Introduction
Biomarkers related to significant clinicopathological parameters of non-small cell lung cancer (NSCLC), particularly survival of patients and ability to predict disease course with a high degree of probability, should be investigated. Proliferation is a basic process that reveals tumor appearance and development; this process is also a factor in the prediction of the biological behavior of tumors. However, reliable evaluation of the proliferative potential of tumors is difficult to achieve because proliferation involves not only a number of proliferative cells (proliferative activity and growth fraction) but also the velocity of cells undergoing phases of cell cycle (duration of cell cycle). Immunohistochemical assay with Ki-67 antigen is among the most common and available methods to estimate tumor proliferative activity. Ki-67 antigen is detected in cells in late G1, S, G2, and М phases; however, the functional role of this nuclear protein in proliferation remains unclear. Argyrophilic proteins associated with nucleolar organizer regions (AgNOR) are markers of cell cycle velocity. Nucleolar organizer regions are ribosomal DNA sequences on the short arms of human acrocentric chromosomes (13, 14, 15, 21, and 22) and encode ribosomal RNA (rRNA). A peculiar group of acidic and highly argyrophilic proteins are also localized at the same sites as...
nucleolar organizer regions, thereby allowing these regions to be very clearly and rapidly visualized by silver nitrate staining procedures. Up to 75% staining of AgNOR consists of two major argyrophilic proteins, namely, C23 (nucleolin) and B23 (nucleosomin). Nucleolin is a 105 kDa phosphoprotein that plays an important role in the transcription of rRNA molecules; nucleosomin is a 38 to 39 kDa phosphoprotein involved in late stages of pre-ribosomal particle organization. Nucleolin and nucleosomin are detected in cell nuclei during the entire cell cycle; these phosphoproteins increase in quantity by 1.5- to 3-fold in S and G2 phases. Notably, the amount of AgNOR in the interphase is related to the speed of cell proliferation. AgNOR is inversely related to cell cycle duration and tumor-doubling time. The relationship between interphase AgNOR quantity and cell doubling time can be attributed to proliferating cells that produce an adequate ribosomal complement for daughter cells; a short cell cycle indicates high ribosomal biogenesis per time unit. The amount of AgNOR in the interphase is also related to cell doubling time because the number of AgNOR in the interphase is related to rRNA transcriptional activity. The evaluation of the quantitative distribution of AgNOR represents a unique tool to obtain information regarding the proliferation rate of tumors during diagnosis from histological sections of routinely processed tissue samples.

Munakata et al. proposed the method of double staining involving Ki-67 antigen and AgNOR to evaluate AgNOR (cell cycle duration) in proliferating cells. However, limited research has been conducted regarding the proliferative potential of tumors by double staining of Ki-67 antigen and AgNOR. Studies have also been performed regarding the importance of AgNOR. Despite these studies, the results of double staining of Ki-67 antigen and AgNOR have not yet been evaluated by computer image analysis and in relation to clinicopathological parameters under TNM system and survival in NSCLC. The TNM system is described as follows. The T status reflects tumor size and collection of size-independent tumor descriptors, such as visceral pleural invasion, main bronchus involvement, atelectasis, or obstructive pneumonitis. The N status describes regional lymph nodes. The M status shows distant metastasis. Therefore, the current study aimed to explore the AgNOR area in MIB-1-positive cells in relation to clinicopathological parameters and survival in NSCLC.

**Materials and methods**

We studied 207 surgical specimens of NSCLC resected from 2007 to 2009 in the Altai Krai Oncology Dispensary. The mean age of patients was 59 years (range, 35-75 years); the patients included 177 males (86%) and 30 females (14%). Lobectomy and pneumonectomy were performed in 145 (70%) and 62 (30%) patients, respectively. Preoperative chemotherapy and radiation therapy were not conducted. Postoperative chemotherapy, most frequently with cisplatin and etoposide, was administered to 30 patients (14%). Postoperative radiation therapy, with a total focal dose ranging from 50 to 60 Gr, was conducted in 64 patients (31%). The clinicopathological parameters of NSCLC were determined in accordance with the TNM classification of seven reviews. In the current study, no cases were found with M1 status under the TNM system, but cases with multiple tumors were detected. The greatest tumor dimension was measured (in cm). This study was examined and approved by the corresponding ethics committee; this study was also performed in accordance with the ethical standards presented in the Declaration of Helsinki. Written informed consent was obtained.

Tissue fragments were fixed for 18 to 24 h in 10% neutral buffered formalin. After standard processing of the surgical material, we prepared histological slices of 4 µm in thickness. Specimens were stained with hematoxylin and eosin to confirm the original pathological diagnosis. For differential diagnostic purposes, histochemical (periodic acid-Schiff-alcian blue and according to Kreiberg) and immunohistochemical staining were applied. Immunohistochemical staining was performed using Ventana Discovery XT automated stainer, as described by the manufacturer (Ventana Medical System, Tucson, AZ, USA). Primary antibodies were used for cytokeratins 7 (clone SP52), 20 (clone SP33), high molecular weight (clone 34βE12), and S/6 (clone D5/16B4). The epidermis and the gastric mucosa were used as stain control.

