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Simultaneous staining of Ki-67 and *chromosome 8* in invasive ductal carcinoma: association with prognosis

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Introduction: This study aimed to evaluate an approach that uses simultaneous staining to estimate malignant potential. This approach combines immunofluorescence (IF) staining for Ki-67 expression with fluorescence in situ hybridization (FISH) for copy number aberrations (CNA) of chromosome 8 in breast cancer cells.

Methods: In 50 specimens of invasive ductal carcinoma (IDC), we examined a method that simultaneously combined immunostaining (Ki-67) and FISH with a chromosome 8 centromere-specific probe. Breast cancer cells were classified into Group 1, Ki-67 positive and chromosomal aberrant; Group 2, Ki-67 negative and chromosomal aberrant; Group 3, Ki-67 positive and chromosomal wild; Group 4, Ki-67 negative and chromosomal wild.

Results: The frequency of Group 1 was significantly associated with nodal metastasis ($p<0.05$) and patient prognosis ($p<0.05$); however, it was not associated with age, tumor size, estrogen receptor status, progesterone receptor status, or histological type. Furthermore, Group 1-positive cases showed a significantly worse prognosis, as shown by the Kaplan–Meier method.

Conclusions: We successfully stained for Ki-67 expression and CNA of chromosome 8 in breast tumor sections (n=50). This approach indicated that Ki-67-positive cells with aberrant chromosome 8 were associated with malignant potential in IDC.

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Introduction

Metastasis is a major cause of death in patients with solid tumors. A variety of genetic alterations have been implicated in tumor progression, including breast carcinogenesis. Recent molecular pathological techniques allow us to examine the molecular abnormalities associated with tumor progression. Combined immunofluorescence (IF) and fluorescence in situ hybridization (FISH) techniques were previously introduced for cytological preparations[1, 2]. Our previous study using this technique demonstrated that the frequency of cells expressing both the immunoactivity of proliferating cell nuclear antigen (PCNA) and CNA of chromosome 17 can be an indicator of the metastatic potential of gastric cancers[3]. Thus, similar approaches may be available to estimate the malignant potential of other types of cancer cells that are pre- or post-surgically obtained from patients. Therefore, we aimed to evaluate a new approach with simultaneous staining to estimate malignant potential, which utilizes a combination of IF for Ki-67 expression and FISH for CNA of chromosome 8 in breast cancer cells.

The Ki-67 labeling index (LI) by immunohistochemistry (IHC), a well-known proliferative indicator, is commonly used to estimate the proliferative activity of cancer cells in formalin-fixed paraffin-embedded (FFPE) tissues from patients. In breast

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cancer, high Ki-67 expression is strongly correlated with high-grade ductal carcinoma[4-6]. Furthermore, CNA of chromosome 8 is frequently demonstrated in a variety of solid tumors, including breast cancer, as evidenced by comparative genomic hybridization (CGH) and FISH analyses[7-14]. The present study revealed a relationship between the simultaneous expression of Ki-67 and CNA on chromosome 8 and clinicopathologic factors in breast cancer cells from patients, suggesting the clinical applicability of this staining method as an ancillary technique to estimate the malignant potential of breast cancers.

Materials and Methods

Patients

We included 50 cases of invasive ductal carcinoma (IDC) from female patients (median age, 56.3- years; range 31-76) who underwent surgery in the First Department of Surgery at the Nagasaki University Hospital from January 1991 to June 1996. Patients who received chemotherapy or radiotherapy before surgery and had bilateral cancers or synchronous multiple breast cancers were excluded from the study. Total axillary clearance (levels I, II, and III) was performed when the size of the breast tumor was 3 cm or larger. The clinicopathologic profiles, including histological type, TNM classification (by the American Joint Committee of Cancer) [15, 16], and hormone receptor status, are summarized in Table 1. The follow-up period for patient prognosis was until October 31, 2002. During the follow-up period, nine out of 50 cases (18%) died owing to tumor-related causes.

Table 1. Characteristics of invasive ductal carcinoma patients (n=50).

