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PRECLINICAL STUDY

Prognostic value of tumor cell DNA content determined by flow cytometry using formalin-fixed paraffin-embedded breast cancer tissues

Hiroki Kusama[1] · Masafumi Shimoda[1] · Tomohiro Miyake[1] · Tomonori Tanei[1] · Naofumi Kagara[1] · Yasuto Naoi[1] · Kenzo Shimazu[1] · Seung Jin Kim[1] · Shinzaburo Noguchi[1]

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Abstract

Purpose The use of formalin-fixed paraffin-embedded (FFPE) tumor tissues in flow cytometry (FCM)-based determination of tumor cell DNA content is more complicated than the use of fresh-frozen tissues. This study aimed to accurately measure tumor cell DNA content from FFPE tissues by separating tumor cells from stromal cells through FCM and investigating its prognostic impact.

Methods We separately measured the DNA contents of tumor cells and stromal cells by gating with pan-cytokeratin and vimentin ($FCM^{C/V}$). We evaluated tumor cell DNA contents [DNA index (DI)] of 290 FFPE tumor tissues and classified them into low and high DI groups, using a cutoff DI value determined through an unbiased computational method.

Results The distribution of DI was bimodal, and a cutoff value was determined at a DI of 1.26. The high-DI tumors were associated with aggressive phenotypes and had significantly worse distant recurrence-free intervals (DRFI) than low-DI tumors. Multivariate analysis revealed that lymph node metastasis, Ki67, and DI were independent factors affecting DRFI. Accordingly, patients with low-DI/low-Ki67 tumors had excellent outcomes compared with other tumor types. Multiploid tumors were associated with increased lymphocytic infiltration and aggressive phenotypes.

Conclusions The DI of FFPE tumors could be precisely determined through $FCM^{C/V}$. A combination of DI and Ki67 analyses may be able to predict the prognoses of breast cancer patients with greater accuracy.

Keywords Breast neoplasms · Ploidies · Keratins · Vimentin · Prognosis · Paraffin-embedding · Ki-67 antigen

Introduction

It has been hypothesized that the extent of DNA content in tumor cells may be linked to genomic instability and associated biological aggressiveness. As a result, the prognostic value of DNA content in breast cancer cells has been extensively studied. Most of these studies measure DNA content of tumor cells by flow cytometry (FCM), using fresh frozen tumor tissues, and their findings indicate that DNA content is an independent prognostic factor for breast cancer [1–4]. Compared with fresh frozen tissues, formalin-fixed paraffin-embedded (FFPE) tissues have the advantage that they can be stored at room temperature for a long period and are tractable for retrospective analyses. However, analysis of tumor cell DNA content by FCM using FFPE tissues is challenging because FFPE tissues are more resistant to enzymatic digestion than fresh frozen tissues, resulting in a high coefficient of variation for a peak in the DNA histogram. This interferes with the identification of the G0/G1 peak of tumor cells [5, 6]. In fact, the European Society for Analytical Cellular Pathology recommends using fresh or fresh frozen tissues for FCM [7]. Additionally, regardless of whether fresh frozen or FFPE tissues are used, analysis of breast tumor tissues has an inherent problem: the tissues are heavily contaminated by stromal cells. These

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✉ Masafumi Shimoda
mshimoda@onsurg.med.osaka-u.ac.jp

[1] Department of Breast and Endocrine Surgery, Osaka University Graduate School of Medicine, 2-2-E10 Yamadaoka, Suita, Osaka 565-0871, Japan

stromal cells interfere with accurate identification of the G0/G1 peak of tumor cells, especially when the peak is close to that of stromal cells.

To overcome these problems, Corver et al. [8] carried out FCM analysis with a separation of tumor cells from stromal cells using anti-cytokeratin (CK; an epithelial marker) and anti-vimentin (VIM; a stromal marker) antibodies. The cells obtained from the FFPE tissues by enzymatic digestion were treated with anti-CK antibody and anti-VIM antibody, and the tumor and stromal cells were separated by FCM to generate each DNA histogram. Dayal et al. also carried out the similar experiments and investigated the association of DNA content with clinicopathological parameters, revealing that the precise evaluation of tumor cell DNA content could identify the four subsets of breast tumors with distinct biomarker expression patterns [9]. The aim of this study was to carry out a novel investigation of the prognostic implications of the DNA content of breast tumor cells, particularly of intrinsic subtypes, determined by FCM with CK/VIM gating.

Materials and methods

Patients

Among patients with invasive ductal and/or lobular carcinoma of the breast who underwent surgery (without any presurgical treatments) between 1994 and 2010 at our institution, those whose FFPE tumor tissues contained enough tumor cells for FCM analysis were included ($N = 334$). Tumor cellularity in the section was approximately 40% (median) and ranged from 5% to 90%. Patients with ductal carcinoma in situ or microinvasive carcinoma were excluded. Histological grading was performed as previously reported [10]. Immunohistochemistry for estrogen receptor (ER), progesterone receptor (PR), and Ki67 were conducted as previously described [11, 12], and their cutoff values were 1%, 1%, and 20%, respectively. ER and/or PR positive tumors were considered as hormone receptor (HR) positive tumors. HER2 status was determined according to the ASCO/CAP guideline [13]. Tumor-infiltrating lymphocytes (TIL) were evaluated according to the method described by Salgado et al. [14], and the cutoff value was provisionally determined as 40%. Survival outcome information was unavailable in two patients (one HER2+ patient and one HR−/HER2− patient). These two patients were excluded from survival analysis. Regimens of adjuvant systemic therapy were indicated in Supplementary Tab. S1. This study was approved by the institutional review board of Osaka University Hospital.