Based on the review of corresponding histological specimens, three tissue cores were obtained from each patient by using paraffin blocks with a needle of 1.5 mm in internal diameter. Three tissue microarrays were prepared, each containing 12×18 cores. Histological slices of 4 µm in thickness were obtained from the tissue microarrays. Slices from the tissue microarrays were immunohistochemically stained following the manufacturer’s protocol for DAKO: streptavidin–biotin method with primary antibodies to Ki-67 (clone MIB-1, DAKO) and chromogen as new fuchsin. The slices were autoclaved at 120 °C for 20 min in 0.01 M citrate buffer (pH=6.0) before staining was performed. The slices were subsequently incubated with chromogen and washed in bidistilled water. The slices were then stained with silver nitrate by one-step method in a humid chamber at 37 °C for 19 min. Further staining of nuclei was not performed, and the slices were placed in a water medium (Faramount, DAKO). Only one representative core from each patient was analyzed.
Silver-stained specimens were examined under OLYMPUS CX-41 microscope equipped with a Plan C ×100/1.25 oil lens. The images were digitized and transferred directly into the computer with OLYMPUS DP72 digital camera and cellSense v.1.1 software with resolution of 1,360x1,024 pixels. The AgNOR area (in µm²) in each nucleus of 100 random MIB-1-positive cells (from 10 to 30 digital images) was measured. Semi-automated image processing was performed with Imagej v.1.42. The images were subjected to conversion into 8-bit image, background, normalization, segmentation, binary processing, and final measurements. To eliminate measurement errors, we excluded granules with a size of <0.1 µm² from analysis. The internal control of stromal lymphoid follicles was attended by MIB-1-positive centroblasts (positive control) and MIB-1-negative small lymphocytes (negative control). The entire AgNOR quantification was performed by one of the authors.

Statistical analysis

Statistical analysis was performed using STATISTICA 6.0. Data values were expressed as median and interquartile range. Data were processed by Kruskal-Wallis test, and significant differences were assessed by Mann–Whitney U test. Comparisons were performed using χ² test. Probabilities of 5-year overall survival were calculated by Kaplan-Meier method (in %), and between-group comparisons were conducted using log-rank test. Univariate and multivariate Cox proportional hazards model was used for the following factors: age at surgery (<59 vs. ≥59 years), gender (male vs. female), type of surgery (lobectomy vs. pneumonectomy), postoperative chemotherapy (yes vs. no), postoperative radiation therapy (yes vs. no), T status (T₁ vs. T₂ to T₃), greatest tumor dimension (<3 vs. ≥3 cm), N status (N₀ vs. N₁ to N₃), TNM stage (I vs. II to III), histology (adenocarcinoma vs. squamous-cell cancer), and differentiation (well vs. moderate to poor). Statistical significance was set at P<0.05.

Results

Immunohistochemical staining results of slides with primary antibodies for MIB-1 and subsequent staining results with silver nitrate were detected in the form of black round granules (AgNOR) located against a red nucleus (MIB-1-positive cells) or against a brown nucleolus or pale yellow nucleus (MIB-1-negative cells; Figure 1). In the nuclei, isolated black round granules (dots) appeared in the nucleoplasm (usually in MIB-1-positive cells) and/or multiple black round granules (dots) as a cluster (usually in MIB-1-positive cells). The morphometric results of the AgNOR area in MIB-1-positive cells in different clinicopathological parameters of NSCLC are shown in Table 1. In NSCLC, the median AgNOR area in MIB-1-positive cells was 10.47 µm² (range, 8.57-12.69 µm²), and this value was chosen as cut-off point. Cases with AgNOR area ≥10.47 µm² in MIB-1-positive cells were counted as large-area cases (101 cases, 49%) and <10.47 µm² as small-area cases (106 cases, 51%). The AgNOR area in MIB-1-positive cells was significantly larger in groups T₂ and T₃ than in T₁. In NSCLC with the greatest tumor dimension of <3 cm, the AgNOR area in MIB-1-positive cells was smaller than in those with tumor size ≥3 cm. A statistically significant increase in the AgNOR area in MIB-1-positive cells of NSCLC with metastases to regional lymph nodes was observed versus non-metastatic tumors (Figure 1A-D).

