The expression of the nucleolar organizer regions (NORs) was quantified in paraffin sections of tumors and lymph node metastasis, by means of digital image analysis, in 75 patients with resected non-small cell lung cancer (NSCLC). Patients were divided in two groups: early stage (stages I and II) and advanced stage (stages IIIa, IIIb and IV). The prognostic significance of AgNOR expression was tested by Cox regression analysis in models controlled for age, sex, vital status, stage and histological type. Tumors at early stages had a lower expression of AgNOR than those at more advanced diseases. The mean values obtained for NORs in advanced disease were almost the same as those in the primary tumors when compared with the corresponding lymph node metastasis ($r = 0.90$; $p < 0.01$; linear regression). The prognostic role of AgNOR was significant only for tumors at stages I and II and not for advanced neoplasms (stages IIIa, IIIb and IV). These results encourage the inclusion of AgNOR quantitation in routine material, especially in early lung cancer.

Key words: nucleolus organizer region – lung neoplasms – cell nucleolus – silver staining – non-small cell lung cancer

INTRODUCTION

Prognosis of patients with non-small cell lung cancer (NSCLC) who have undergone surgical treatment is highly dependent on tumor staging at the moment of diagnosis (1–4). Although the TNM (tumor–node metastasis) staging is useful in determining tumor staging and grading, it does not provide information about the intrinsic biological aggressiveness of tumors within each stage, since different clinical outcomes are observed for tumors at the same stage, especially in those cases of early phases of the disease (5). According to this point of view, it would be of interest to have an indicator of tumor biological behavior, just after resection, in order to provide basis for some adjuvant therapy to surgery in those cases with greater probability of recurrence.

Our group has been interested in determining prognostic markers derived from routine cytological and histopathological material, using morphometric procedures applied to hematoxilin–eosin-stained slides (6) or AgNOR-stained preparations (7).

In previous studies, the AgNOR technique was shown to be a practical and efficient tool to gather information not only in order to distinguish reactive mesothelium from neoplastic cells (7–9), but also to establish prognostic markers of survival in squamous cell carcinoma of the lung (6).

NORs are segments of DNA coding ribosomal genes and are situated on the short arms of acrocentric chromosomes. They can be demonstrated in formalin-fixed paraffin-embedded tissues by one-step silver staining, the resulting black dots being termed AgNORs (10–13).

In the present study, we decided to explore the role of AgNOR measurements as a prognostic marker in NSCLC, computing Cox proportional hazard models and considering different histopathological subtypes in the analysis. In addition, the series of patients studied were distributed within a wide range of tumor stages, in order to verify the role of AgNOR in predicting survival in subpopulations regardless of the degree of the tumor extension.

Moreover, we compared the amount of AgNOR expression in the primary tumors with that of the corresponding lymph node metastasis in cases that presented advanced disease. This procedure was followed in order to know if prognostic markers could be obtained from material sampled during mediastinoscopy.
instead of using tissues from lung resections. Such information is
not available in any current literature.

MATERIALS AND METHODS

SELECTION OF PATIENTS

Seventy-five patients treated by surgical resection of NSCLC at the
Department of Surgery of the Universidade de Mogi das Cruzes
Hospital, from 1988 to 1995, were retrospectively identified. The
data collected included race, age and sex of the patients, type of
operation performed, post-surgical follow-up time, including recurrences of the disease, vital status or date of death.

There were 60 men and 15 women at ages ranging from 39 to
79 years old (mean = 60.6 years). Patients were split into two
groups: early stage (stages I and II; n = 36) and advanced stage
(stages IIIa, IIIb and IV; n = 39). Seven deaths in the first 30 days
after surgery were regarded as post-operative deaths and were not
included in the survival analysis. Post-surgical follow-up ranged
from 4 to 83 months (mean = 25.4 months).

Staging classification for lung cancer was made according to
the TNM criteria (1). Histopathological classification was
determined according to the World Health Organization
classification (14). The diagnosis of NSCLC was agreed upon by
two independent pathologists, based on the examination of
conventional hematoxilin–eosin staining. There were 27 patients
at stage I, 9 at stage II, 22 at stage IIIa, 11 at stage IIIb and 6 at
stage IV. There were 30 patients with adenocarcinoma, 30 with
squamous cell carcinoma, 10 with adenosquamous cell
carcinoma and 5 with large-cell carcinoma (Table 1).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Number of cases (total 75)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>60</td>
</tr>
<tr>
<td>Female</td>
<td>15</td>
</tr>
<tr>
<td>Cell type</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>30</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>30</td>
</tr>
<tr>
<td>Adenosquamous carcinoma</td>
<td>10</td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>5</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>27</td>
</tr>
<tr>
<td>Stage II</td>
<td>9</td>
</tr>
<tr>
<td>Stage IIIa</td>
<td>22</td>
</tr>
<tr>
<td>Stage IIIb</td>
<td>11</td>
</tr>
<tr>
<td>Stage IV</td>
<td>6</td>
</tr>
</tbody>
</table>