Single cell preparation from FFPE tissues

This procedure was performed based on a previously reported method [8, 9]. Briefly, invasive lesions were macrodissected from two or three 50-μm slices of an FFPE tissue block to minimize contamination by normal tissue. Deparaffinization with xylene for 5 min was repeated twice because preliminary studies showed that additional deparaffinization does not improve the cell yield or immunostaining intensity of tumor and stromal cells. Rehydration was conducted with 100% ethanol for 5 min twice, followed by 95% ethanol for 1 min, 70% ethanol for 1 min, 50% ethanol for 1 min, 30% ethanol for 1 min, and distilled water for 1 min. Antigen retrieval was performed using 10 mM Tris–EDTA (pH 9.0; DAKO, Glostrup, Denmark) at 80 °C for 60 min in a block incubator (AccuBlock; Labnet, Edison, NJ, USA). Tissues were dissociated to single cells with 255 U/mL collagenase type III (Worthington, Lakewood, NJ, USA), 300 PU/mL dispase (Godo Shusei, Tokyo, Japan), and 0.25 mg/mL RNase (Sigma-Aldrich, Tokyo, Japan) in RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for 60 min. Cells were passed through a 40-μm strainer and washed thrice.

Immunostaining and flow cytometry (FCM)

Cells were stained with the anti-pan-CK antibody conjugated with Alexa Fluor 647 (clone C11; Cell Signaling Technology, Danvers, MA, USA), and the anti-VIM antibody conjugated with phycoerythrin (clone RV202; BD Biosciences, San Jose, CA, USA) at room temperature overnight. For DNA staining, 4′,6-diamidino-2-phenylindole (DAPI; Dojindo, Kumamoto, Japan) was added to the samples and incubated at 4 °C for 30 min. After washing twice, the cells were subjected to flow cytometry using FACSCanto II (BD Biosciences). Cases in which fewer than 10,000 events except for debris could be recorded; cases in which the fluorescence intensities of pan-CK and VIM were insufficient, and cases in which the coefficient of variation of the highest peak exceeded 8 were excluded from further analysis. DNA contents were analyzed using the ModFit 5.0.9 software (Verity Software House, Topsham, ME, USA). Doublets were excluded using the forward scatter height vs. forward scatter width gate. Tumor cells were considered as a pan-CK+/VIM− population, while stromal cells were considered as a pan-CK−/VIM-strongly positive population (Fig. 1a). We selected cells from an off-centered area of the cluster of VIM+ cells, which included VIM strongly positive cells to avoid contamination by tumor cells because these cells were used as a diploid control. This procedure for measuring the DNA

