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Multicenter retrospective study on the use of Curebest™ 95GC Breast for estrogen receptor-positive and node-negative early breast cancer

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Abstract

Background: The benefits of postoperative chemotherapy in patients with estrogen receptor (ER)-positive breast cancer remain unclear. The use of tumor grade, Ki-67, or ER expression failed to provide an accurate prognosis of the risk of relapse after surgery in patients. This study aimed to evaluate whether a multigene assay Curebest™ 95GC Breast (95GC) can identify the risk of recurrence and provide more insights into the requirements for chemotherapy in patients.

Methods: This single-arm retrospective multicenter joint study included patients with ER-positive, node-negative breast cancer who were treated at five facilities in Japan and had received endocrine therapy alone as adjuvant therapy. The primary lesion specimens obtained during surgery were analyzed using the 95GC breast cancer multigene assay. Based on the 95GC results, patients were classified into low-risk (95GC-L) and high-risk (95GC-H) groups.

Results: The 10-year relapse-free survival rates were 88.4 and 59.6% for the 95GC-L and 95GC-H groups, respectively. Histologic grade, Ki-67, and PAM50 exhibited a significant relationship with the 95GC results. The segregation into 95GC-L and 95GC-H groups within established clinical factors can identify subgroups of patients using histologic grade or PAM50 classification with good prognosis without receiving chemotherapy.

Conclusions: Based on the results of our retrospective study, 95GC could be used to evaluate the long-term prognosis of ER-positive, node-negative breast cancer. Even though further prospective validation is necessary, the inclusion of 95GC in clinical practice could help to select optimal treatments for breast cancer patients and identify those who do not benefit from the addition of chemotherapy, thus avoiding unnecessary treatment.

Keywords: ER-positive breast cancer, Curebest™ 95GC breast, DNA microarrays, Breast cancer staging, Node-negative breast cancer, Prognostic prediction

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Background

Breast cancer is the most common cancer among women worldwide [1]. The overall survival and prevention of relapse after surgery are improving owing to the increase in the number of available treatment options. Estrogen receptor (ER)-positive breast cancers account for > 70% of all breast cancers and have favorable outcomes compared with ER-negative breast cancers [2]. Moreover, 10–20% of patients experience relapse after surgery [2, 3], and although chemotherapy improves breast cancer survival, its benefits in ER-positive breast cancers are being questioned lately [4].

In the past, the risk of breast cancer relapse was assessed by taking into account the patient's age, tumor diameter, tumor grade, Ki-67, and ER expression. However, the risk of relapse and the need for chemotherapy are difficult to predict accurately based solely on clinicopathological factors.

Recently, it has been possible to more accurately predict recurrence risk in breast cancer patients by supplementing information from clinicopathological factors used in previous breast cancer multigene assays, with some already incorporated in clinical practice. Oncotype DX® (Exact Sciences Corporation, Madison, WI) is currently a widely used multigene classifier that can be used to stratify patients with node-negative ER-positive breast cancer into low-, intermediate-, and high-risk groups based on the expression of 21 genes [5–8]. Another widely used multigene assay is the MammaPrint® (Agendia, Inc., Amsterdam, Netherlands), which can analyze 70 genes to predict recurrence in patients with both ER-positive and ER-negative node-negative breast cancer. The PAM50 (NanoString Technologies, Inc., Seattle, WA) was developed to classify patients based on intrinsic subtypes, but it has also been used for recurrence prediction [9, 10]. The analysis of patient samples by various multigene assays revealed that risk categorization differs between tests [11].

Curebest™ 95GC Breast (95GC, Sysmex Corporation, Kobe, Japan) is a breast cancer multigene assay that uses DNA microarray to analyze the expression of 95 genes [12, 13] and has been available in Japan for research use only since 2013. In this assay, RNA is extracted from frozen or formalin-fixed paraffin-embedded tissue samples and the expression of 95 genes is analyzed by microarray. After this, the 95GC score is calculated from the expression of the 95 genes using a unique algorithm. By setting a specific cut-off value for the 95GC score, patients with ER-positive, node-negative breast cancer can be classified into low-risk (95GC-L) and high-risk (95GC-H) groups without the intermediate-risk group. According to a report by Naoi et al., in patients who received endocrine therapy alone as an adjuvant therapy, the recurrence rate was remarkably low in the low-risk

group and significantly more favorable than that in the high-risk group, with 10-year recurrence-free survival rates of 93% for 95GC-L and 53% for 95GC-H [12]. From these results, owing to the extremely low risk of recurrence after surgery, adjuvant chemotherapy may be considered omissible in patients who are categorized as 95GC-L based on 95GC.

