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Significance of AgNORs Ratio as a Prognostic Factor of Breast Cancer

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The numbers and the sizes of Argyrophilic Nucleolar Organizer Regions (AgNORs) were analysed in 76 cases of mammary invasive ductal carcinoma for the evaluation of biologic behaviour of breast cancer. For the measurement of AgNORs size, a total of AgNORs area per nucleus and the ratio of AgNORs area to the nuclear area were measured by automatic image analyzer as "AgNORs area" and "AgNORs ratio", respectively. Concerning the tumor grade, the mean AgNORs area and ratio were significantly increased in high grade tumor (area: $p < 0.001$, ratio: $p < 0.05$). A significant increase ($p < 0.05$) of the mean AgNORs ratio was shown in patients with nodal metastasis. The survival time was analysed among four groups separated according to the lymph node status and the mean value of AgNORs amounts. As a result in patients with nodal metastasis, the group that showed high AgNORs ratios of more than the mean value had significantly ($p < 0.05$) shorter survival time as compared with the group of less than the mean AgNORs ratio. There was a tendency in the group of larger AgNORs areas toward poorer prognosis than that of smaller AgNORs areas. On the contrary, the AgNORs number was of no value to predict the prognosis. In the patients without nodal metastasis, the survival time was not affected by any of the AgNORs parameters.

In conclusion, the AgNORs ratio was disclosed to be an important variable in predicting the prognosis of the patients with lymph node metastasis of breast cancer.

INTRODUCTION

Nucleolar Organizer Regions (NORs) are organized as tandem repeats of 800 rDNA genes together with non-transcribed spacers in five pair of acrocentric chromosomes: 13, 14, 15, 21 and 22[1]. During interphase, these regions were shown to be loops of rDNA working continuously for transcription of rRNA genes to supply ribosomes which are closely related to protein synthesis. When cells are in the proliferative state, activation of the rRNA transcription in the NORs and increase of protein synthesis are seen in a series of cell cycles.

Argyrophilic Nucleolar Organizer Regions (AgNORs) are a silver stain of NORs-associated protein which is identified as a black dot in nucleus. The numbers of AgNORs tend to be more numerous in the malignant tumors than they are in those of benign neoplasms. Enumeration of AgNORs have attracted much attention because of claims that their numbers assist in the distinction between high- and low-grade lymphoma[2], benign melanocytic lesions from malignant melanoma[3] and benign breast lesions from malignant lesions[4].

A great number of studies have shown the importance of clinical prognostic factors of breast cancer for the choice of appropriate operation; which could be the employment of adjuvant chemo-hormonal therapy and/or radiotherapy. It is recognized that the prognosis of breast cancer largely depends on the lymph node involvement. On the other hand, there has been a constant search for molecular biological indicators that would predict the prognosis of breast cancer. AgNORs is one of the methods which has been studied as a prognostic indicator. However, it has been shown that the AgNORs number in breast cancer has no correlation with the disease free interval or survival rate[5,6].

Since it has been proven that not only the number of AgNORs but also the size of AgNORs correlate with cell duplication activity[7], the size of AgNORs is supposed to have a predictable potential for the prognosis of breast cancer. A concept of AgNORs size could be divided into two categories. One is "AgNORs area", which represents the total amount of AgNORs area per nucleus. Another is "AgNORs ratio", which is the proportion of AgNORs area to the nuclear area. Up to the present, very few reports evaluating the prognosis of breast cancer were published by the AgNORs area. There has never been a report published in the study pertaning to the AgNORs ratio.

The present study was undertaken to determine if the number or the size of AgNORs are useful variables as prognostic factors of breast cancer with the study of lymph node involvement.

MATERIALS AND METHODS

Paraffin blocks from 76 cases of breast cancer operated at the First Department of Surgery, Nagasaki University School of Medicine from 1981 to 1993 were obtained.
Patients with distant metastasis were excluded from the study (Table 1).