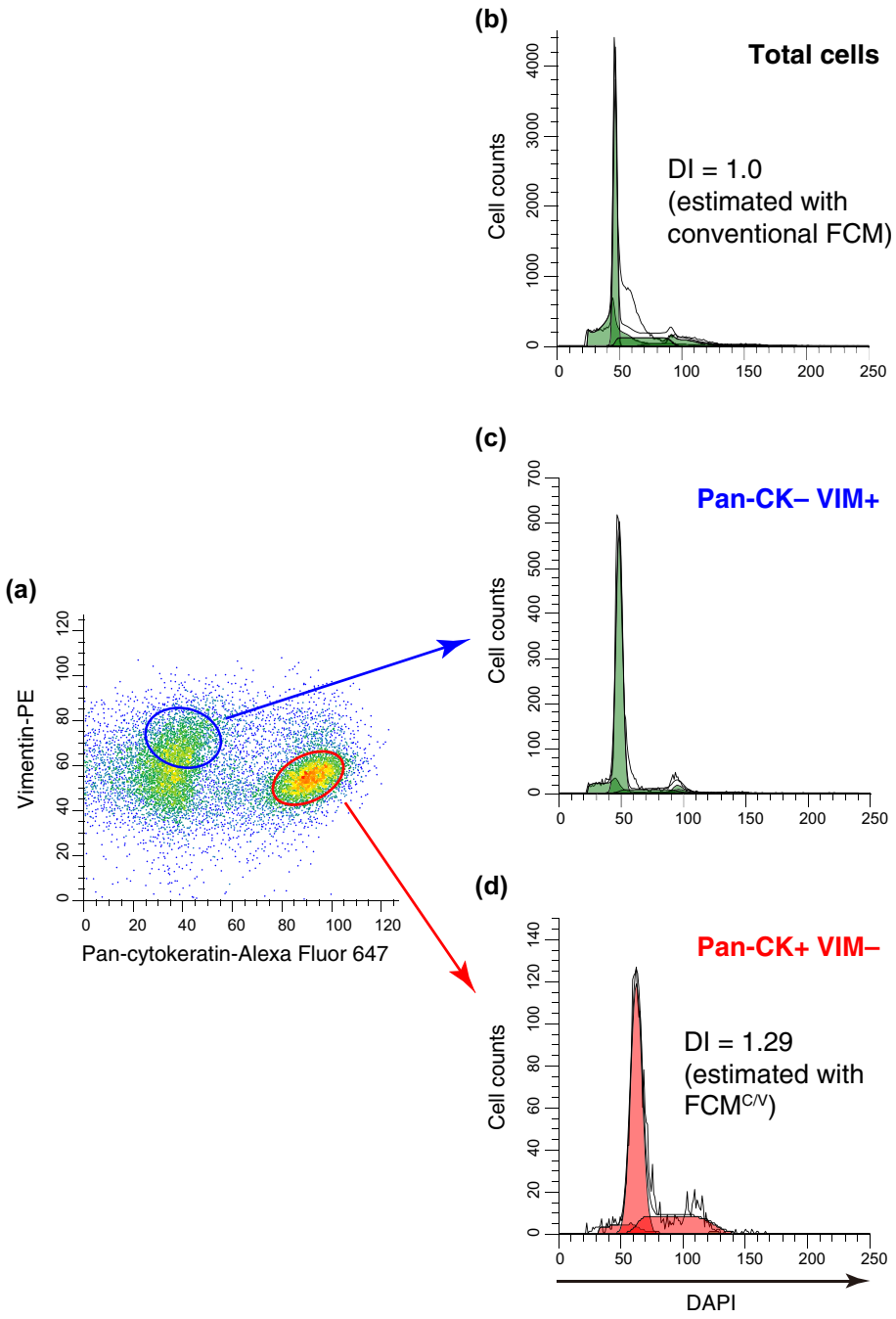

Fig. 1 A representative case of FCM analysis of gated or ungated cells with pan-CK and VIM immunostaining. **a** Scatter plot of cells stained with anti-pan-CK and anti-VIM antibodies. The red circle indicates the gate for tumor cells expressing pan-CK. The blue circle denotes the gate for stromal cells strongly expressing VIM. **b** Histogram showing the intensity of DAPI staining of ungated cells. When cells were ungated, only one peak was recognized, and thus DI was estimated to be 1.0. **c**, **d** Histograms showing the intensity of DAPI staining of gated cells with pan-CK and VIM. The peak DAPI intensity of pan-CK+/VIM− tumor cell population (**d**) differed from that of the pan-CK−/VIM+ stromal cell population (**c**), and thus, the DI of this tumor was estimated to be 1.29. These panels **a**–**d** were all derived from the same tumor

content of tumor cells by FCM and gating with pan-CK and VIM to distinguish tumor cells from stromal cells was denoted as FCM$^{C/V}$. The representative DNA content of a tumor was estimated by calculating DNA index (DI) as follows: [the peak DAPI intensity of the largest tumor cell population]/[the peak DAPI intensity of the G0/G1 stromal cell population]. A tumor showing two or more G0/G1 peaks of tumor cells was considered as multiploid [1] because stromal cells were removed by FCM$^{C/V}$ and only tumor cells were analyzed.

Statistics

The JMP 13.2.1 software (SAS Institute, Cary, NC, USA) was used for statistical calculations. Contingency testing was done using Pearson's Chi-square test or Fisher's exact test, as indicated in Table footnotes. Distant recurrence-free interval (DRFI) was defined as the interval between surgery and death from breast cancer or the development of distant metastases, regardless of the presence or absence of loco-regional relapse. The median observation period for the

patients was 100 months (range: 1–256 months). Survival curves were drawn using the Kaplan–Meier method, and log-rank testing was used for comparison. Univariate and multivariate analyses were conducted using likelihood ratio tests with the Cox proportional hazard regression model. The multivariate analysis included only explanatory variables found to be statistically significant by univariate analysis. $P < 0.05$ was considered significant.

Results

DNA content analysis using FFPE tissues by FCM

The FFPE samples from 334 cases were analyzed by $FCM^{C/V}$, which was successful in 290 samples (86.8%) for determining the DI of tumor cells (=DNA content of tumor cells/DNA content of stromal cells). The reasons for unsuccessful $FCM^{C/V}$ were as follows: insufficient immunostaining (35 tumors), large (more than 8) coefficient of variation (7 tumors), and insufficient cell counts (2 tumors).

A representative result showing the superiority of $FCM^{C/V}$ compared to FCM without gating by CK and VIM expressions is shown in Fig. 1. When CK/VIM-immunostained cells were analyzed by FCM with CK/VIM-gating ($FCM^{C/V}$), the tumor cells were found to have a DI = 1.29 by comparing the G0/G1 peak of tumor cells (Fig. 1d) to that of stromal cells (Fig. 1c). However, when the CK/VIM-immunostained cells were analyzed by FCM without CK/VIM-gating (equivalent to FCM analysis of unstained cells) (Fig. 1b), the G0/G1 peak of tumor cells disappeared in the G0/G1 peak of large numbers of stromal cells and DI of this tumor was misjudged as 1.0.

Additionally, DI of normal breast epithelial cells derived from 25 mastectomy specimens was measured by $FCM^{C/V}$. DI of normal epithelial cells was 1.024 ± 0.032 (mean $\pm$ 2SD).

DNA content analysis can classify breast cancer patients into two subsets

The distribution of DI of breast tumors was clearly bimodal (Fig. 2), which was consistent with previous reports [9, 15,

