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Prognostic value of nucleolar organiser regions (AgNORs) in laryngeal cancer

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Key words. Laryngeal cancer; AgNORs; prognosis

Abstract. Prognostic value of nucleolar organiser regions (AgNORs) in laryngeal cancer. The argyrophilic nucleolar organizer regions (AgNORs) of 154 biopsy specimens of laryngeal squamous cell carcinoma were examined. The silver colloid staining technique was used. For the control, normal laryngeal epithelium obtained from 25 patients with benign lesions of the larynx was examined. There was significant difference in mean AgNORs count between control group and cancer patients. No significant correlation was found between AgNORs count and patients' age and sex, tumour site, T stage and lymph node metastasis. There was a significant correlation between AgNORs number per cell and tumour histological grading. The mean number of AgNORs per nucleus was $3.85 \pm 2.09$ in patients with poor and $2.47 \pm 0.71$ in patients with satisfactory clinical outcome after treatment. Significant correlation between the mean AgNORs number and survival in univariate and multivariate analysis was documented. Among clinical features the lymph node status and the T stage were important prognostic factors. We conclude that AgNORs may be a potential prognostic marker in laryngeal cancer.

Introduction

Squamous cell carcinoma of the larynx is clinically the most significant malignant neoplasm in the head and neck region. In Poland, according to epidemiological investigations, the incidence of this tumour has been increasing continuously in the past twenty years.

The estimation of a patient's prognosis is very important as the selection of individual therapy may be based on it. Various cellular markers have been reported to be valuable in predicting the malignant potential of neoplastic lesions (1, 2, 3, 4). One such marker is the number of nucleolar organizer regions (NORs).

The nucleolus is the site of synthesis of ribosomal RNA. Nucleolar organizer regions are equivalent to genes on which the ribosomal RNA is coded. These are large loops of DNA which are localised on the short arms of the five acrocentric chromosomes (5).

Methods of assessing cell proliferation in routinely fixed and processed tissues are of great interest in histopathology, because they preserve tissue architecture and allow retrospective studies.

The method of determination of NORs is based on their argentophilic property. These regions and their associated proteins may be selectively stained within interphase nuclei using a simple silver colloid technique, thus the term silver-staining nucleolar organizer regions AgNORs (5).

The mean AgNORs number per nucleus has been found to be a useful marker of tumour aggressiveness in squamous cell carcinomas (6, 7) and some other tumours (8). Other research has failed to show this feature for adenoid cystic carcinoma of the parotid gland and for thyroid tumours (9, 10).

The purpose of this study was to estimate the prognostic value of the AgNORs count per nucleus in laryngeal cancer. The study was
based on the examination of 154 routinely processed biopsy specimens. The relation between the AgNORs number and clinicopathological features was studied.

**Materials and methods**

One hundred and fifty four patients who underwent biopsy for carcinoma of the larynx were selected for this study. All patients were diagnosed between 1991 and 1994. There were 26 females and 128 males. The mean age of patients was 61.3 ± 10.1 years. Tumour staging was performed according to TNM criteria. In forty-six cases cervical lymph node metastases were detected (Table 1).

All cases were squamous carcinomas. The histopathological grading of tumours (G1-G3) was done on the hematoxylin-eosin sections. There were 39 cases - G1, 61 cases - G2 and 54 cases - G3. The grading is applied only to malignant tumours. Two major factors are considered: the degree of anaplasia or undifferentiation of the tumour and an estimate of the rate of growth. Customarily, tumours are graded numerically into three grades with the low number implying a lesser degree of malignancy. Evidence of differentiation is based on the resemblance of the tumour to the normal tissue prototype. The estimate of rapidity of growth is based on the number of mitoses per unit of tissue and the increase of nuclear chromatin (11).