AgNOR STAINING TECHNIQUE

Sections of 3 µm, obtained from each paraffin block of the main
tumor and its corresponding lymph node metastasis, were
selected and stained by the one-step silver colloid method.
Briefly, the sections were dewaxed in xylene and rehydrated
through decreasing concentrations of ethanol to distilled, deionized water. The AgNOR solution was freshly prepared by
dissolving gelatin at a concentration of 2 g/dl in 1 g/dl aqueous
formic acid. This solution was added to 50 g/dl aqueous silver
nitrate solution (1:2, v/v ). This final solution was then
immediately poured on to the slides, which were left in the dark
at room temperature for 45 min. The silver colloid was washed
from the sections with distilled, deionized water and the sections
were dehydrated through a graded series of ethanol to xylene
(12,13).

AgNOR QUANTITATION

The quantitative analysis of the AgNOR expression was
performed by means of digital image analysis, using the
Biосan-Optimas software. The images were generated by a
Zeiss Axiosplan (Zeiss, Germany) microscope connected to a
Sony camera (Sony, Japan) and fed into the computer through an
Oculus TCX frame grabber (Coreco, Canada) for off-line
processing.

A total of 100 cells/case were analyzed in primary tumor and
in metastatic lymph nodes, with the aid of a 1000x magnification
and an oil-immersion lens. A mean of 10 different areas of tumor
was chosen in order to determine the homogeneous AgNOR
quantitation throughout the main tumor and its corresponding
metastasis. The quantitations were always performed in well
preserved cells, excluding areas of tumor necrosis, staining
artifacts or overlapped cells. The threshold for AgNOR dots was
selected for individual cases, after enhancing the contrast up to a
point at which the AgNOR dots were easily identified as black
points within the nuclei. The results were expressed in terms of
area/nuclei (15,16). Concerning the reproducibility of the
AgNOR quantitation method, consecutive measurements of the
same cases showed excellent agreement (0.9–4% variation
between two measures).

STATISTICAL ANALYSIS

The results were analyzed by means of parametric and
non-parametric tests (17), as follows:

Student’s paired t-test and Pearson correlation coefficient to
compare the values of AgNOR observed for each patient in the
tumor and in its corresponding lymph node metastasis.

Student’s unpaired t-test to compare the AgNOR values in
tumors when patients were split into two groups (early and
advanced stages).

One-way analysis of variance to compare the AgNOR values
for different tumor stages and histological types. When the
difference was significant, this analysis was complemented by a
Scheffé test.

The χ² test for statistical comparisons of proportions.

In order to identify independent factors which had a significant
influence on survival, we used Cox proportional hazard models
employing categorical non-linear indicators of the AgNOR
expression as predictive variables, such as sex, age, staging,
histological type and vital status (18).

Survival analysis of 68 patients was made, excluding 7 patients
whose causes of death were other than lung cancer.

For statistical analysis of the survival rate in the early stage
group, a cut-off point of 7 µm²/nucleus was considered. The

Table 1. Characteristics of the population studied with primary NSCLC
designations ‘low AgNOR content’ was used for patients with ≤7 µm²/nucleus AgNOR area and those with >7 µm²/nucleus AgNOR area were considered as having ‘high AgNOR content’. The difference was considered significant when \( p < 0.05 \).

The statistical procedures were followed with the aid of SPSS (Statistical Package for Social Science) v 6.0 statistical software (19).

RESULTS

Black silver-stained dots for AgNORs were clearly identified in all cell nuclei of tumor and nodal metastasis, as shown in Fig. 1a and b.

In 39 cases of advanced NSCLC, the mean values of AgNOR showed no statistical differences between the primary tumor and its nodal metastasis, as determined by means of a paired Student’s \( t \)-test. Fig. 2 shows the results of the linear regression analysis of AgNOR scores in the primary tumor and the nodal metastasis. There was a significant correlation between the two samples (Pearson correlation coefficient \( r = 0.90; p < 0.01 \)), suggesting that cancer cells in the mediastinal lymph node metastasis had a similar proliferative activity when compared with the primary lesion.