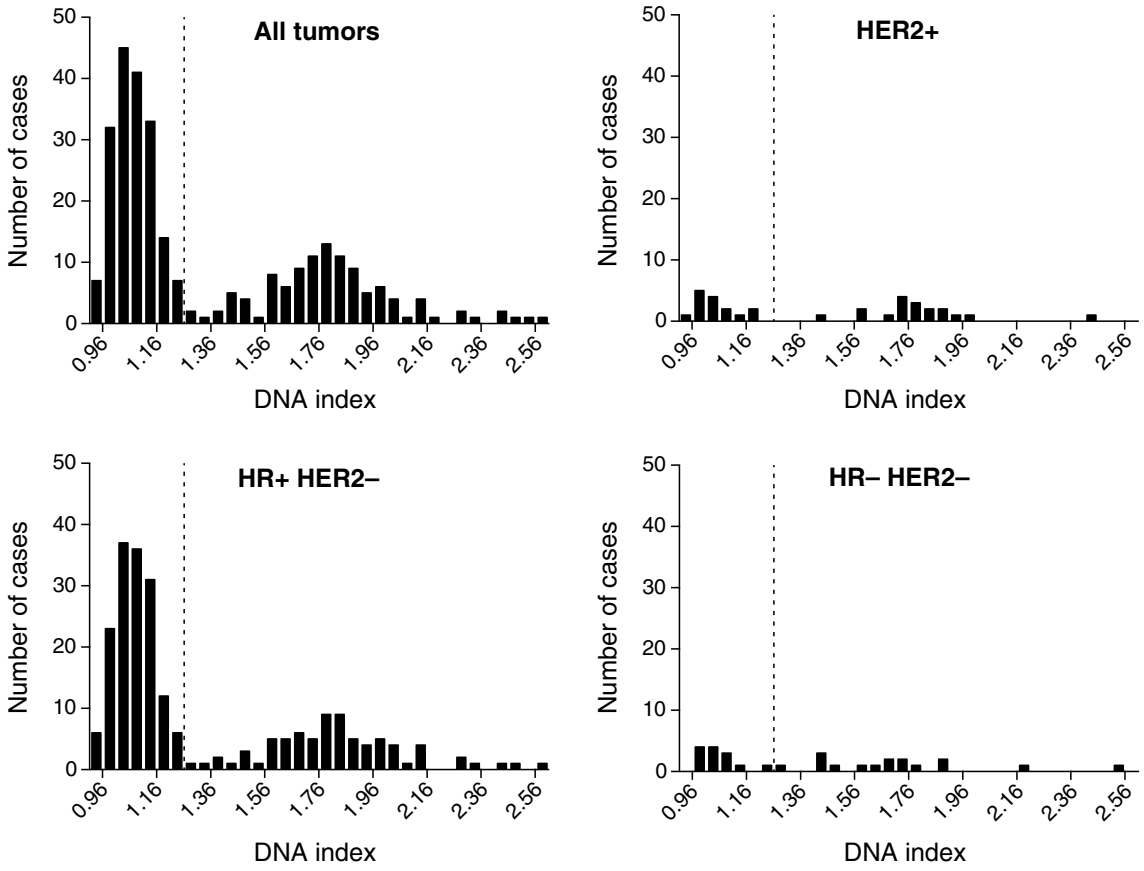

Fig. 2 Histograms of DI of all tumors, HR+HER2− tumors, HER2+ tumors, and HR−HER2− tumors. Broken lines (DI = 1.26) represent the cutoff value

16]. Therefore, we determined the cutoff value for DI to be 1.26, using an unbiased method developed by Budczies [17] to classify the tumors into the low-DI and high-DI groups. Consequently, 179 patients (61.7%) were classified into the low-DI group, while 111 patients (38.3%) were classified into the high-DI group. The high-DI tumors were significantly associated with histological grade 3, increased lymphocytic infiltration, ER negativity, high Ki67 index, and adjuvant chemotherapy (Table 1). High-DI tumors were significantly more prevalent than low-DI tumors in HR±/HER2+ subset (high-DI/low-DI = 18/15 cases) and HR−/HER2− subset (high-DI/low-DI = 16/13 cases) than HR+/HER2− subset (high-DI/low-DI = 77/151 cases; $P = 0.01$, Chi-square test) (Fig. 2).

Table 1 Association of DI with clinicopathological factors

Variables	DI low $N = 179$ Cases (%)	DI high $N = 111$ Cases (%)	P value
Age, years			
< 50	68 (38)	35 (32)	
≥ 50	111 (62)	76 (68)	0.313[a]
Tumor size			
pT1	123 (69)	74 (67)	
pT2 and 3	56 (31)	37 (33)	0.796[a]
Lymph node metastasis			
Negative	120 (67)	74 (67)	
Positive	59 (33)	37 (33)	1.000[a]
Histological grade			
1	75 (42)	29 (26)	
2	87 (49)	50 (45)	
3	17 (9)	32 (29)	< 0.001[b]
Lymphocytic infiltration (%)			
< 40	170 (95)	97 (87)	
≥ 40	9 (5)	14 (13)	0.025[a]
ER status			
Positive (≥ 1%)	162 (90)	89 (80)	
Negative	17 (10)	22 (20)	0.020[a]
PR status			
Positive (≥ 1%)	136 (76)	77 (69)	
Negative	43 (24)	34 (31)	0.222[a]
HER2 status			
Positive	15 (8)	18 (16)	
Negative	164 (92)	93 (84)	0.056[a]
Ki67 (%)			
< 20	137 (77)	65 (59)	
≥ 20	42 (23)	46 (41)	0.002[a]
Adjuvant therapy			
No or endocrine therapy	119 (66)	54 (49)	
Chemotherapy	60 (34)	57 (51)	0.003[a]

DI DNA index, *ER* estrogen receptor, *PR* progesterone receptor, *HER2* human epidermal growth factor receptor 2

[a]Fisher's exact test

[b]Chi-square test

Prognostic impact of tumor cell DNA contents

Patients with high-DI tumors showed a significantly worse DRFI than those with low-DI tumors ($P = 0.007$) (Fig. 3). In the HR+/HER2− subset, the result was similar ($P = 0.005$). However, no prognostic significance of DI was demonstrated in patients with HR±/HER2+ tumors ($P = 0.93$) or HR−/HER2− tumors ($P = 0.84$). Univariate analysis indicated that lymph node metastasis, Ki67, histological grade, PR, HER2, and DI were significant prognostic factors (Table 2) in all patients. Multivariate analysis indicated that lymph node metastasis, Ki67, and DI were significant and independent prognostic factors. Similarly, in the HR+/HER2− subset, lymph node metastasis, Ki67, and DI were identified as significant and independent prognostic factors (Table 3). DI was not significantly associated with breast cancer-specific survival in all the patients or in any subset of patients (data not shown).