Figure 1 AgNOR in MIB-1-positive and MIB-1-negative cells of NSCLC in squamous cell cancer with absence (A, B) and presence (C, D) of metastases to lymph nodes and in adenocarcinoma with well (E, F) and poor (G, H) differentiation. Double staining for Ki-67 (clone MIB-1) by immunohistochemistry and for AgNOR with silver nitrate, ×1,000. AgNOR, argyrophilic nucleolar organizer region; NSCLC, non-small-cell lung cancer.
The AgNOR area in MIB-1-positive cells was smaller in TNM stage I than in stages II to III. No difference was observed in the AgNOR area in MIB-1-positive cells of adenocarcinoma and squamous cell cancer. The AgNOR area in MIB-1-positive cells was larger in moderately and poorly differentiated tumors than in well-differentiated tumors (Figure 1E-H). The AgNOR area in MIB-1-positive cells of NSCLC was correlated with T status (<0.001), greatest tumor dimension (<0.001), N status (<0.001), TNM stage (<0.001), and differentiation (<0.001).

The 5-year overall survival of patients with NSCLC was (39.3±3.8)%. The overall survival of patients with NSCLC exhibited statistically significant difference (<0.001) based on the AgNOR area in MIB-1-positive cells at (61.2±5.4)% in small area vs. (16.2±4.2)% in large area (Figure 2). The overall survival of patients with NSCLC was also stratified based on the AgNOR area in MIB-1-positive cells, and the clinicopathological parameters showed statistically significant difference (Table 2).

### Table 1 AgNOR area in MIB-1-positive cells and clinicopathological parameters of NSCLC

| Clinicopathological parameters | n (%) | AgNOR area (in µm²) | P   |
|-------------------------------|-------|---------------------|-----|
| T status                      |       |                     |     |
| T₁                            | 55 (27) | 8.95 (7.60 to 10.91) | <0.001|
| T₁ to T₃                      | 152 (73) | 11.08 (8.92 to 13.16) |     |
| Greatest tumor dimension      |       |                     | <0.001|
| <3 cm                         | 87 (42) | 8.93 (7.60 to 10.92) |     |
| ≥3 cm                         | 120 (58) | 11.60 (9.37 to 13.42) |     |
| N status                      |       |                     | <0.001|
| N₀                            | 132 (64) | 9.43 (8.04 to 11.88) |     |
| N₁ to N₃                      | 75 (36)  | 11.89 (10.37 to 14.23) |     |
| TNM stage                     |       |                     | <0.001|
| I                             | 107 (52) | 9.35 (7.89 to 11.87) |     |
| II to III                     | 100 (48) | 11.41 (9.21 to 13.75) |     |
| Histology                     |       |                     | 0.8  |
| Adenocarcinoma                | 94 (45)  | 10.20 (8.79 to 12.53) |     |
| Squamous cell cancer          | 113 (55) | 10.58 (8.37 to 12.69) |     |
| Differentiation               |       |                     | <0.001|
| Well                          | 52 (25)  | 8.79 (7.73 to 10.95) |     |
| Moderate to poor              | 155 (75) | 11.01 (8.97 to 13.04) |     |

n, number of cases; %, percentage of cases; T, T status under TNM system; N, N status under TNM system; P, P value (statistical significance). AgNOR, argyrophilic nucleolar organizer region; NSCLC, non-small-cell lung cancer.

### Table 2 Five-year overall survival stratified according to cut-off point of AgNOR area in MIB-1-positive cells and clinicopathological parameters of NSCLC

| Clinicopathological parameters | AgNOR area, n (%) | P   |
|-------------------------------|------------------|-----|
| T status                      |                   |     |
| T₁                            | 40 (54.9±9.3) | 15 (22.9±12.1) | 0.006|
| T₁ to T₃                      | 66 (63.1±6.7) | 86 (14.7±4.2) | <0.001|
| Greatest tumor dimension      |                   |     |
| <3 cm                         | 62 (63.9±7.2) | 25 (26.5±9.2) | <0.001|
| ≥3 cm                         | 44 (54.3±8.8) | 76 (12.5±4.3) | <0.001|
| N status                      |                   |     |
| N₀                            | 87 (63.6±5.9) | 45 (34.7±8.5) | <0.001|
| N₁ to N₃                      | 19 (37.1±15.6) | 56 (0) | <0.001|
| TNM stage                     |                   |     |
| I                             | 73 (65.4±6.4) | 34 (37.6±10.0) | 0.002|
| II to III                     | 33 (46.8±11.0) | 67 (4.2±2.7) | <0.001|
| Histology                     |                   |     |
| Adenocarcinoma                | 50 (45.0±9.0) | 44 (7.9±4.8) | <0.001|
| Squamous cell cancer          | 56 (72.3±6.7) | 57 (19.6±6.2) | <0.001|
| Differentiation               |                   |     |
| Well                          | 37 (68.6±9.2) | 15 (44.4±13.5) | 0.03|
| Moderate to poor              | 69 (54.9±6.8) | 86 (10.9±3.7) | <0.001|