Thus far, the benefits of 95GC as a prognostic factor in patients with ER-positive, node-negative breast cancer have been evaluated only in retrospective studies conducted in a single facility or from public databases. Here, we aimed to assess the use of 95GC in patients from five Japanese facilities with ER-positive, node-negative breast cancer who received endocrine therapy alone as adjuvant therapy. To examine the relationships between 95GC and pathological factors associated with low reproducibility or inter-pathologist agreement, a repeat test was conducted with a central pathology review for Ki-67 and histologic grade and repeated analysis using array data for HER2.

This study aimed to evaluate relapse-free survival between the 95GC-L and 95GC-H groups and the differences in the clinical features of each group. It also aimed to analyze the outcome of 95GC regarding individual clinical features, such as histologic grade and intrinsic subtypes.

Methods

Study design

This non-interventional, non-invasive, and single-arm retrospective multicenter joint study included patients from five facilities in Japan (National Cancer Center Hospital, Shikoku Cancer Center, Shinshu University Hospital, Hiroshima University Hospital, and JCHO Osaka Hospital). The surgery was performed between January 19, 1999, and October 22, 2010, with a median follow-up period of 90 months. We followed the STROBE guidelines (checklist is included as Additional file 1).

Female patients aged ≥20 years with confirmed ER-positive invasive breast cancer with T1 or T2 primary lesions, no lymph node involvement, and no distant metastasis were included in the study. Patients who received adjuvant endocrine therapy regardless of drug classification, such as selective ER modulators (tamoxifen and toremifene), aromatase inhibitors (letrozole and exemestane), luteinizing hormone-releasing hormone agonist (goserelin and leuprolide), and a combination thereof (combination or sequential therapy) were also included. The primary lesion specimens were collected 30 min after surgery and stored at − 80 °C until analysis. Patients who received neoadjuvant therapy (prior to surgery) and adjuvant therapy other than endocrine therapy were excluded. In principle, only endocrine therapy is

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administered for T1/T2, lymph node-negative, and ER-positive breast cancer. Chemotherapy is recommended for patients with histologic grade 3, but it may not be administered at the patient's request.

Specimen standards

Frozen specimens from patients were delivered on dry ice from the participating facilities to Sysmex Corporation and cryosectioned using Tissue-Tek® O.C.T. compound (Sakura Finetek, Osaka, Japan). Sections measuring 6-μm thick were prepared from the top and bottom edges of the specimen and stained using hematoxylin and eosin, and the tumor cell percentage was calculated. The remaining specimen was reserved for later use for microarray measurement.

RNA was extracted and purified from the preserved specimens using the RNeasy® Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). Only specimens that yielded > 17 ng/μL RNA, determined using the NanoDrop™ 2000 (Thermo Fisher Scientific Inc., Waltham, MA), and with RIN value > 5.0, calculated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), were considered valid for the study. Microarray measurements were performed using the GeneChip™ 3'IVT PLUS Reagent Kit (Thermo Fisher Scientific) and GeneChip™ Human Genome U133 Plus 2.0 Array (Thermo Fisher Scientific). All procedures were performed in accordance with the manufacturer's protocol.

The microarray data obtained were evaluated for quality using MAS5 normalization, and only the data that complied with the following conditions were considered: GAPDH3'/5′ ratio < 3.0, average background value in the range of 20–100, scaling factor ≤ 15, percent $P ≥ 30$, spike-in labeling control lys_3_signal < phe_3_signal < dap_3_signal, and spike-in hybridization control BioB_3_signal < BioC_3_signal < BioD_3_signal < cre_3 signal. After normalizing the microarray data to analyze recurrence in patients using the RefRMA model, the 95GC algorithm was used to obtain the classification result of 95GC-L or 95GC-H, as described by Naoi et al. [12].