Table 1. Characteristics of 76 patients

variables	number of patients (%)
Age	
mean :52.4 years	
range :25-79	
Menopausal status	
pre	27(36)
post	49(64)
Histopathology	
papillo-tubular	21(28)
solid-tubular	25(33)
scirrhous	30(39)
Clinical stage (JBCS)	
I	21(28)
II	33(43)
III	22(29)
Lymph node status	
negative	42(55)
positive	34(45)
Total	76

AgNORs silver staining

Four-micron sections of representative blocks were prepared in 76 cases of invasive ductal carcinoma and the staining was carried out using a solution of one volume of 2% gelatin in 1% aqueous formic acid and two volumes of 50% silver nitrate. The resulting colloid was placed on the sections and allowed to react for 20 min at room temperature in the absence of light.

Measurement of AgNORs numbers and sizes

In each patient, 100 of cancer cells were examined using a × 100 oil-immersion lens. The numbers of AgNORs "dots" were counted according to the recommendation of Crocker et al.[8], i.e., where dots were seen separately within a nucleolus, each dot was included in the count together with those lying outside the nucleolus. The numbers of AgNORs were assessed with careful focusing. At the same time, the AgNORs areas and the AgNORs ratios were measured using a specific program of an automatic image analyzer (IBAS 2000, KONTRON) with a light microscope (Carl Zeiss).

Histopathological grading

Tumor grading was done by the Scarff-Bloom-Richardson scheme[9] for 33 cases of invasive ductal carcimoma according to the following histological criteria: 1) Tuble formation; 2) Hyperchromatism and mitosis; and 3) Irregularity of size, shape and staining of nuclei. For this classification, grade 1 tumor is considered indicative of low malignancy, grade 2 tumor, intermediate malignancy, and grade 3 tumors, high malignancy.

RESULTS

AgNORs and tumor grade

Concerning the tumor grade, significant increases of the mean AgNORs area and ratio were observed in tumors with higher malignancy grades (area: $p<0.001$, ratio: $p<0.05$). On the contrary, there was no correlation between the AgNORs numbers and the tumor grades (Table 2).

AgNORs and lymph node status

Lymph nodes were examined histologically. The mean value of AgNORs numbers, areas and ratios in patients without nodal metastasis were 4.26, 3.56 μm^2 and 6.45%, respectively. Patients with nodal metastasis had higher mean AgNORs in all the parameters as 4.38, 3.95 μm^2 and 7.18%, respectively. A significant increase ($p<0.05$) of the mean AgNORs ratio was shown in patients with nodal

Table 2. The mean value of AgNORs parameters in each tumor grade

	n	number	area(μm^2)	ratio(%)
Grade 1	9	4.71±1.17	2.81±0.42**	5.63±0.83*
Grade 2	16	4.38±1.41	3.58±1.00**	6.71±1.40*
Grade 3	8	5.42±1.63	5.30±0.54**	7.72±1.76*

*$p<0.05$, **$p<0.001$: Significantly different among the groups (Kruskal-Wallis test)

Table 3. The mean value of AgNORs parameters and lymph node status

	n	number	area(μm^2)	ratio(%)
LN(−)	42	4.26±1.31	3.56±1.25	6.45±1.37*
LN(+)	34	4.38±1.55	3.95±1.01	7.18±1.47*

*$p<0.05$: Significantly different between the groups (Wilcoxon-Mann-Whitney test)

metastasis (Table 3).

Lymph node status and survival

Fig.1. shows the survival curves of the 76 patients and illustrates its position with regard to the two resulting curves of lymph node status. The overall test of significance among these two groups shows a markedly significant difference ($p<0.001$). This result shows that the lymph node status is a greatly influential factor on the prognosis of breast cancer.

AgNORs and survival

Most of the mortal cases possessed larger sizes of AgNORs amounts as compared to survival cases (Fig.2, 3). A statistical analysis were carried out to confirm this phenomenon. Survival time was analysed among four groups separated according to the lymph node status and the mean value of AgNORs amounts. As the results of the analysis in patients with nodal metastasis, the group with higher AgNORs numbers than the mean value had a better survival time as compared to that with lower AgNORs numbers. This result expalains that the AgNORs number is of no usefulness for the prediction of the prognosis (Fig.4). The group with larger AgNORs areas had a tendency toward poorer prognosis than that with smaller AgNORs areas (Fig.5). As to AgNORs ratio, the survival time was significantly ($p<0.05$) worse in the group with higher AgNORs ratios than that with lower AgNORs ratios (Fig.6). Subsequently, patients without nodal metastasis were analysed in the same way. The result showed that the survival time was not affected by any of the AgNORs parameters (Fig.4, 5 and 6).