After diagnosis the patients were treated by surgery and/or radiotherapy. Eighty-nine patients underwent complete surgical excision of the tumour with free margins. Thirty-two patients were treated by radiotherapy alone and thirty-three patients underwent surgery and subsequent radiotherapy. Different treatment procedures were applied according to the site and the extension of the tumour, lymph node

### Table 1

Clinicopathological findings and AgNORs number in laryngeal cancer

<table>
<thead>
<tr>
<th>Features</th>
<th>Number of cases</th>
<th>AgNORs no. (mean ± SD)</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>26</td>
<td>2.84 ± 1.09</td>
<td>NS</td>
</tr>
<tr>
<td>Male</td>
<td>128</td>
<td>3.19 ± 1.67</td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 55</td>
<td>41</td>
<td>3.29 ± 1.67</td>
<td>NS</td>
</tr>
<tr>
<td>56-65</td>
<td>47</td>
<td>3.16 ± 1.48</td>
<td></td>
</tr>
<tr>
<td>&gt; 65</td>
<td>66</td>
<td>3.15 ± 1.62</td>
<td></td>
</tr>
<tr>
<td>Site of tumour</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epiglottic</td>
<td>56</td>
<td>3.31 ± 1.81</td>
<td>NS</td>
</tr>
<tr>
<td>Glottic</td>
<td>65</td>
<td>3.01 ± 1.53</td>
<td></td>
</tr>
<tr>
<td>Subglottic</td>
<td>8</td>
<td>3.24 ± 1.78</td>
<td></td>
</tr>
<tr>
<td>Transglottic</td>
<td>25</td>
<td>3.21 ± 1.71</td>
<td></td>
</tr>
<tr>
<td>T stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>32</td>
<td>2.95 ± 1.45</td>
<td>NS</td>
</tr>
<tr>
<td>T2</td>
<td>28</td>
<td>3.12 ± 0.98</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>35</td>
<td>3.21 ± 1.52</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>59</td>
<td>3.38 ± 1.63</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>108</td>
<td>3.01 ± 1.51</td>
<td>NS</td>
</tr>
<tr>
<td>N1-3</td>
<td>46</td>
<td>3.32 ± 1.95</td>
<td></td>
</tr>
<tr>
<td>Histopathological grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>39</td>
<td>2.32 ± 1.16</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>G2</td>
<td>61</td>
<td>3.22 ± 1.58</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>54</td>
<td>3.89 ± 0.93</td>
<td></td>
</tr>
</tbody>
</table>
involvement and agreement from the patients for the proposed method of treatment.

A minimum follow-up of 3 years or to a patient's death was available for all the cases.

For the control, normal laryngeal epithelium obtained from 25 patients with benign lesions of the larynx was examined.

The tissue specimens, in the form of paraffin blocks, were available in the Department of Pathology, Medical University of Wroclaw.

For estimation of AgNORs the staining technique described by Ploton et al. (5) was used. Paraffin sections were cut at 7 µm, deparaffinized, and dehydrated by absolute ethanol. Then specimens were fixed in Carnoy's solution (3 parts absolute ethanol to 1 part acetic acid) for 10 minutes and were hydrated in distilled water. Slides were incubated with a silver solution for 30 min at room temperature. The staining solution was prepared by adding 1 volume of 2% gelatin in 1% formic acid to 2 volumes of 50% aqueous silver nitrate. The silver colloid was then washed off with deionized distilled water and the slides were postfixed in 5% sodium thiosulfate for 5 minutes. No counterstain was used. The AgNORs were presented as dark or black intranuclear bodies. AgNORs in benign cells appeared regular in size and round in shape whereas those in malignant tissue often appeared less uniform in size and sometimes took on odd forms (Figure 1a, b).

The dots of AgNORs were counted using an Olympus microscope (ocular magnification × 40). In the process of counting, the semi-automatic computer system of image analysis was applied (MultiScan, Poland). In each specimen, random fields containing 100 nuclei were examined. When AgNORs were in the form of clusters they were counted as a single dot.

Prognostic factors analysed for their influence on survival were age, sex, site and size of tumour, lymph node metastasis, histopathological grading, and AgNORs number. Any deaths resulting from causes other than the primary cancer were excluded from the statistical analysis.

Association between AgNORs score and tumour clinico-pathological features was estimated by one-way analysis of variance (ANOVA).

Univariate survival analyses were based on the Kaplan-Meier product-limit estimates of survival distribution.

Differences between survival curves were tested statistically using generalised Wilcoxon test. The relative importance of multiple prognostic factors on survival was estimated using the Cox proportional hazards regression model.
All data were processed with SPSS and S-PLUS statistical software. A p value less than 0.05 was considered significant.