Statistical analysis

Using the Kaplan–Meier method, the relapse-free survival curves of the 95GC-L and 95GC-H groups were plotted and evaluated using the log-rank test.

The correlation between clinical factors and 95GC was evaluated using Fisher's exact test. The hazard ratio for recurrence was evaluated for each factor based on the Cox proportional hazard model, and multivariate analysis was performed using factors with statistically significant hazard ratios in the univariate analysis. Each clinical factor was analyzed, excluding the missing data values. Multivariate analysis only included patients with available data on all the evaluated factors. The sample size was determined by calculating the number of cases required to verify the predicted 5-year recurrence-free survival rate of Curebest 95GC Breast [12].

For patients with histologic grade 2 and those with the PAM50 luminal B subtype, relapse-free survival curves were evaluated for each 95GC risk group using the Kaplan–Meier method and log-rank test.

All statistical tests were performed using two-tailed tests, with a significance level of 5%. We used the following applications: for Fisher's exact test, R ver. 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria); for the Cox proportional hazard model, Kaplan-Meier method, and log-rank test, MedCalc ver. 12.7.0.0 (MedCalc Software Ltd., Ostend, Belgium).

Results

Patient characteristics

A total of 102 patients met all the selection criteria. Samples for which 95GC assessment results could not be obtained owing to problems with RNA quality were excluded, and ultimately 75 patients were selected for analysis. We followed the method described in the study by Parker et al. [14] to determine the breast cancer subtype (luminal A, luminal B, HER2-enriched, basal, or normal-like) using the PAM50 classification technique (Fig. 1).

The relationships between the distribution of each clinical factor and the 95GC results in the analyzed patients are shown in Table 1. Histologic grade, Ki-67, and PAM50 exhibited a significant relationship with the 95GC result as evaluated by Fisher's exact test. Patients included in the 95GC-L group had mostly grade 2 luminal A breast cancer, and only 8.5% of the patients had Ki-67 staining of ≥20%. In contrast, patients in the 95GC-H group presented with grade 3, luminal B subtype, and 28.6% presented with Ki-67 staining of ≥20%. The three cases of pathological HER2-positive breast cancer were included in the five cases of PAM50 HER2-positive breast cancer but, because of this, the results of the pathological examination and classification by PAM50 may not completely match. This is because PAM50 is a classification based on gene expression levels using microarray data, and the method is different from that of pathological examination.

Prognostic performance of 95GC

The relapse-free survival between the 95GC-L and 95GC-H groups showed a statistically significant difference ($p = 0.0017$). The 5-year relapse-free survival rates of the analyzed patients were 97.8% for the 95GC-L group and 81.0% for the 95GC-H group. The 10-year relapse-free survival rates were 88.4% for the 95GC-L group and 59.6% for the 95GC-H group (Fig. 2). Furthermore, luminal B tumors could be divided into
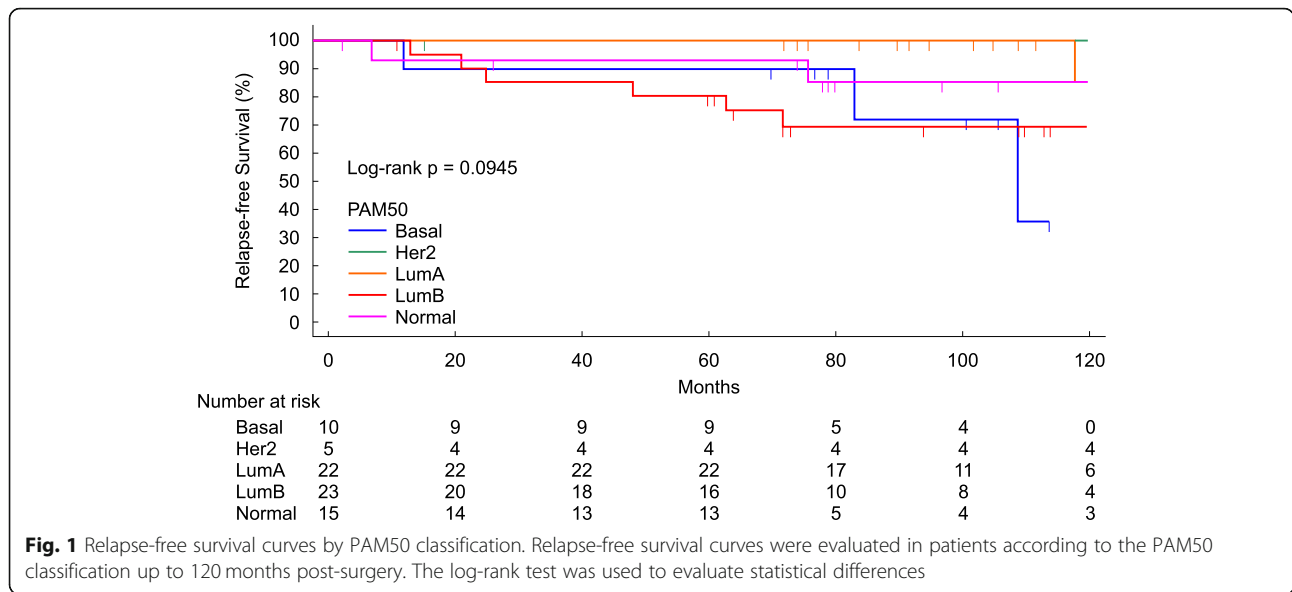
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Fig. 1 Relapse-free survival curves by PAM50 classification. Relapse-free survival curves were evaluated in patients according to the PAM50 classification up to 120 months post-surgery. The log-rank test was used to evaluate statistical differences