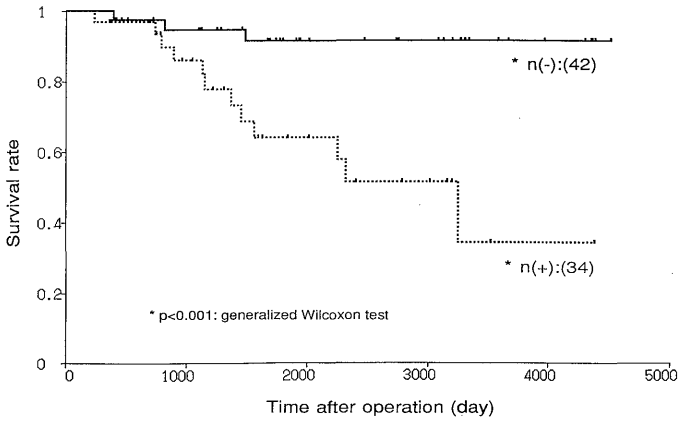

Fig.1. Survival curves (Kaplan-Meier method) of 76 patients distributed by their lymph node status. n (+) and n (−): the status of lymph node metastasis proven histologically.

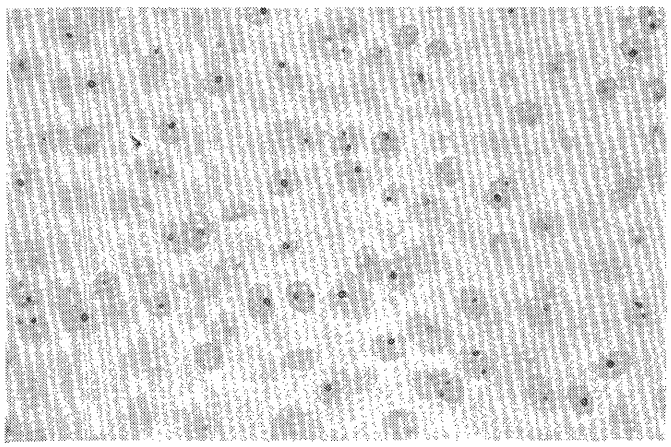

Fig.2. Survival case of breast cancer. Comparatively small sizes of AgNORs are seen in nuclei.

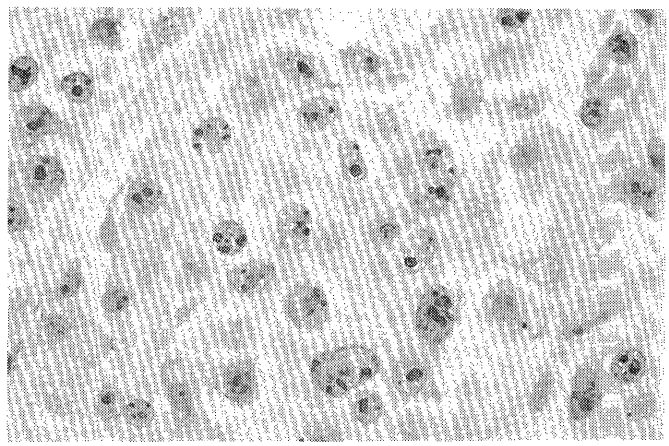

Fig.3. Mortal case of breast cancer. Most of the mortal cases possessed large sizes of AgNORs.