n, number of cases; T, T status under TNM system; N, N status under TNM system; P, P value (statistical significance). AgNOR, argyrophilic nucleolar organizer region; NSCLC, non-small-cell lung cancer.
In univariate analysis, the following factors affected the survival of patients: AgNOR area in MIB-1-positive cells ($\chi^2=59.9$, $P<0.001$), N status ($\chi^2=52.2$, $P<0.001$), TNM stage ($\chi^2=44.2$, $P<0.001$), differentiation ($\chi^2=21.7$, $P<0.001$), greatest tumor dimension ($\chi^2=21.3$, $P<0.001$), type of surgery ($\chi^2=8.7$, $P=0.002$), T status ($\chi^2=6.9$, $P=0.01$), and histological characteristics ($\chi^2=5.2$, $P=0.02$). Age, gender, postoperative chemotherapy, and radiation therapy did not affect the survival of patients with NSCLC in univariate analysis. In multivariate analysis ($\chi^2=120.2$), the survival of patients was affected by the AgNOR area in MIB-1-positive cells ($\beta=1.05$, standard error =0.23, $P<0.001$), greatest tumor dimension ($\beta=0.94$, standard error =0.35, $P=0.007$), metastases to regional lymph nodes ($\beta=0.79$, standard error =0.34, $P=0.02$), histology ($\beta=0.23$, standard error =0.11, $P=0.03$), and differentiation ($\beta=0.66$, standard error =0.29, $P=0.02$). Likewise, age, gender, type of surgery, postoperative chemotherapy, radiation therapy, T status, and TNM stage did not influence the survival of patients with NSCLC in multivariate analysis.

**Discussion**

Our study found the relationship between the AgNOR area in MIB-1-positive cells of NSCLC and the following clinicopathological parameters under the TNM system: T status, greatest tumor dimension, N status, TNM stage, and differentiation. Other studies have also shown the relation of individual clinicopathological parameters under the TNM system with AgNOR in MIB-1-positive cells. Yamaguchi\(^{13}\) found that AgNOR increases in MIB-1-positive cells of NSCLC in T\(_1\) vs. T\(_0\), T\(_2\) to T\(_1\), and in N\(_0\) to N\(_1\), N\(_0\) to N\(_3\). Kidogawa et al.\(^{10}\) also found that AgNOR increases in MIB-1-positive cells of breast cancer with the largest size of >2 cm vs. tumor size <2 cm. Tomobe et al.\(^{12}\) found a sequential increase in AgNOR in MIB-1-positive cells of bladder cancer in T\(_p\), T\(_2\), and T\(_3\). In contrast to our current results obtained from objective analysis (computer image analysis and measurement of AgNOR area), these previous results were obtained from subjective analysis (visual counting of AgNOR number). Other studies have shown the relationship between clinicopathological parameters under TNM system and AgNOR\(^{4,16}\). Thus, our study showed that the AgNOR area in MIB-1-positive cells was correlated with clinicopathological parameters, thereby reflecting the relationship among molecular, biological, and clinicopathological parameters of NSCLC.

The survival of patients with NSCLC with small AgNOR area in MIB-1-positive cells is better than that of those with greater AgNOR area, as well as in homogeneous groups of clinicopathological parameters. In univariate and multivariate regression analyses, the AgNOR area in MIB-1-positive cells independently affected the survival of patients with NSCLC.

Our results correlate with those of studies on NSCLC\(^{9,10,11}\), breast cancer\(^{7,8,16,17}\), and bladder cancer\(^{12}\). Studies on AgNOR in malignant tumors have also revealed that the content of AgNOR is an independent prognostic factor\(^{16,19}\). The prognostic value of AgNOR in MIB-1-positive cells is related to different rates of NSCLC proliferation. A large AgNOR area in MIB-1-positive cells indicates short cell cycle of proliferating cells and high proliferation speed. By contrast, small area implies long cell cycle of proliferating cells and low proliferation speed. Thus, our study showed that the AgNOR area in MIB-1-positive cells was correlated with the survival of patients with NSCLC; this result revealed the relationship of molecular and biological parameters, as well as biological behaviors of tumors. The current results provide further insights into an accurate assessment of actual survival curves and prognosis of patients with NSCLC.

**Conclusion**

In NSCLC, clinicopathological parameters under the TNM system are related to molecular and biological parameters, including AgNOR area in MIB-1-positive cells. The survival of patients with NSCLC with a small AgNOR area in MIB-1-positive cells is better than that of patients with a large AgNOR area. Molecular, biological (AgNOR area in MIB-1-positive cells), and clinicopathological (greatest tumor dimension, metastases to regional lymph nodes, histology, and differentiation) parameters are independent prognostic factors of NSCLC.

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**Conflict of interest statement**

No potential conflicts of interest are disclosed.

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