Since both DI and Ki67 were independent prognostic factors in all cases and the HR+/HER2− subset, we examined whether the combination of DI and Ki67 could predict the outcome more accurately. Patients were classified into four groups based on DI and Ki67 (high-DI/high-Ki67, low-DI/high-Ki67, high-DI/low-Ki67, and low-DI/low-Ki67 groups). In all patients, the high-DI/high-Ki67, low-DI/high-Ki67, and high-DI/low-Ki67 groups showed a significantly ($P < 0.001$, $P < 0.001$, and $P = 0.013$, respectively) worse DRFI as compared to the low-DI/low-Ki67 group. In the HR+/HER2− subset, the high-DI/high-Ki67 and low-DI/high-Ki67 groups showed a significantly ($P < 0.001$ and $P < 0.001$, respectively) worse DRFI compared to the low-DI/low-Ki67 group (Fig. 4). Multivariate analysis showed that the combination of DI and Ki67 was a significant and independent prognostic factor in all patients (Supplementary Tab. S2) and the HR+/HER2− subset (Supplementary Tab. S3).

The performance of DI estimated with $FCM^{C/V}$ was compared to that of DI estimated by conventional FCM. In fact, 41 high-DI and 8 low-DI tumors classified by $FCM^{C/V}$ were misclassified by conventional FCM as low-DI and high-DI tumors, respectively (Supplementary Fig. S1a). Moreover, although DRFI was significantly different between low-DI and high-DI tumors classified by $FCM^{C/V}$, this difference was not observed between low-DI and high-DI tumors classified by conventional FCM (Supplementary Fig. S1b).

Significance of multiploid tumors

Although some studies treat them as a distinct subset, in the aforementioned analysis, multiploid tumors were classified

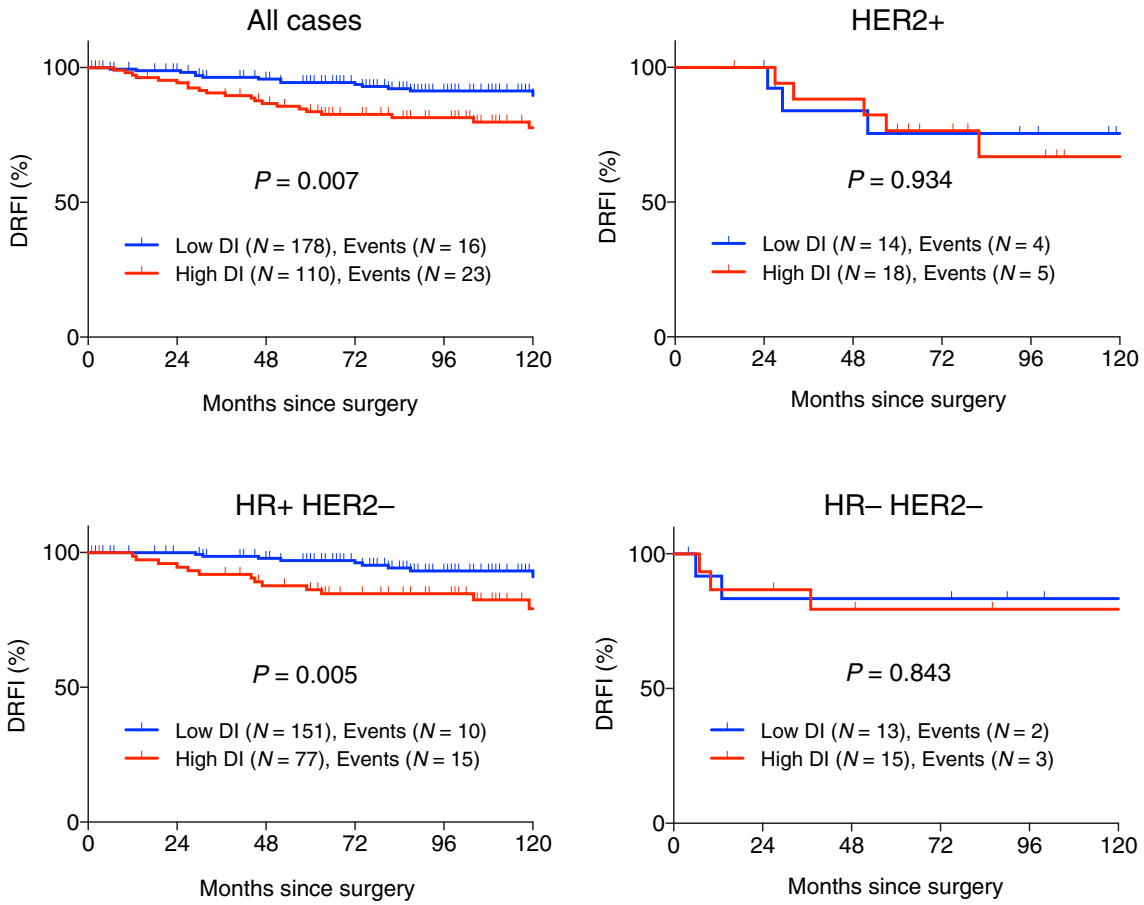

Fig. 3 Kaplan–Meier curves of DRFI of high-DI and low-DI groups in all cases, HR+HER2− subset, HER2+ subset, and HR−HER2− subset. Log-rank *P* values are shown. Although log-rank testing was applied to all events, DRFI between 0 and 120 months have been shown for clarity

