0.8cm in length, of six colonic adenocarcinomas were used for the purpose of this study. For the assessment of the effect of delayed fixation on AgNOR staining, samples of tumor tissue were left in air for the following times: thirty minutes, one hour, three hours, six hours, twelve hours, twenty-four hours and forty-eight hours. Samples were also fixed immediately. The tissue blocks were fixed in 10% formol saline for twenty-four hours. After fixation the tissues were processed on a Miles Scientific V.I.P. tissue processor. The tissues were embedded in paraplast and 3 μm sections were cut on a Leitz rotary microtome. The sections were then dried for three hours in a 60°C incubator. The paraffin sections were dewaxed in xylene and hydrated in deionized water. Cryostat sections (5μm) were cut on a Bright cryostat. The sections were then placed for one minute in 10% formol saline. They were then washed in deionized water. The sections were stored in deionized water prior to AgNOR staining. The slides were reviewed by all of the authors. They were then scored blind by two of the authors under two headings - intensity of staining and lack of background. The results were then averaged. A high score under the first heading (intensity of staining) demonstrated good AgNOR intensity. A high score under the second heading (lack of background stain) demonstrated minimal background staining. A score of 20 indicated good AgNOR intensity and no background staining. There were no significant differences between inter-observer scores.

**Modified AgNOR staining technique.**

1. Paraffin sections. Sections were taken to water and

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then placed in three changes of deionized water (two minutes per change). They were then immersed in absolute alcohol for thirty minutes. The sections were then washed in deionized water and placed in the staining solution for thirty five minutes at 20°C. The staining solution consisted of a filtered 50% silver nitrate solution (2 parts) and 1% fresh formic acid (90% solution) in 2% gelatin (1 part). The sections were then thoroughly washed in deionized water and placed in a Coplin jar of 5% sodium thiosulphate for 5 minutes. The sections were then washed in deionized water and finally dehydrated, cleared and mounted. The AgNORs are seen as brown-black dots within the nucleolus. The staining solution was similar to the solution prepared for Howell and Black with two exceptions. The standard technique did not use the pre-alcohol bath prior to staining or the sodium thiosulphate bath post staining. (2) Following extensive washing in deionized water the sections were placed in the staining solution and treated as for paraffin sections.

Results. Qualitative assessment of paraffin and cryostat sections. The results for this assessment are shown in Table 1. They show that cryostat sections fixed in 10% formol saline prior to staining show comparable results to paraffin sections that have been subjected to immediate fixation. However, the cryostat sections did not have any background staining unlike the paraffin sections. The paraffin and cryostat sections were stained together with the same silver staining solution. Hematoxylin and eosin sections were prepared of all test samples for identification of the tumor tissue in colorectal carcinoma (Fig 1). The modified staining technique carried out on a cryostat section is shown in (Fig. 2).

Qualitative assessment of AgNOR following a delay in fixation. The results for the effect of delay in fixation are also shown in Table 1. Optimum AgNOR staining results were obtained using cryostat sections that were fixed in 10% formalin for one minute prior to staining (Fig. 3). Optimum AgNOR results were also seen with paraffin sections of tissues subjected to no more than a thirty minute delay in fixation (Fig. 4). There was a highly significant decline in AgNOR intensity after a sixty minute fixation delay. This manifested itself as blurred AgNORs with indistinct borders. It was not possible to identify distinct AgNORs following a twenty four hour fixation delay (Fig. 5).

Discussion. Nucleolar organizer regions are loops of ribosomal DNA (rDNA) which contain the genes that transcribe to ribosomal RNA (rRNA). Nucleolar organizer regions reside on the short arm of the acrocentric human chromosomes 13,14,15,21 and 22 and have been identified using in situ hybridization with radiolabelled rRNA. The nucleolar organizer regions can also be demonstrated by the argyrophilia of their associated proteins. It is only transcriptionally active nucleolar organizer regions that are argyrophilic. AgNORs, which are visualized
as black dots, are an integral part of the nucleolus. The main application of the AgNOR technique is the assessment of the AgNOR numbers and AgNOR areas in neoplastic tissue. There is a hypothesis that in certain tumors there is a direct correlation between the degree of malignancy and the number of AgNORS. More recent studies have shown that in certain tumors AgNOR area measurements differentiate benign and malignant lesions more accurately than simple counting. The AgNOR expression has also been used recently as a prognostic indicator in certain neoplastic lesions. There have also been conflicting reports on the prognostic value of AgNORS on the same lesions. Negative studies indicating no value for AgNORS as a prognostic indicator on breast lesions were reported by Sacks et al. Positive studies indicating value as a prognostic indicator on breast lesions were published by Herir et al.

The results of our study have shown clearly that immediate or prompt fixation of tissues is essential for AgNOR demonstration. Frozen sections compared favorably with paraffin sections. Tissues left unfixed for periods of more than thirty minutes are not suitable for AgNOR assessment. In the present study the modification of the AgNOR technique of including a pre-alcohol bath before staining appeared to give consistently good AgNOR staining. This may be because the protein chains turn outwards after treatment with alcohol. This makes the sulphydryl and disulphide groups more available to the silver ions. The fact that an alcohol pre-staining bath improves AgNOR intensity highlights the fact that alcohol based fixatives are not an essential prerequisite for optimal AgNOR demonstration. In the present study the use of a 5% sodium thiosulphate post-staining bath maintained the intensity of the AgNOR staining.

Table 1 - Results of the assessment of paraffin and cryostat sections

<table>
<thead>
<tr>
<th>Results</th>
<th>Frozen Sections</th>
<th>Paraffin sections</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Delay in fixation (hours)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>AgNOR intensity</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Lack of Background</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Total score</td>
<td>20</td>
<td>17</td>
</tr>
</tbody>
</table>

A high score (20) indicates good AgNOR intensity and no background staining.

References


10. Sacks NP, Robertson JF, Ellis IO, Nicholson RI, Crocker J, Blamey RW. Silver-stained nucleolar organizer region counts are of no prognostic value in primary breast cancer.