The experimental values are given as a mean ± SD (standard deviation).

Results

The positive staining of AgNORs was obtained in all cases of laryngeal cancer and in all control specimens.

Mean AgNORs counts was 1.25 ± 0.88 for benign laryngeal lesions and 3.15 ± 1.62 for laryngeal cancer. This was statistically significant (p < 0.01).

The mean AgNORs number showed an increase from T1 through T2, T3 to T4 categories but the differences among them were not significant (Table I).

There was no statistically significant difference in AgNORs number regarding the patient’s other clinical background, such as sex, age, site of tumour and lymph node metastasis (Table I).

Comparing the results of the grade of malignancy, there was an increase in AgNORs score with the increasing grade of malignancy. The differences between G1 vs G3 and G2 vs G3 were significant (p < 0.05) (Table I).

The number of AgNORs per cell correlated with the patients clinical course. The mean number of AgNORs per cell was 3.85 ± 2.09 in cases with recurrence and 2.47 ± 0.71 in cases free of disease after treatment (p < 0.05).

Univariate analysis revealed strong correlation between AgNORs counts and survival rates. The three and five year survival rates for the whole group of the patients were 62 and 41% for cases with AgNORs > 3 in contrast to 79 and 73% for cases with AgNORs ≤ 3 (Figure 2). When we take into consideration only the patients with T4 supraglottic tumours the three and five year survival rates were 31 and 20% for cases with AgNORs > 3 as opposed to 80 and 69% for cases with AgNORs ≤ 3.

Apart from AgNORs count, T stage, N status and site of the tumour and method of treatment significantly correlated with a patient’s survival in univariate analysis.

T4 stage cases had 3 and 5 year survival rates of 51 and 28% respectively vs 76-85% and 69-82% for other stages.

N1-3 patients had 3 and 5 year survival rates of 49 and 31% vs 79 and 61% for N0.

Transglottic carcinomas had 3 and 5 year survival rates of 54 and 24% vs 55-65 and 40-54% for epiglottic or subglottic and 91 and 82% for glottic carcinomas.

The patients with stage T3 laryngeal carcinoma treated only by radiotherapy had 3 and 5 year survival rates of 50% vs 85-90% for both the patients treated by surgery alone and those who underwent surgery with subsequent radiotherapy.

The age and sex of the patients and histopathologic staging of tumours did not significantly correlate with prognosis.

To determine if AgNORs was an independent prognostic variable in laryngeal carcinoma, a multivariate analysis was performed. By testing the association of response with covariates in the Cox model, only three variables showed significant correlation with prognosis: AgNORs counts (p < 0.01) and, to a lesser degree, the N status and T stage (p < 0.05) (Table II).

![Figure 2](image-url)

**Fig. 2**

Kaplan-Meier survival curves for laryngeal carcinoma with values: ≤ 3 and > 3 AgNORs/nucleus. The survival time of patients with a high AgNORs number was significantly shorter than those with a low AgNORs number (p < 0.01).
Table II
Multivariate analysis of Cox’s proportional hazards model in patient’s with laryngeal cancer

<table>
<thead>
<tr>
<th>Variables</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>NS*</td>
</tr>
<tr>
<td>Sex</td>
<td>NS</td>
</tr>
<tr>
<td>Site of tumour</td>
<td>NS</td>
</tr>
<tr>
<td>T status</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>N status</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Grading</td>
<td>NS</td>
</tr>
<tr>
<td>AgNORs</td>
<td>p &lt; 0.01</td>
</tr>
</tbody>
</table>

* NS : not significant

Discussion

The biologic role of the AgNORs is still controversial. Morita et al. (12) documented the existence of a significant correlation between the cellular DNA content and AgNORs number. Other studies suggested that the AgNORs number is related to ploidy (13, 14).

There are several studies which demonstrated a positive correlation between AgNOR number and rate of cell proliferation (6, 15, 16).

The AgNORs expression was used to differentiate benign from malignant lesions of gastrointestinal tract (17, 18), and for grading tumours (8).

Correlation between AgNORs count and prognosis has been found in childhood fibrosarcoma (19), pharyngeal carcinoma (20) and oesophageal carcinoma (12).