Table 1 Relationship between clinicopathological factors and 95GC results

Factors		95GC-L (N = 47) N (%)	95GC-H (N = 28) N (%)	Total (N = 75) N (%)	*p*-value
Age	≤50 years	14 (29.8)	11 (39.3)	25 (33.3)	*p* = 0.453
	> 50 years	33 (70.2)	17 (60.7)	50 (66.7)	
T stage	T1	30 (63.8)	16 (57.1)	46 (61.3)	*p* = 0.628
	T2	17 (36.2)	12 (42.9)	29 (38.7)	
Histology	Ductal	43 (91.5)	28 (100.0)	71 (94.7)	*p* = 0.384
	Lobular	1 (2.1)	0 (0.0)	1 (1.3)	
	Other	3 (6.4)	0 (0.0)	3 (4.0)	
Histologic grade	1	8 (17.0)	2 (7.1)	10 (13.3)	**p < 0.001**
	2	33 (70.2)	10 (35.7)	43 (57.3)	
	3	6 (12.8)	15 (53.6)	21 (28.0)	
	NA	0 (0.0)	1 (3.6)	1 (1.3)	
PR	Negative	9 (19.1)	7 (25.0)	16 (21.3)	*p* = 0.572
	Positive	38 (80.9)	21 (75.0)	59 (78.7)	
HER2	Negative	46 (97.9)	26 (92.9)	72 (96.0)	*p* = 0.552
	Positive	1 (2.1)	2 (7.1)	3 (4.0)	
Ki-67	< 20%	37 (78.7)	16 (57.1)	53 (70.7)	**p = 0.024**
	≥20%	4 (8.5)	8 (28.6)	12 (16.0)	
	NA	6 (12.8)	4 (14.3)	10 (13.3)	
PAM50	Luminal A	22 (46.8)	0 (0.0)	22 (29.3)	**p < 0.001**
	Luminal B	7 (14.9)	16 (57.1)	23 (30.7)	
	HER2	1 (2.1)	4 (14.3)	5 (6.7)	
	Basal	2 (4.3)	8 (28.6)	10 (13.3)	
	Normal	15 (31.9)	0 (0.0)	15 (20.0)	

NA not available

95GC-L and 95GC-H based on 95GC, with more favorable outcomes for 95GC-L than for 95GC-H (Fig. 3).

Univariate Cox regression analysis showed that Ki-67 and 95GC were the factors with significant hazard ratios for recurrence, whereas multivariate Cox regression analysis using these two factors showed that 95GC was a significant factor (Table 2). The three HER2-positive patients showed no recurrence; therefore, the hazard ratio was difficult to calculate and is not presented.

