Clinical Significance of Double Staining of MIB-1 and AgNORs in Primary Breast Carcinoma

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Abstract. Background: Argyrophilic nucleolar organizer regions (AgNORs) and MIB-1 as proliferating activities have been applied separately to assess the malignant potential of cancer cells. We conducted staining of AgNORs and MIB-1 in 42 surgically-resected invasive breast carcinomas. Materials and Methods: Paraffin-embedded sections were used for double staining and the mean AgNOR counts in 100 MIB-1-positive and -negative cells were calculated. Results: The mean AgNOR count in MIB-1-positive cells was significantly higher than in MIB-1-negative ones. AgNOR counts in MIB-1-positive tumors were significantly higher in tumors ≥2 cm and those with positive nodes. Multivariate analysis identified the AgNOR count in MIB-1-positive tumors as the only independent factor related to node metastasis. Survival of patients with lower counts of AgNORs in MIB-1-positive tumors was significantly better compared to those with higher counts. Conclusion: Double staining of MIB-1 and AgNORs is useful for predicting lymph node metastasis and prognosis of patients with breast carcinoma.

The proliferative activity is an important component of the metastatic potential of tumors or prognosis in cancer patients. Therefore, it is crucial to identify the extent of such activity so that the most appropriate therapy can be selected and the prognosis can be predicted.

Based on the pathological criteria, molecular biology techniques may be useful in staging the process of malignancies and to know their biological behavior. The argyrophilic nucleolar organizer regions (AgNORs) are loops of DNA present on the short arms of acrocentric chromosomes that have been associated with protein synthesis and ribosomal activity (1). AgNORs have been successfully visualized in the later interphase period on metaphase spreads by colloidal silver staining technique (2), in routinely processed histological and cytological preparations (3). The number of AgNORs is thought to reflect the proliferative activity of tumor cells. Crocker et al. (4) reported that AgNOR counts per nucleus could differentiate high- from low-grade non-Hodgkin’s lymphomas. Other studies have shown informative results in a variety of other neoplasmas (5-8). In general, the AgNOR counts in malignant cells are greater than in histologically less malignant and benign tumors (5-8). In breast carcinomas, the number of AgNORs is significantly related to tumor size (9, 10), DNA ploidy or S-phase fraction (9, 11), and tumor stage (12). However, it has been reported that there were no correlations between the AgNOR count and any clinicopathological parameters (13). The MIB-1 (Ki-67) monoclonal antibody seeks different epitopes of the same proliferation-related antigen presenting on non-resting cells, which usually identifies cells in the G1-, S-, G2- and M-phases of the cell cycle (14-17). At present, MIB-1 staining can be applied to paraffin-embedded fixed sections (18). Janmohamed et al. examined proliferative activity in non-Hodgkin’s lymphoma using Ki-67 and AgNORs, in which the AgNOR counts in Ki-67-positive cells proved to be significantly higher than those in negative cells (19).

The above background prompted us to assess the characteristics of proliferating and non-proliferating cells in solid tumors by AgNORs-MIB-1 double staining. The main hypothesis of the present study was that the proliferation activity of the synthetic phase of the cell cycle in cancer cells reflects the malignant behavior of the tumor. To evaluate the hypothesis, we examined 42 surgically-resected MIB-1-AgNORs double-stained invasive breast carcinomas. Our goal was to determine the correlation between proliferating activity in the synthetic phase and clinicopathological parameters or patient prognosis.

Materials and Methods

Patients and surgical specimens. Specimens were obtained from 42 female patients with primary invasive breast cancer who underwent surgical resection at the Division of Surgical Oncology, Nagasaki
University Graduate School of Biomedical Sciences, Japan, between 1996 and 1998. Patients who preoperatively received anticancer drugs were excluded from this study. The patient demographics are shown in Table I. All patients were followed-up for at least 6 years. The experimental protocol was approved by the Humans Ethics Review Committee of our hospital. After surgery, the specimens were fixed in formalin and processed in paraffin. Histological diagnosis was carried out by light microscopy of hematoxylin- and eosin-stained tissue sections by an experienced pathologist. Histological subtype and tumor stage were described according to the General Rules for Clinical and Pathological Recording of Breast Cancer (20).