Esser et al. (21) revealed a statistically significant correlation between the AgNORs count and the extent of lymphogenic formation of metastases in pharyngeal carcinoma.

Yamamoto et al. (22) applied AgNORs staining to supraglottic carcinomas. The results were correlated with the degree of histological differentiation and the TNM classification. There was no significant correlation between AgNORs count and either the histological differentiation or the T stage. The AgNORs number was significantly higher in the presence than in the absence of lymph node metastasis.

Kuwabara et al. (16) found statistically significant differences between the number of AgNORs in sinus squamous cell carcinoma and that found in the normal mucosa, but there was no difference between recurrent and non-recurrent tumours.

The prognostic significance of AgNORs in 30 patients with squamous carcinomas of the larynx was assessed by Bockmühl et al. (7). Considering the mean number of AgNORs, the statistical analysis revealed significant differences between carcinomas in patients, who were alive of at least 3 years without local or distant recurrence and those who had died of laryngeal cancer.

The value of AgNORs in the diagnosis of head and neck tumours was examined by Hirsch et al. (6). The number of AgNORs was significantly higher in carcinomas than in benign epithelium. The AgNORs number increased with the stage of the disease, but there was no correlation with histologic grade.

Teixeira et al. (23) analysing 43 cases of oral cancer demonstrated in multivariate analysis that two variables showed significant negative correlation with the recurrence free interval: high AgNORs area and compromised surgical margins.

The prognosis of laryngeal carcinoma in its early stages is generally good. Unfortunately in Poland the diagnosis is often delayed. As a result we must face the challenge of treating advanced cases. In such a situation the additional prognostic factors are very important for selecting the therapeutic strategy.

In this series of 154 squamous cell carcinomas of the larynx, we examined AgNORs number per cell versus clinical status, histopathologic grade and clinical outcome.

The mean number of AgNORs per nucleus was significantly less for benign lesions than for malignant epithelium, in accordance with the findings of Hirsch et al. (6) and Kuwabara et al. (16). This suggests that AgNORs number might be of value for differentiating benign from malignant squamous epithelial lesions.

The average AgNORs number for squamous cell carcinomas in our study was smaller than in other reports (16, 20). This may be attributed to many factors, including section
thickness, staining reaction time, and convention of counting AgNORs.

We have not observed positive association between AgNORs number and T stage, in agreement with the findings of Pich et al. (20) in pharyngeal carcinomas and Yamamoto et al. (22) in laryngeal carcinoma, but in contrast with Hirsch et al. (6) in different head and neck carcinomas.

Since the precise function of AgNORs has not yet been clarified it is difficult to assess the correlation between lymph node status and AgNORs count. Yamamoto et al. (22) and Esser et al. (21) suggest that this is the most important relation between AgNORs and clinicopathological features but other authors have not confirmed this correlation (7, 20).

There is no agreement in the literature about the relation between cell proliferation and lymph node metastasis formation. Some authors claim that this relation exists (24, 25) but others haven’t proved it (26, 27). Our results indicate that cell proliferation and potential for metastasizing may be two independent features of the tumour.

We have demonstrated a significant correlation between AgNORs number per cell and tumour grading. The results concerning correlation between markers of cell proliferation and histological grading are conflicting. Some authors confirm such correlation (28, 29, 30) and others do not (16, 20). The explanation of the relation between histopathological grading and AGNORs count may be based on the fact that cell proliferation is one of the factors to consider in estimation of the grade of histopathological malignancy (11).

There are several clinicopathological prognostic factors in laryngeal cancer. In our study, among the clinical features, the lymph node status and the T stage were important prognostic factors both in univariate and multivariate analysis. Stell (31) stated that the drop in survival with increasing T stage is almost entirely due to the increasing incidence of lymph node metastases.

This paper documents the existence of a significant correlation between the mean AgNORs number and prognosis in univariate and multivariate analysis. Characterisation of laryngeal carcinoma with AgNORs number may be of potential use as a guideline for selecting therapeutic strategy.

The fact that the technique of AgNORs determination is time and cost effective adds to its potential usefulness. More standardised analysis methods could improve the diffusion of AgNORs as a diagnostic and prognostic tool.

References


Nucleolar organiser in laryngeal cancer


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