Table 2 Univariate and multivariate analyses of the clinicopathological factors and DI in association with DRFI of all patients

Explanatory variables	Categories	Univariate			Multivariate		
		HR	95% CI	*P* value[a]	HR	95% CI	*P* value[a]
Age	≥ 50 years vs. < 50 years	0.67	0.36–1.29	0.227			
Tumor size	pT2-3 vs. pT1	1.50	0.78–2.82	0.223			
Lymph node metastasis	Positive vs. negative	3.35	1.78–6.46	< 0.001	3.77	1.98–7.36	< 0.001
Histological grade	Grade 2 vs. Grade 1	1.44	0.67–3.36	0.355			
	Grade 3 vs. Grade 1	2.65	1.09–6.57	0.031	1.06	0.49–2.46	0.881
Lymphocytic infiltration	≥ 40% vs. < 40%	0.27	0.02–1.25	0.108			
Ki67	≥ 20% vs. < 20%	3.13	1.66–5.94	< 0.001	2.62	1.33–5.16	0.005
ER	Positive vs. negative	0.89	0.40–2.38	0.801			
PR	Positive vs. negative	0.52	0.27–1.00	0.049	0.59	0.29–1.23	0.155
HER2	Positive vs. negative	2.79	1.24–5.68	0.015	1.80	0.77–3.82	0.165
DI	High vs. low	2.35	1.21–4.40	0.008	1.93	1.00–3.81	0.049

HR hazard ratio, *DI* DNA index, *DRFI* distant recurrence-free interval, *CI* confidence interval, *ER* estrogen receptor, *PR* progesterone receptor, *HER2* human epidermal growth factor receptor 2

[a]Likelihood ratio test

Table 3 Univariate and multivariate analyses of the clinicopathological factors and DI in association with DRFI in hormone receptor-positive and HER2-negative subset

Explanatory variables	Categories	Univariate			Multivariate		
		HR	95% CI	*P* value[a]	HR	95% CI	*P* value[a]
Age	≥ 50 years vs. < 50 years	0.77	0.35–1.77	0.522			
Tumor size	pT2-3 vs. pT1	1.15	0.49–2.56	0.735			
Lymph node metastasis	Positive vs. negative	2.86	1.30–6.45	0.009	2.52	1.13–5.74	0.024
Histological grade	2 vs. 1	1.78	0.75–4.66	0.196			
	3 vs. 1	1.86	0.40–6.69	0.392			
Lymphocytic infiltration	≥ 40% vs. < 40%	1.22	0.07–5.78	0.852			
Ki67	≥ 20% vs. < 20%	6.07	2.75–14.0	<0.001	4.94	2.19–11.6	<0.001
PR	Positive vs. negative	0.42	0.18–1.08	0.069			
DI	High vs. low	2.95	1.34–6.79	0.007	2.65	1.19–6.15	0.017

HR hazard ratio, *DI* DNA index, *DRFI* distant recurrence free interval, *ER* estrogen receptor, *CI* confidence interval, *PR* progesterone receptor, *HER2* human epidermal growth factor receptor 2

[a]Likelihood ratio test

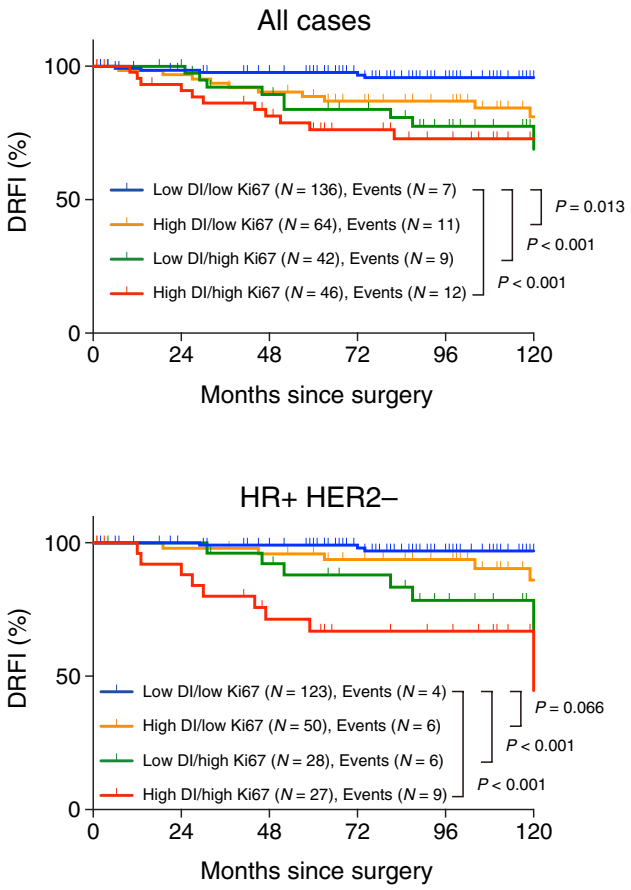

Fig. 4 Kaplan–Meier curves of DRFI of four subpopulations stratified by DI and Ki67 in all cases and HR+HER2− subset. Log-rank *P* values are shown

into high-DI or low-DI tumors, depending on the DI of the major population of tumor cells in each case [9, 18–20]. Therefore, in the following analysis, tumors were classified into three groups, i.e., low-DI/non-multiploid tumors ($N = 164$), high-DI/non-multiploid tumors ($N = 63$), and multiploid tumors ($N = 62$). A representative DNA histogram of multiploid tumors is shown in Fig. 5a, b. Multiploid tumors were significantly associated with histological grade 3, increased lymphocytic infiltration, ER-negativity, PR-negativity, HER2-positivity, high-Ki67, and adjuvant chemotherapy (Table 4). Besides, the frequency of multiploid tumors was the lowest in the HR+/HER2− subset (Chi-square $P < 0.001$; Fig. 5c). Prognosis (DFRI) of the low-DI/non-multiploid tumors was significantly better than that of the high-DI/non-multiploid tumors ($P = 0.0023$); the prognosis of the multiploid tumors was between those of these two groups (Fig. 5d). Multivariate analysis showed that a combination of DI and ploidy was a significant and independent prognostic factor in all patients (Supplementary Tab. S4) and the HR+HER2− subset (Supplementary Tab. S5).