When the population of patients was distributed into stages I, II, IIIa, IIIb and IV, AgNOR values showed considerable overlap among the groups. No significant difference was found among the ratios for stages I and II or among stages IIIa, IIIb and IV. However, analysis of data collected by one-way analysis of variance showed that the AgNOR scores in earlier stages (I and II) were significantly lower than in advanced stages (stage I vs IIIa, IIIb, IV; stage II vs stages IIIa, IIIb, IV) (Table 2).

When patients were split into early and advanced stages, the values of AgNOR allowed the discrimination between these two groups. Analysis of data collected by means of a Student’s unpaired \( t \)-test showed that the NOR scores were significantly lower in early cancer (stages I + II) than in advanced stages (IIIa + IIIb + IV) (Fig. 3).

When the different histopathological types were considered, the one-way analysis of variance showed no statistical differences in the AgNOR expression among the various cell types. These results suggested that the AgNOR expression has no use for histological diagnosis (Fig. 4).
Table 2. Relationship between mean AgNOR area and various clinical and pathological variables in 75 patients with NSCLC

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>n</th>
<th>Mean ± SD AgNOR area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Female</td>
<td>15</td>
<td>8.21 ± 3.01</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>60</td>
<td>8.80 ± 2.09</td>
</tr>
<tr>
<td>Stage</td>
<td>I</td>
<td>27</td>
<td>7.22 ± 1.48</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>9</td>
<td>6.04 ± 0.87</td>
</tr>
<tr>
<td></td>
<td>IIIa</td>
<td>22</td>
<td>10.52 ± 2.28</td>
</tr>
<tr>
<td></td>
<td>IIIb</td>
<td>11</td>
<td>11.17 ± 2.64</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>6</td>
<td>10.52 ± 2.13</td>
</tr>
<tr>
<td>Group</td>
<td>Early cancer (I + II)</td>
<td>36</td>
<td>6.92 ± 1.43</td>
</tr>
<tr>
<td></td>
<td>Advanced cancer (IIIa + IIIb + IV)</td>
<td>39</td>
<td>10.71 ± 2.46</td>
</tr>
<tr>
<td>Cell type</td>
<td>Adenocarcinoma</td>
<td>30</td>
<td>8.02 ± 2.47</td>
</tr>
<tr>
<td></td>
<td>Squamous cell carcinoma</td>
<td>30</td>
<td>9.58 ± 3.08</td>
</tr>
<tr>
<td></td>
<td>Adenosquamous carcinoma</td>
<td>10</td>
<td>9.60 ± 2.43</td>
</tr>
<tr>
<td></td>
<td>Large cell carcinoma</td>
<td>5</td>
<td>8.58 ± 2.35</td>
</tr>
<tr>
<td>Vital status</td>
<td>Alive</td>
<td>31</td>
<td>7.58 ± 2.09</td>
</tr>
<tr>
<td></td>
<td>Dead</td>
<td>44</td>
<td>9.82 ± 2.80</td>
</tr>
</tbody>
</table>

ns, not significant; SD, standard deviation; *, p < 0.05. Vital status refers to May 31, 1996.

Figure 3. Mean and standard deviation (SD) of AgNOR in 75 cases of NSCLC according to two groups, early and advanced stages.

We also analyzed the capacity of AgNOR expression to predict the survival of patients by performing a survival analysis of 68 patients.

Table 3 represents the coefficients of the Cox regression model for AgNOR categories after controlling the model according to tumor stage, histological type, sex and age for all cases considered. In this simultaneous analysis, the only significant predictive variable was tumor staging. No significant effect of histological subtyping was observed, although large cell carcinoma tended to present a lower survival rate in comparison with other types. The AgNOR expression was not significantly associated with survival of the overall population. However, there was a clear tendency for a progressive effect on survival reducing among the four quartiles of AgNOR, as observed by the B coefficient of the Cox model.

The 5-year predicted survival as a function of tumor stage, which was adjusted for age, sex, histological type and four quartiles of AgNOR in overall cases, showed that there are two distinct subgroups of patients in terms of survival: those with limited disease (stages I + II) and those with advanced disease (stages IIIa + IIIb + IV) (Fig. 5).

In another proportional hazard model, a cut-off point of 7 µm² was chosen in order to analyze only the patients restricted to early stages (I and II).

In patients whose AgNOR values were >7 µm² the 5-year survival rate was ~23% and in those with ≤7 µm² the 5-year survival rate was ~65%, with a significant difference between the two groups (Table 4).