Association with histologic grade

Relapse-free survival rates correlated with the histologic grade (Fig. 4a). Furthermore, the relapse-free survival for the grade 2 subgroup was significantly different (*p* = 0.0480) between the 95GC-L and 95GC-H groups (Fig. 4b).

Discussion

Although breast cancer outcomes have improved owing to recent advances in adjuvant chemotherapy, it is possible that patients who do not require chemotherapy also receive superfluous treatment. From this perspective, the possibility of separately evaluating the appropriateness of adjuvant chemotherapy for individual patients using multigene assays has garnered attention. The 95GC multigene assay is useful for the prognostic assessment of ER-positive, node-negative breast cancer [12, 13, 15]. However, previous studies regarding the usefulness of 95GC as a prognostic factor have been based only on patients from a single facility or public databases.

In the present investigation, we performed a retrospective study of the usefulness of 95GC in patients from five Japanese facilities with ER-positive, node-negative breast cancer who received endocrine therapy
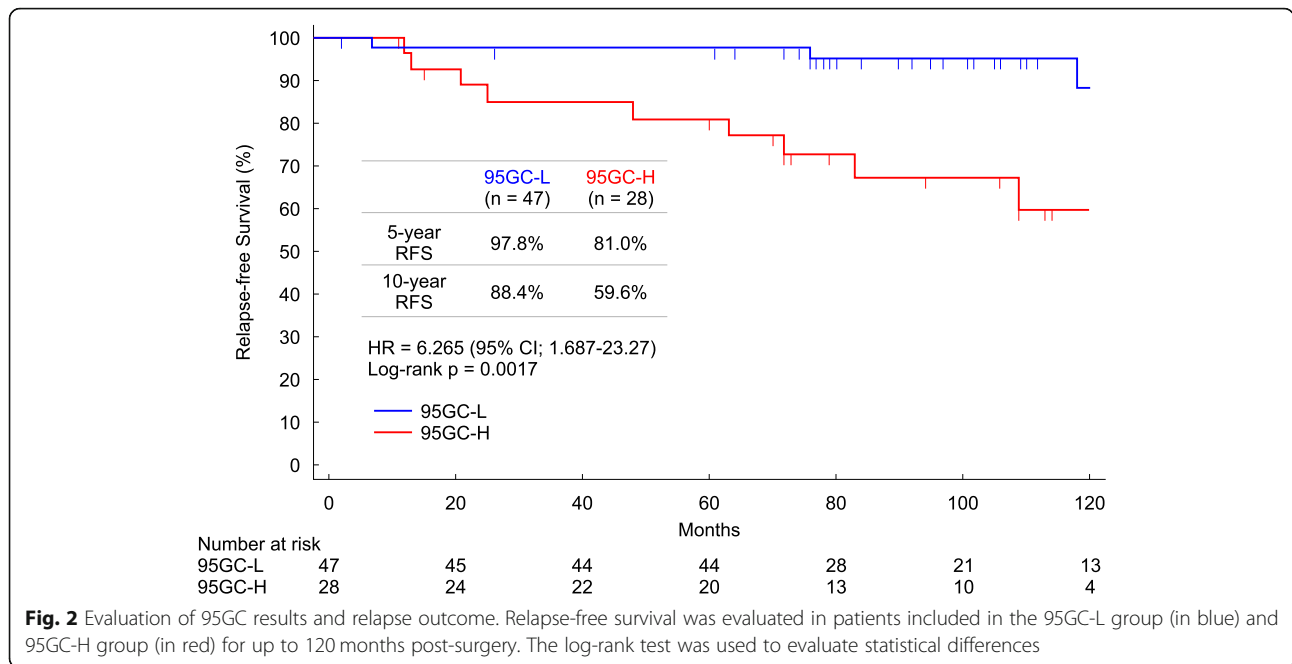
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Fig. 2 Evaluation of 95GC results and relapse outcome. Relapse-free survival was evaluated in patients included in the 95GC-L group (in blue) and 95GC-H group (in red) for up to 120 months post-surgery. The log-rank test was used to evaluate statistical differences

alone as adjuvant therapy. The 10-year relapse-free survival rate for patients diagnosed with 95GC-L was 88.4%, suggesting that favorable outcomes can be expected for these patients even if chemotherapy is omitted and only endocrine therapy is administered.