Discussion

We were able to confirm that $FCM^{C/V}$ analysis using FFPE breast tumor tissues was feasible. Association of high-DI tumors with aggressive phenotypes such as histological grade 3, ER-negativity, high Ki67, and poorer prognosis is consistent with previous reports [4, 15, 18, 21]. One noteworthy result is that the low-DI/low-Ki67 tumors showed an excellent prognosis, i.e., 10-year DRFI rates of 96.9% (Fig. 4). When only the low-DI/low-Ki67 tumors with negative nodes that were treated with adjuvant hormonal therapy alone ($N = 79$) were considered, 10-year

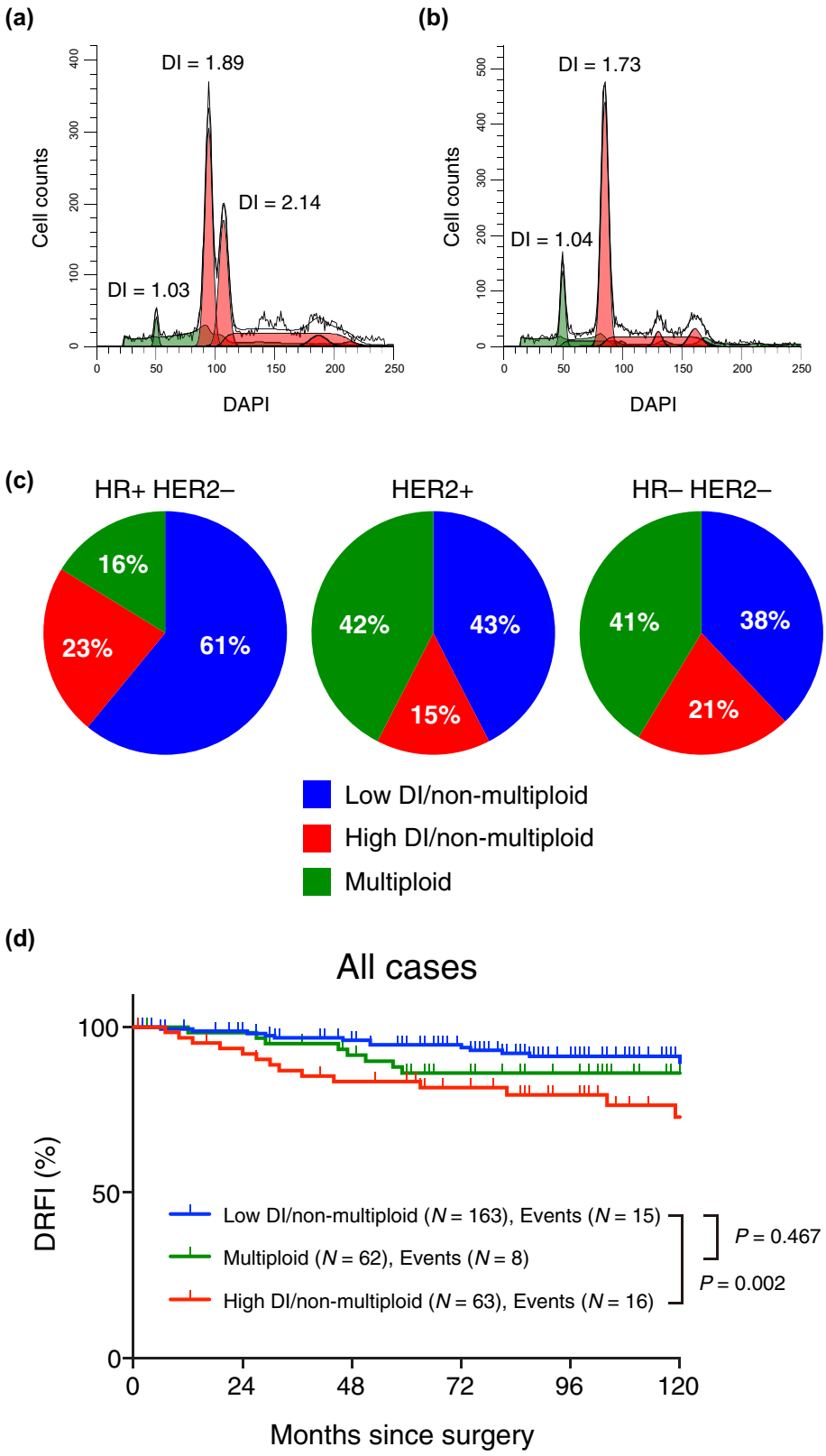

Fig. 5 Prognostic significance of multiploid tumors. **a**, **b** Examples of histograms of DAPI intensities in multiploid tumor cells. **c** Frequency of multiploid tumors in each subset. **d** Kaplan–Meier curves of DRFI of high-DI/non-multiploid group, low-DI/non-multiploid group, and multiploid group in all cases. Log-rank *P* values are shown

DRFI was as high as 96.9% in the HR+/HER2− subset ($N = 136$) (Supplementary Fig. S2). These results suggest a possibility that DI and Ki67 might be clinically useful for the selection of patients that do not need chemotherapy in the HR+/HER2− subset, though the frequency of low risk tumors in our study (79 cases/136 cases = 58.1%) is lower than that reported by Oncotype DX (85%) [22].