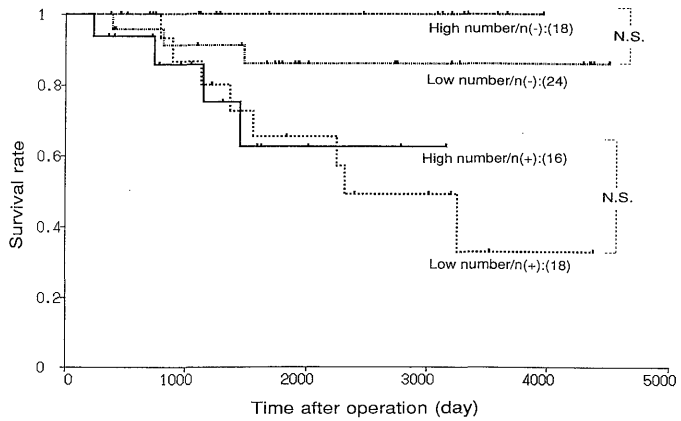

Fig.4. Survival curves (Kaplan-Meier method) and AgNORs number. Patients were separated into four groups according to the lymph node status and the mean value of AgNORs number. High- and Low -number : more or less than the mean value of AgNORs number.

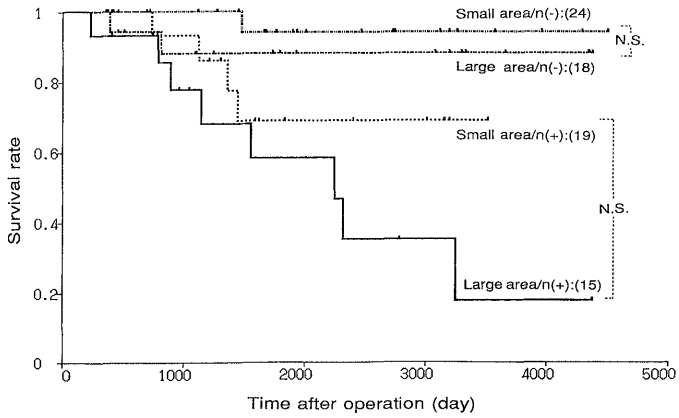

Fig.5. Survival curves (Kaplan-Meier method) and AgNORs area. Separated according to the lymph node status and the mean value of AgNORs area. Large- and Small- area: more or less than the mean value of AgNORs area.

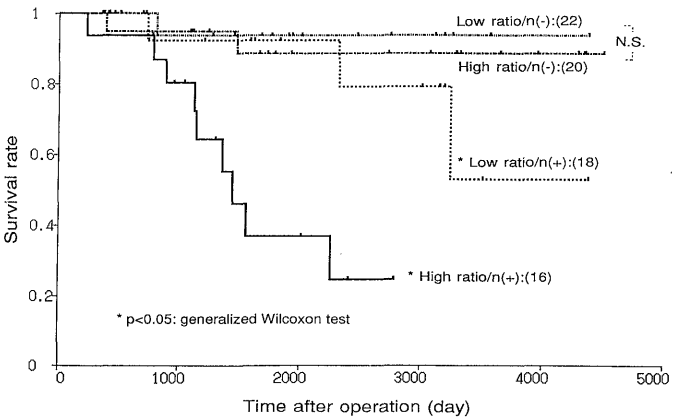

Fig.6. Survival curves (Kaplan-Meier method) and AgNORs ratio. Separated according to the lymph node status and the mean value of AgNORs ratio. High- and Low -ratio : more or less than the mean value of AgNORs ratio.

DISCUSSION

AgNORs reaction was first described in 1975 as a three-step silver staining technique[10,11]. Recently, the method was subsequently abbreviated to a one-step sequence which can be carried out easily at room temperature[12]. Protein C23, B23[13] and RNA polymerase I [14], called NORs-associated protein, have been considered responsible for the silver staining component. The increased number of AgNORs has been shown to correlate with the activation of the rRNA transcription[15] and cell proliferation level[16].

While the efforts have been made by several investigators to establish AgNORs number as a reliable prognostic factor, the high number of AgNORs has not indisputably been a poor prognostic sign. Sivridis and Sims[17] showed in their study among 20 cases of breast cancer that patients with metastasis in four or more lymph nodes possessed significantly high AgNORs numbers. On the contrary, inverse result was reported by Sacks et al.[5] that no correlations were observed between AgNORs number and disease-free interval or survival. Toikkanen et al.[6] reported that AgNORs number had no predictive value on the 8-year survival rate and didn't relate to any other prognostic variables studied including histologic type, tumor grade, mitotic count, tumor size or lymph node status. The result seems to be more reliable because of the large number of patients (230 cases) in their study. A similar result was shown in the present study that the AgNORs number was not related to tumor grade, lymph node status or survival time.