Fig. 6 shows the predictive survival function for tumors with low and high AgNOR expression, based on the model presented in Table 4.
Table 3. Coefficients obtained for the Cox proportional hazard model relating stage, histological type, quartiles of AgNOR, sex and age with survival of 68 patients with NSCLC

<table>
<thead>
<tr>
<th>Variable</th>
<th>B</th>
<th>SE</th>
<th>Sig</th>
<th>Exp B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>0.0834</td>
<td>0.7150</td>
<td>0.9071</td>
<td>1.0870</td>
</tr>
<tr>
<td>Stage IIIa</td>
<td>1.1645</td>
<td>0.5803</td>
<td>0.0448*</td>
<td>3.2043</td>
</tr>
<tr>
<td>Stage IIIb</td>
<td>1.6034</td>
<td>0.7752</td>
<td>0.0386*</td>
<td>4.9700</td>
</tr>
<tr>
<td>Stage IV</td>
<td>1.3146</td>
<td>0.6756</td>
<td>0.0517</td>
<td>3.7233</td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large cell</td>
<td>1.2824</td>
<td>0.7424</td>
<td>0.0841</td>
<td>3.6053</td>
</tr>
<tr>
<td>Adenosquamous cell</td>
<td>0.22133</td>
<td>0.7182</td>
<td>0.0386*</td>
<td>1.2378</td>
</tr>
<tr>
<td>Squamous cell</td>
<td>0.0376</td>
<td>0.4580</td>
<td>0.9346</td>
<td>1.0383</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>57–62</td>
<td>0.1644</td>
<td>0.5009</td>
<td>0.7427</td>
<td>1.1787</td>
</tr>
<tr>
<td>63–66</td>
<td>0.5032</td>
<td>0.3536</td>
<td>0.4381</td>
<td>1.6540</td>
</tr>
<tr>
<td>67–79</td>
<td>–0.5335</td>
<td>0.6126</td>
<td>0.3838</td>
<td>0.5866</td>
</tr>
<tr>
<td>Sex</td>
<td>–0.6759</td>
<td>0.4841</td>
<td>0.1626</td>
<td>0.5087</td>
</tr>
<tr>
<td>Quartiles of AgNOR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.57–7.80</td>
<td>0.6114</td>
<td>0.7050 ns</td>
<td>0.3858</td>
<td>1.8431</td>
</tr>
<tr>
<td>7.82–10.45</td>
<td>0.9080</td>
<td>0.7702 ns</td>
<td>0.2384</td>
<td>2.4793</td>
</tr>
<tr>
<td>10.67–16.76</td>
<td>1.1380</td>
<td>0.8165 ns</td>
<td>0.1634</td>
<td>3.1205</td>
</tr>
</tbody>
</table>

B, coefficient; SE, standard error; Sig, significance; *, p < 0.05; ns, not significant. Overall $\chi^2 = 29.63; p < 0.01$.

Table 4. Coefficients obtained for the Cox proportional hazard model relating histological type, age, sex and an AgNOR cut-off point value of 7 $\mu$m$^2$/nucleus to survival of 36 patients with early stages (I + II) of NSCLC

<table>
<thead>
<tr>
<th>Variable</th>
<th>B</th>
<th>SE</th>
<th>Sig</th>
<th>Exp B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large cell</td>
<td>–0.7568</td>
<td>1.2517</td>
<td>0.5454</td>
<td>0.4692</td>
</tr>
<tr>
<td>Adenosquamous cell</td>
<td>–0.9896</td>
<td>1.2851</td>
<td>0.4413</td>
<td>0.3717</td>
</tr>
<tr>
<td>Squamous cell</td>
<td>–0.7678</td>
<td>0.9388</td>
<td>0.4134</td>
<td>0.4640</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>61–62</td>
<td>0.6279</td>
<td>1.1335</td>
<td>0.5796</td>
<td>1.8736</td>
</tr>
<tr>
<td>63–68</td>
<td>–1.0465</td>
<td>0.9960</td>
<td>0.2934</td>
<td>0.3512</td>
</tr>
<tr>
<td>69–79</td>
<td>0.6374</td>
<td>1.0097</td>
<td>0.5279</td>
<td>1.8915</td>
</tr>
<tr>
<td>Sex</td>
<td>1.0877</td>
<td>0.9880</td>
<td>0.2709</td>
<td>2.9674</td>
</tr>
<tr>
<td>AgNOR &gt;7 $\mu$m$^2$</td>
<td>2.1701</td>
<td>0.8708</td>
<td>0.0127*</td>
<td>8.7590</td>
</tr>
</tbody>
</table>

B, coefficient; SE, standard error; Sig, significance; *, p < 0.05. Overall $\chi^2 = 10.35; p = 0.2409$.

DISCUSSION

Many studies have been reported relating NORs and correlated argyrophilic proteins with lung cancer (20–24).