Clinicopathologic variables	Number of patients (n=50)
Tumor size (T)	
T1	9 (18%)
T2	35 (70%)
T3	5 (10%)
T4	1 (2%)
Lymph node metastasis (N)	
Positive	24 (48%)
Negative	26 (52%)
Distant metastasis (M)	
Positive	2 (4%)
Negative	48 (96%)
Histological type	
Differentiated	32 (64%)
Scirrhous	18 (36%)
Estrogen receptor (ER)	
Positive	31 (62%)
Negative	19 (38%)
Progesterone receptor (PR)	
Positive	30 (60%)
Negative	20 (40%)

Simultaneous IF staining for Ki-67 expression and FISH analysis for chromosome 8 aberrations

This procedure was performed as described in our previous report[3]. Touch preparation samples of freshly snap-frozen tissues from resected IDCs were prepared according to the procedure reported by Kovach *et al*.[17] and were subsequently subject to staining. Briefly, after washing in phosphate-buffered saline (PBS) twice for 5 min each at 4˚C, snap-frozen cancer tissue was gently touched on coated glass slides (MAS coated, MATSUNAMI Glass Ind. Ltd. Osaka, Japan), immersed in PBS for 10 min, and fixed in acetone for 15 min at -20˚C. Fixed slides were then air-dried and treated with 10% normal goat serum for 30 min at 24˚C. After washing in PBS twice for 5 min each, the slides were incubated with anti-Ki-67 mouse monoclonal antibody (Dianova, Hamburg, Germany) at a 1:20 dilution in PBS for 60 min at room temperature. The slides were subsequently incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Dianova) at a 1:20 dilution in PBS for 60 min. After washing twice in 4× standard sodium citrate (SSC)/0.05% Tween20 for 5 min, the slides were re-fixed with 4% paraformaldehyde for 3 min at 4˚C, followed by dipping in distilled water. The slides were dehydrated in a series of ethanol solutions (70%, 90%, and 100%), air-dried, and then processed for FISH analysis of chromosome 8 by a previous report by Pinkel *et al*.[18]. This analysis employed a biotin-labeled DNA probe for the chromosome 8 alpha-satellite (D8Z2, Oncor, Gaithersburg, MD). Briefly, 10 μL of hybridization mixture, which consisted of 1 μL labeled probe, 1 μL human placental DNA (Sigma Co., St. Louis, MO, USA), 10% dextran sulfate, and 50% formamide/2x SSC, was denatured in 70% formamide/2x SSC, pH 7.0, at 70˚C. The samples were denatured in 70% formamide/2x SSC (pH 7.0) for 2.5 min at 70˚C and then incubated with a denatured probe for 2 h at 37˚C in a humidified chamber. After hybridization, slides were washed three times for 10 min each time in 50% formamide/2x SSC, pH 7.0 at 45˚C, 2x SSC, pH 7.0 at 45˚C, and 2x SSC, pH 7.0, at 24˚C. After washing three times, the slides were treated with Texas Red-conjugated avidin (EY Laboratories, Inc., San Mateo, CA) in 4x SSC/1% bovine serum albumin (BSA) for 60 min at 24˚C. Finally, slides were

counterstained with an antifade solution containing 0.2 μg/ml 4',6-diamidino-2-phenylindole (DAPI) (Bio-Rad Laboratories, Hercules, CA, USA). Microscopic observation was performed using a Nikon FXA epifluorescence microscope (Nikon, Tokyo, Japan). FITC signals for Ki-67 expression and Texas Red signals for chromosome 8 were simultaneously visualized using dual bandpass filters for green and red. A total of 200 cancer cells per slide were examined to determine the number of signals.

Classification of cancer cells

Chromosome 8 aberrations were numerically evaluated as loss (single signal) or gain (triple or more signals) of each nucleus. The types of cancer cells were classified into the following four groups as described previously[3]; Group 1, Ki-67 positive and chromosomal aberrant; Group 2, Ki-67 negative and chromosomal aberrant; Group 3, Ki-67 positive and chromosomal wild; Group 4, Ki-67 negative and chromosomal wild. A total of 200 nuclei per case were examined and evaluated by calculating the frequency (%) of the above groups.

Statistical analysis

The Mann–Whitney U test (two groups) and Kruskal–Wallis test (three or more groups) were used for statistical analysis. The probability of survival was calculated using the Kaplan–Meier method, and survival differences were assessed using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards regression model. Differences were considered significant when the probability value (p-value) was less than 0.05. These statistical studies were performed using StatView-J 5.0 software (Abacus Concepts, Inc., Berkeley, CA, USA) for the Macintosh computer.