**Immunohistochemistry.** Specimens of approximately 1 cm³ were cut from the resected tumor, fixed in 10% neutral buffered formalin for 24 hours and then embedded in paraffin. In the next step, 3-μm-thick sections were cut for staining (one section was stained with hematoxylin/eosin). Paraffin was removed with xylene and the sections were rehydrated with serial dilutions of ethanol solution. After microwaving in 0.01 M citric acid buffer for 15 minutes, the slides were sequentially stained for MIB-1 and AgNORs. The anti-Ki-67 mouse monoclonal antibody (MIB-1; Immunotech, Marseille, France) was used as the primary antibody at a dilution of 1:100. MIB-1 was reacted with biotinylated anti-immunoglobulin and reagent using the labeled streptavidin-biotin (LSAB) kitPeroxidase® (DAKO Co., Carpenteria, CA, USA) alkaline phosphatase with red as the first chromogen. Thereafter, the preparations were thoroughly washed with distilled water, and the AgNORs were visualized by one-step silver staining as described by Ploton et al. (3). Briefly, 2% gelatin in 1% formic acid was mixed at a ratio of 1:2 with 50% silver nitrate and reacted in the dark for 30 minutes. For comparison, single AgNORs stain was performed in the same manner. Slides were decolorized with 5% sodium thiosulfate and washed by distilled water.

**Evaluation.** All sections were observed under a light microscope (VANOX®, Olympus, Tokyo, Japan) at a magnification of X1000. For MIB-1-stained sections, the nuclei were stained in red and all nuclei with detectable staining above the background were scored as positive. Five hundred nuclei per specimen were counted to determine the MIB-1 labeling index, i.e., the proportion of MIB-1-positive cells. AgNOR-stained cells were counted as described by Crocker et al. (21), i.e., where AgNORs were seen separately within a nucleolus, each AgNOR was counted as a unit, together with the smaller AgNORs seen outside the nucleolus. Figure 1 shows a photograph of a representative section simultaneously stained for MIB-1 and AgNORs. The MIB-1-positive nuclei were stained in red. A total of 200 nuclei were counted for evaluating the results of simultaneous staining for AgNORs and MIB-1. The number of AgNOR grains in each cell, and the mean number of AgNOR granules in 100 MIB-1-positive cells and 100 MIB-1-negative cells were determined.

**Estrogen and progesterone receptors.** Estrogen and progesterone receptors were examined by conventional immunohistochemistry using paraffin-embedded tissue fixed in 10% neutral buffered formalin (Biomedical Laboratories Inc., Tokyo, Japan).

**Statistical analysis.** The data were expressed as mean±SD. The data of different groups were compared using one-way analysis of variance (ANOVA) and examined by Student’s t-test. The relationship between AgNOR counts and clinicopathological parameters was examined with the Mann-Whitney test. Correlations between two parameters were examined by calculating Pearson’s correlation coefficient. Multivariate analysis was performed to predict lymph node metastasis using logistic regression analysis. Survival curves were calculated by the product limit estimate of Kaplan and Meier. Statistical significance between curves was assessed using the log-rank test. All statistical analyses were carried out using Stat View-J 5.0 software (Abacus Concepts, Inc., Berkeley, CA, USA).

**Results**

**Single staining for MIB-1 and AgNORs.** In single staining for each protein, the MIB-1 labeling index did not correlate with the AgNOR counts (Figure 2). The number of MIB-1-positive cells ranged between 2.1% and 59.3%, with a mean of 16.2%. The AgNOR counts and MIB-1 labeling index did
not correlate with distant metastasis, expression of estrogen-receptor and progesterone-receptor and histological differentiation or types (data not shown).