Table 4 Association of DI and multiploidy with clinicopathological factors

Variables	DI low, non-multiploid $N=164$ Cases (%)	DI high, non-multiploid $N=63$ Cases (%)	Multiploid $N=63$ Cases (%)	P value[a]
Age, years				
< 50	64 (39)	18 (29)	21 (33)	
≥ 50	100 (61)	45 (71)	42 (67)	0.311
Tumor size				
pT1	112 (68)	42 (67)	43 (68)	
pT2 and 3	52 (32)	21 (33)	20 (32)	0.970
Lymph node metastasis				
Negative	111 (68)	42 (67)	41 (65)	
Positive	53 (32)	21 (33)	22 (35)	0.932
Histological grade				
1	73 (44)	19 (30)	12 (19)	
2	77 (47)	29 (46)	31 (49)	
3	14 (9)	15 (24)	20 (32)	< 0.001
Lymphocytic infiltration (%)				
< 40	158 (96)	62 (98)	47 (75)	
≥ 40	6 (4)	1 (2)	16 (25)	< 0.001
ER status				
Positive (≥ 1%)	150 (91)	56 (89)	45 (71)	
Negative	14 (9)	7 (11)	18 (29)	< 0.001
PR status				
Positive (≥ 1%)	128 (78)	47 (75)	38 (60)	
Negative	36 (22)	16 (25)	25 (40)	0.025
HER2 status				
Positive	14 (9)	5 (8)	14 (22)	
Negative	150 (91)	58 (92)	49 (78)	0.009
Ki67 (%)				
< 20	127 (77)	41 (65)	34 (54)	
≥ 20	37 (23)	22 (35)	29 (46)	0.002
Adjuvant therapy				
No or endocrine therapy	110 (67)	35 (56)	28 (44)	
Chemotherapy	54 (33)	28 (44)	35 (56)	0.006

DI DNA index, *ER* estrogen receptor, *PR* progesterone receptor, *HER2* human epidermal growth factor receptor 2

[a]Chi-square test

$FCM^{C/V}$ is advantageous because the DI of tumor cells and stromal cells can be separately evaluated to more precisely measure the DI of tumor cells compared to conventional FCM (FCM without gating), which evaluates the DI of a mixture of tumor cells and stromal cells. In fact, 17% (49/290) of DI in all cases, as determined by $FCM^{C/V}$, were misclassified by conventional FCM without gating, showing that DI was a non-significant prognostic factor. These results demonstrate the superiority of $FCM^{C/V}$ over conventional FCM.

Multiploid refers to divergent karyotypes within a tumor. We found that multiploid tumors were present at a frequency of 21.7% (63/290), and these tumors were characterized by histological grade 3, ER-negativity, PR-negativity, HER2-positivity, and high Ki67. The frequency of multiploid tumors was therefore significantly lower in the HR +/HER2− subset than the other two subsets ($P < 0.001$). These results seem to suggest that the multiploid tumors are biologically more aggressive. But the comparison of prognoses among these three groups demonstrates that prognoses of the multiploid tumors were between the low-DI/non-multiploid tumors and the high-DI/non-multiploid tumors. This result may be explained, at least in part, by a higher incidence of lymphocytic infiltration in multiploid tumors than the other tumors, since lymphocytic infiltration serves as a marker

of a good prognosis, especially in triple negative and HER2+ tumors [23]. As for the intrinsic subtypes, the frequency of multiploid tumors was lower in the HR+/HER2− subset than the other two subsets. The finding that the multiploid tumors were associated with lymphocytic infiltration might indicate that the multiploid tumors are highly immunogenic and therefore can be a candidate for immune-checkpoint therapy.

The limitation of our study is that it is a retrospective study without a large number of patients. Thus, it is difficult to draw a firm conclusion about the prognostic impact of DI determined by FCM using FFPE tissues from this study, but our results are consistent with previous reports [9]. The results seem to suggest, at least, that the determination of DI using FFPE tissues is feasible and that DI has the potential to be used as a prognostic tool, especially in luminal tumors. This potential needs to be explored by a future prospective study on a larger number of patients. Additionally, the number of patients in the HR±/HER2+ and HR−/HER2− subsets was too small to draw meaningful conclusions on the prognostic impact of DI in these subsets.

In conclusion, DI can be determined by FCM that separates tumor cells from stromal cells in FFPE tumor tissues by using anti-CK and anti-VIM antibodies, and this method outperforms unseparated FCM. High-DI tumors are associated with a more biologically aggressive phenotype and poorer prognoses than low-DI tumors. A combination of DI and Ki67 seems to classify the breast tumors more accurately with respect to prognosis. In addition, among the breast tumors, multiploid tumors are found to have different characteristics from the other tumors and are more frequently seen in triple negative breast tumors and HER2+ breast tumors than luminal tumors.

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Compliance with ethical standards

Conflict of interest Noguchi S. has been an adviser for Taiho, AstraZeneca and Novartis and has received honoraria and research funding for the other studies from AstraZeneca, Pfizer, Novartis, Chugai, Takeda, Nippon-Kayaku, Nittobo, and Sysmex.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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