Results

Representative images of cancer cells expressing Group 1 are shown in Figure 1. All patients expressed two or more groups. The frequencies of Group 1, 2, 3, and 4 cells were 24.81±10.59% (range: 9-53%, median: 23.0%), 21.22±9.32% (range: 7-52%, median: 18.0%), 23.46±9.90% (range: 5-45%, median: 22.0%), and 30.51±11.31% (range: 11-53%, median: 28.0%) respectively, in our series of 50 breast cancers, indicating that a total of 10,000 cells were counted.

If 23%, which was equivalent to the median frequency of Group 1, was considered as the cut-off value for Group1-positive in each case for estimating a greater likelihood of malignancy, Group 1 was significantly associated with nodal metastasis ($p<0.05$), and patient prognosis ($p<0.05$); however, it was not associated with age, tumor size, estrogen receptor status, progesterone receptor status, or histological type. Furthermore, Group 1-positive cases showed a significantly worse prognosis, as shown by the Kaplan–Meier method (Figure 2). The frequencies of the other groups were not associated with any clinicopathological factors. In our series, there were no significant associations between lymph node metastasis and clinicopathological variables (age, tumor size, estrogen or progesterone receptor status, or histological type).

Our univariable analysis suggested that Group 1 was the only significant factor in estimating prognosis among clinicopathologic factors in this study ($p<0.05$). In addition, multivariate analysis with the Cox proportional hazards regression model suggested that Group 1 was an independent factor in estimating prognosis (Table 2).

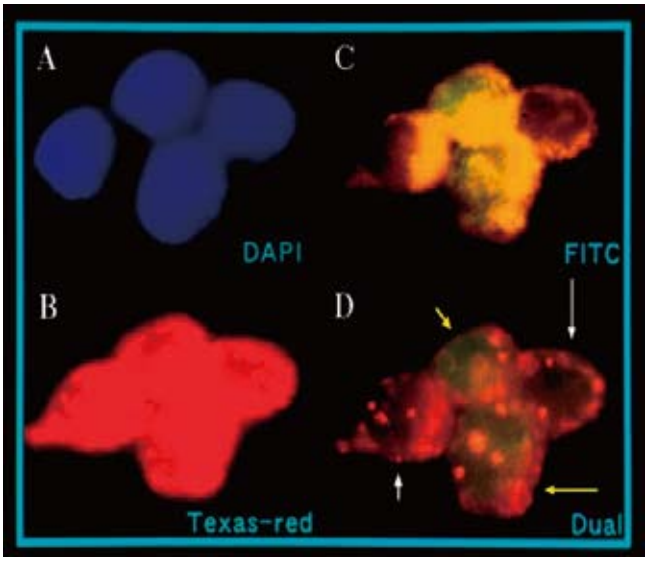

Figure 1. Representative images of breast cancers by combined immunofluorescence (IF) for Ki-67 expression and fluorescence in situ hybridization (FISH) for detecting aberrant *chromosome 8*. Original magnification, x1000.
(A) Nuclear staining with 4', 6-diamidino-2-phenylindole. (B) FISH signals with Texas-Red fluorescence. (C) IF with fluorescein isothiocyanate. (D) Merged image. A long yellow indicates a double-positive cell (Group 1). A short white arrow indicates a FISH single-positive cell (Group 2). A short yellow arrow indicates IF single-positive cell (Group3). A long white arrow indicates a double-negative cell (Group 4).