In recent years, AgNOR determinations have been considered to provide an objective prognostic parameter to predict the clinical behavior of surgically resected lung cancer patients (23–26). More recently, studies carried out on lung cancer have demonstrated a positive correlation between AgNOR and other markers of cellular proliferation, such as tumor volume doubling time (22,27,28), DNA content (29–31), PCNA index (24,26,32), Ki67 index (33) and cathepsin B activity (30,34). The potential prognostic value of AgNOR in NSCLC has been considered since the work of Kaneko et al. (25), who found significant AgNOR scores and survival of patients at stage I of the disease.

Other studies have proved the value of the morphometric evaluation of AgNOR in the differentiation of hyperplasia from incipient cellular alterations (35,36) or in the detection of premalignant lesions of bronchial epithelium and carcinoma (37–39).

Hence the amount of AgNOR proteins can be used as a marker of cell proliferation (40,41). AgNOR quantification by image analysis is a useful tool in the evaluation of the rate of cancer cell proliferation (42,43).

The present study was designed to verify the usefulness of AgNOR quantification in predicting survival in NSCLC. The reasons for such a study are based both on previous studies by our group and on the evidence reported in the literature. Initially we reported that AgNOR measurements were useful in discriminating benign from malignant pleural effusions (7).
Recently we have found a significant effect of AgNOR expression on the survival rate of squamous cell carcinoma of the lung (44). Consequently, we decided to explore further the prognostic value of AgNOR in lung cancer by studying a different group of patients. In the analysis, other histological types of NSCLC were considered and patients having a wide spectrum of tumor staging at the moment of treatment were included.

Since we included patients with an advanced disease in this study, we considered that tumor size and extension could be playing a limiting role in the satisfactory evaluation of the effect of AgNOR expression on survival. Additional studies were carried out considering only the subgroup of patients with limited disease, especially those at stages I and II at the moment of surgery. For this purpose we included in the analysis, as predictive variables, histological types, age, sex and two quartiles of AgNOR values, based on the mean of this population: tumors with low (<7 µm²) and high (>7 µm²) AgNOR expression. The coefficients of this model indicated that patients who had limited disease and tumors with lower expression of AgNOR showed longer survival than those with higher AgNOR quantities.

The presence, in this series, of patients with lymph node metastasis also allowed us to perform simultaneous AgNOR measurements in the primary tumor and its corresponding metastasis. This procedure was designed not only to test the reproducibility of AgNOR measurements but also to verify if prognostic markers could be obtained from material sampled during mediastinoscopy, thus helping to select patients with advanced disease who are better candidates to benefit from a surgical resection.

Although there is a tendency for a little longer survival in patients with advanced disease presenting tumors with low AgNOR expression, this effect was not significant in this series. Consequently, the use of AgNOR in evaluating the behavior of advanced NSCLC was of limited value. However, the good agreement between AgNOR quantity in the primary tumor and the corresponding lymph node metastasis indicates that, at least in terms of AgNOR, the proliferative activity of NSCLC is equally increased in the main lesion and in its metastasis in comparison with tumors at lower stages. Similar results were reported by Kakeji et al. (45) in gastric cancer.

Considering that the AgNOR expression is increased in advanced disease, it would be possible to conclude that AgNOR has no intrinsic prognostic value, since it may be similar to tumor staging. We tried to explore this possibility by restricting our analysis to patients with limited disease. This procedure indicated that the AgNOR expression has prognostic implications in this subgroup of patients. Similar findings were reported by Kaneko et al. (25), Oyama and co-workers (23,24) and Rodrigues (46). They found a significant prognostic role of AgNOR in stage I of NSCLC.

Therefore, multicentric studies with larger casuistry may be necessary in order to evaluate the true role of AgNOR as a prognostic marker in advanced NSCLC before dismissing the role of AgNOR in the prognosis of such patients.

In addition, we reported a significant effect of the AgNOR expression and survival in NSCLC at stages I and II. In those cases of more advanced disease the prognostic role of AgNOR was not significant. However, advanced cancer showed a higher expression of AgNOR in comparison with cases at early stages of the disease. This situation may impair the statistical evaluation of the role of AgNOR in advanced cancer, since there are few cases fulfilling the requirements of both low AgNOR expression and advanced tumor staging.

We also observed a remarkable overlapping of the AgNOR scores within each histological type. Their value was not significant in discriminating among the cell types except for large cell carcinoma. These results are in agreement with previous reports (33,47,48).

Finally, we suggest that increased cell proliferative activity, measured by AgNOR expression, occurs simultaneously in the primary tumor and in its lymph node metastasis. Further studies are necessary to verify whether the same finding is presented by hematogenous metastasis.
Acknowledgments

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References