**Relationship between immunohistochemistry and clinico-pathological parameters.** AgNOR counts in MIB-1-positive cells were significantly higher than in MIB-1-negative cells and AgNOR counts in single-stained cells (Figure 3). Figure 4 shows the relationships between MIB-1 and AgNOR counts, and tumor size. Only in MIB-1-positive cells, the mean AgNOR count in tumors with a diameter >2 cm was significantly greater than in tumors <2 cm in diameter. Figure 5 shows the relationships between MIB-1 and AgNOR counts, and lymph node status. Only in MIB-1-positive cells, the AgNOR counts in node-positive tumors were significantly greater than node-negative tumors.
predict the factors that correlate with lymph node metastasis, multiple logistic regression analysis was performed using five parameters: tumor size, estrogen- and progesterone-receptor status, MIB-1 labeling index, and mean AgNOR count in MIB-1-positive cells. Such analysis identified the mean AgNOR counts in MIB-1-positive cells as the only independent factor that correlated with lymph node metastasis (Table II).

**Relationship between immunohistochemistry and patient prognosis after resection.** The predictive cut-off value of AgNOR counts in MIB-1-positive cells was set at 4.5 (median value) in the present study. The survival rate in patients with high AgNOR counts in MIB-1-positive cells was significantly poorer than in patients with lower counts (Figure 6). There were no significant relationships between other factors, such as tumor size, estrogen- or progesterone-receptor, only MIB-1 labeling index and only AgNOR counts, and patient survival (data not shown).

**Discussion**

The malignancy evaluation of carcinoma cells based on their proliferating potential using growth-related markers such as PCNA, Ki-67 and DNA polymerase-alpha has been studied extensively in various organs by various molecular techniques (9-12, 14-18, 21). The AgNOR count has been studied as an indicator of biological aggressiveness in primary breast cancer, in addition to other markers (9-12). Crocker *et al.* have shown that higher AgNOR counts reflected increased proliferative activity (22). Other studies showed, however, that the number of AgNORs did not correlate with any of the prognostic variables and AgNOR counts did not appear to be of prognostic value in breast cancers (11, 13). In contrast, Ohri *et al.* showed an inverse relationship between AgNOR counts and lymph node status (12). Opposing results in single staining of AgNORs have hitherto been described by other (23). Thus, the significance of AgNOR counts in breast cancer is still controversial,
Ki-67 for predicting patient survival after surgery. The showed the usefulness of double staining of AgNORs and evaluating tumor aggressiveness. The present result also using the double staining technique seems to be useful for Thus, our results indicate that simultaneous evaluation could lead to tumor growth and lymph node metastasis. Positive seem to be growth/metastasis aggressive, which tumor cells with higher AgNOR counts that are MIB-1- comparison with tumor characteristics. Our results indicated correlation between the AgNOR count in MIB-1-positive cells and any clinicopathological parameters. In the present study, the AgNOR counts in MIB-1-positive cells only, but not in MIB-1-negative cells, correlated significantly with tumor size and lymph node metastasis. Furthermore, this result was independently associated with node metastasis in comparison with tumor characteristics. Our results indicated that tumor cells with higher AgNOR counts that are MIB-1-positive seem to be growth/metastasis aggressive, which could lead to tumor growth and lymph node metastasis. Thus, our results indicate that simultaneous evaluation using the double staining technique seems to be useful for evaluating tumor aggressiveness. The present result also showed the usefulness of double staining of AgNORs and Ki-67 for predicting patient survival after surgery. The simultaneous evaluation of proliferating activities should be more useful to identify the malignant behavior of breast carcinomas in comparison with each single staining analysis. Our present study was the first report to show the usefulness of double staining of AgNORs and Ki-67.

In conclusion, we examined the relationship between MIB-1-positive cell and AgNOR counts, and clinicopathological findings in breast carcinomas using a simultaneous double staining technique. Higher AgNOR counts in MIB-1-positive cancer cells significantly correlated with larger tumor size, lymph node metastasis and poor patient survival after resection. This parameter of proliferating activity using a double staining technique is a potentially useful indicator of malignant activity compared with a single AgNOR stain.

Acknowledgements

The authors thank Dr. F. G. Issa (www.word-medex.com.au) for the critical reading and editing of the manuscript. This article is dedicated to the former Professor Hiroyoshi Ayabe, who also contributed to this work.

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Received April 7, 2005
Accepted August 30, 2005