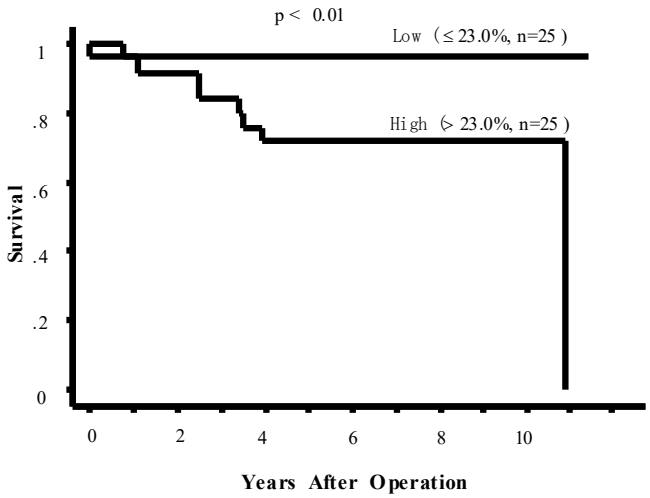

Figure 2. Kaplan–Meier actuarial survival curves of patients were subdivided according to the frequency of cells positive for Ki-67 with chromosome 8 aberrations (Group 1).Survival curves reveal that patients with a low frequency of Ki-67-positive cells with chromosome 8 numerical aberrations (Group 1) survive significantly longer than those with a high frequency of Ki-67-positive cells with chromosome 8 numerical aberrations (Group 1) when the median ratio (23%) of the frequencies of cells in Group 1 is used as the cut-off value ($p<0.01$).

Table 2. Multivariate analysis to determine the hazard ratio of each variable for tumor-related death by the Cox proportional hazards regression model.

Variables	Hazard ratio	P value
Group 1	16.7	0.033
Group 2	2.26	0.519
Group 3	1.56	0.705
Group 4	1.32	0.775
Age older than 58 years	1.82	0.598
T factor	1.70	0.361
Nodal metastasis	4.03	0.274
Estrogen receptor	2.04	0.553
Progesterone receptor	1.41	0.795
Scirrhous type	2.48	0.279

Group 1: the frequency of cells with Ki-67 positive and aberrant chromosome 8 over 23%, Group 2: the frequency of cells with Ki-67 negative and aberrant chromosome 8 over 18%, Group 3: the frequency of cells with Ki-67 positive and wild type chromosome 8 over than 22%, Group 4: the frequency of cells with Ki-67 negative and wild type chromosome 8 over than 28.0%.

Discussion

The present study clarified the usefulness of a combined IF for Ki-67 expression and FISH technique for numerical alteration of chromosome 8, using 50 cases of primary breast cancers as an ancillary technique to estimate the prognosis of patients. Using this technique, we were able to identify cancer cells with both Ki-67 expression and aberrant chromosomal 8. Our results indicated that cases of IDC in Group 1, defined as the frequency of double-positive cells over 23%, had nodal metastasis and a poor prognosis of tumor-related death. Immunohistochemistry for Ki-67 LI in breast cancer is a well-established technique for evaluating proliferative ability and estimating sensitivity to chemotherapy. Additionally, aberrations of chromosome 8 are frequently observed in primary breast cancers by CGH analyses, such as 22% of cases with a loss of 8p and 50% of cases with a gain of 8q[8-11]. Some previous reports showed that the gain of chromosome 8q is associated with adverse clinicopathologic factors[8, 11]. Particularly, amplification of the *c-MYC* oncogene located at 8q24 is commonly found in breast cancer[19, 20] as well as in other malignancies[21]. Thus, because of the clinicopathologic significance of the two factors in breast cancer, simultaneous detection of Ki-67 expression and aberrant chromosome 8 in cancer cells should be useful for estimating the prognosis of patients with breast cancer.

Solid tumors are thought to consist of heterogeneous cells in terms of proliferative capability and invasive and metastatic potential. Indeed, our simultaneous staining demonstrated heterogeneous staining profiles, including a variety of frequencies of breast cancer cells, in terms of group classification. In this study, we found an association between Group 1-positivity and tumor-related death in patients with breast cancer. This study used cytological preparations from surgically resected and snap-frozen cancer samples, which allowed us to utilize fresh materials for analysis before formalin fixation. However, from a technical point of view, we needed to re-fix cancer cells with 4% paraformaldehyde after IF, before conducting the FISH procedure, to maintain both the antigenicity for Ki-67 expression and access of the detection probe to chromosome 8 during the two-color staining procedure. Preoperative assessment by fine-needle aspiration cytology of the breast is currently available to evaluate the malignant potential of tumorous lesions. The present technique may be available not only postoperatively but also in preoperative cytological assessments of the malignant potential of tumor cells after optimizing fixative conditions during the procedure.

In conclusion, this preliminary study first utilized a double staining technique of IF for Ki-67 expression and FISH for aberrant chromosome 8 to evaluate the clinicopathological prognosis of breast cancer. The obtained results suggested that a high frequency of double-positive (Group1) cells, identified using this technique, was a significant indicator of poorer prognosis in cases of breast cancer. Further studies are needed to elucidate the applicability of this method as an

ancillary technique to estimate malignant potential in cytological samples from breast cancers.

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