There are two different ways to count AgNORs numbers. 1) The clusters of AgNORs dots both inside and outside the nucleoli are taken as one structure. 2) AgNORs dots within a nucleolus are counted separately. The latter method naturally gives higher counts in the same sample. In the present study, the counting of AgNORs numbers was carried out according to the latter method which was recommended by Croker et al. that any separation could be obtained between benign and malignant breast tumors[8]. However, the enumeration method has some problem. Since stored tissue samples have difference of silver stainability even within the same staining time, some specimens are stained so much that whole nucleolus is seen as a single silver stained structure. In those cases, each AgNORs dot can't be discernible within a nucleolus. The variability of argyrophilia between different tissue blocks may be due to the lack of standardization of formalin fixation time. Besides, when the specimen was rather argyrophilic, the background outside the AgNORs is simultaneously stained by silver. Consequently, it sometimes makes us troublesome to discriminate non-specific silver stains from the AgNORs dots. An observer's standard to identify the true AgNORs dots sometimes depends on his subjectivity. Thus, these problems may have hampered from counting the true AgNORs numbers.

It is well-known that not only the numbers of AgNORs but also the sizes of AgNORs vary among the various malignant cells. The morphometric approach using automatic image analyzer in the evaluation of AgNORs has the advantage over simply counting by eyes as it involves the determination of the size of AgNORs objectively. Derenzini et al.[7] have compared AgNORs areas of 12 different types of human tumor cell lines with their proliferation rate. In their report, a linear relationship was shown between cell duplication activity and AgNORs areas independently of the type of cancer cell lines.

Relationship between the clinical behaviour of breast cancer and the size of AgNORs was reported by two authors. Kolar et al.[18] examined the diameters of AgNORs in 109 cases of breast cancer. In their report, the significant short survival were observed in patients with AgNORs larger than 5 μm in diameter. However, they commented that the measurement of AgNORs diameters visually was not very accurate because the selected

magnification ×400 could not reliably distinguish between associated and dissociated silver granules. Eusebi et al.[19] introduced automatic image analysis to quantify the AgNORs areas in 14 cases of sarcomatoid carcinoma of breast. They showed that patients who died early of the disease had larger AgNORs areas than patients who survived longer than 3 years. Although this is the only report that has disclosed the relationship between the AgNORs area and survival of breast cancer, the problem seems to remain in their methodology that they analysed absolute quantity of AgNORs areas among mixed types of histopathology of the carcinomatous component, invasive ductal carcinoma and squamous carcinoma. Since the area of AgNORs may depend on its nuclear area and the type of histopathology to some extent, the ratio of AgNORs area to the nuclear area is considered to be more proper parameter than the absolute quantity of AgNORs area for the evaluation of malignancy of the disease. It is speculative as the reality on the basis of a result in this study that the AgNORs ratio influences significantly on the survival time but the AgNORs area does not so much. Besides, it is worth while noticing this fact that usefulness of AgNORs ratio was found in patients with lymph node metastasis. Since it was shown that the prognosis of the patients with nodal metastasis is poor, such a patient with high AgNORs ratio suggests the necessity of more aggressive therapy.

Up to the present, the reports concerning to survival of breast cancer evaluated by AgNORs ratio have not been reported. Under the circumstances, the present author apllied image cytometry for the evaluation of AgNORs ratio in correlation with clinicopathological factors in breast cancer for the first time.

It is of great value to introduce AgNORs method in the clinical works because of not only its predictive usefulness of patient's prognosis, but also the inexpensiveness of reagents and simplicity of the technique.

This study clarified the fact that assessment of AgNORs ratio is an important clue to knowing the patient's prognosis and also provides a valuable information of the malignant biological behavior in patients with lymph node metastasis of breast cancer.

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