We examined the relationship between 95GC and intrinsic subtypes. The proportion of luminal A tumors was significantly higher among 95GC-L patients than among 95GC-H patients, whereas that of luminal B tumors was lower. Furthermore, luminal B tumors can be divided based on 95GC, with 95GC-L showing more favorable outcomes than 95GC-H. This finding demonstrates the possibility that patients with tumors classified by PAM50 as luminal B include a few for whom the addition of chemotherapy will have little beneficial effect on the outcome and that unnecessary chemotherapy for such patients may be reduced by combining PAM50 and 95GC results.
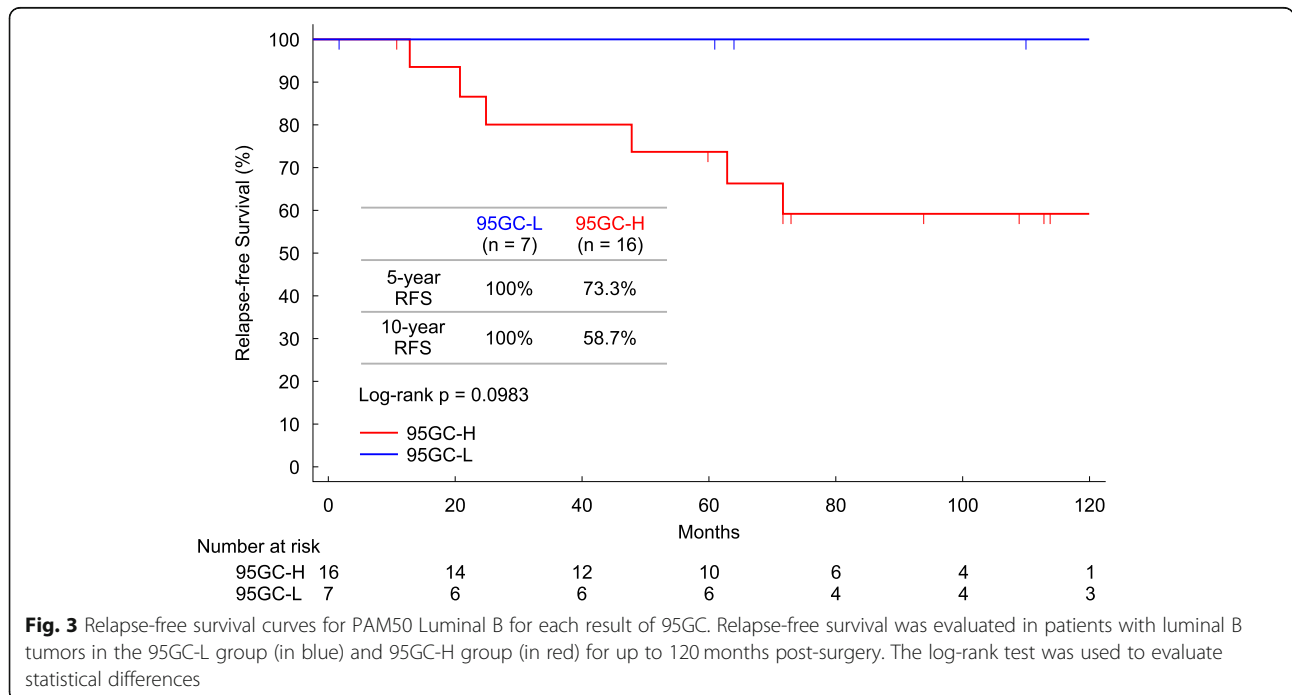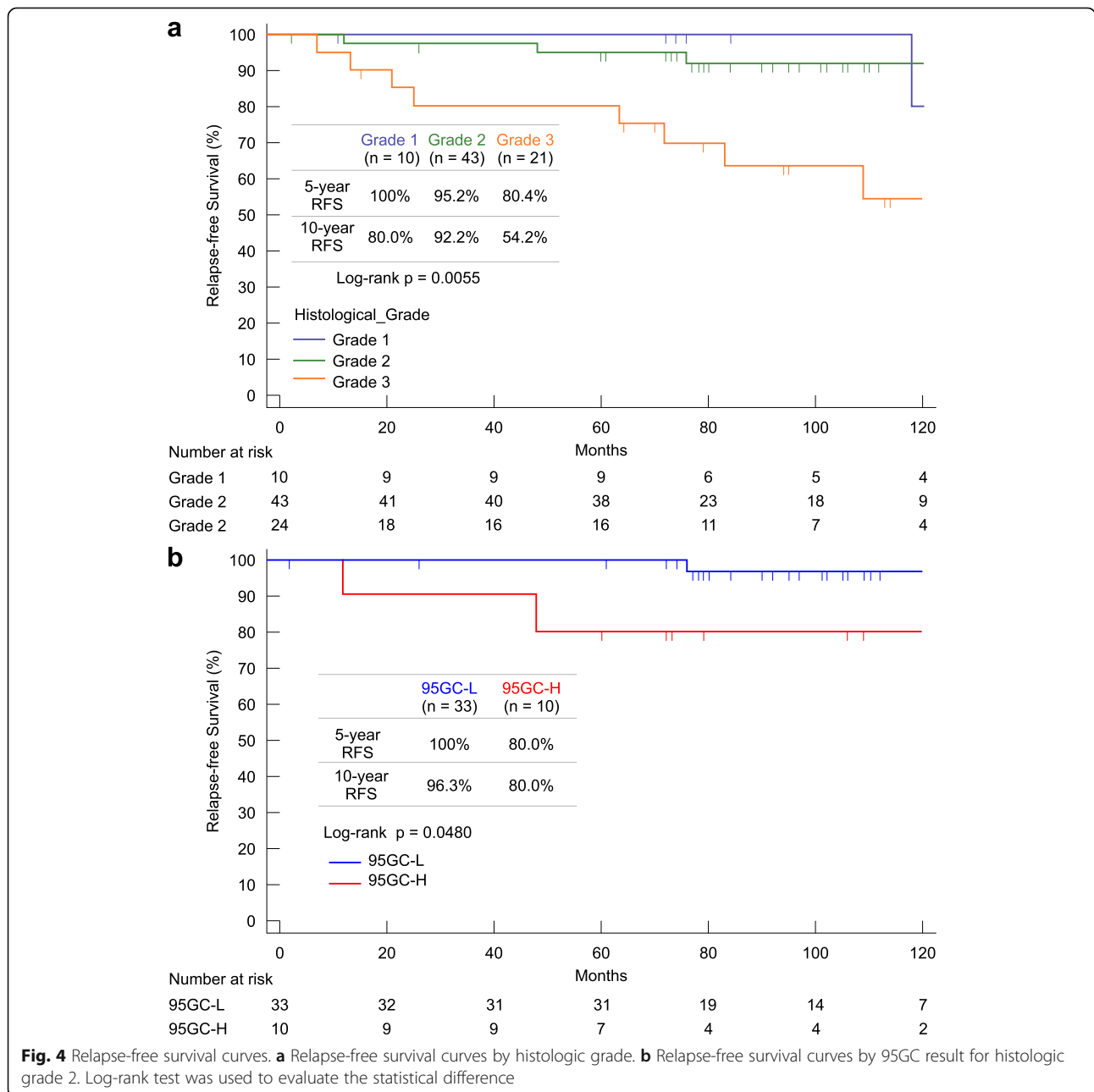

Fig. 3 Relapse-free survival curves for PAM50 Luminal B for each result of 95GC. Relapse-free survival was evaluated in patients with luminal B tumors in the 95GC-L group (in blue) and 95GC-H group (in red) for up to 120 months post-surgery. The log-rank test was used to evaluate statistical differences

Table 2 Univariate and multivariate analyses of 95GC and other clinicopathological factors

Variable		Univariable analysis			Multivariable analysis		
		HR	95% CI	P	HR	95% CI	P
Age	≤50 vs > 50 years	0.60	0.19–1.90	0.3845			
T stage	T2 vs T1	1.44	0.45–4.55	0.5385			
Histologic grade	2 and 3 v 1	2.03	0.26–15.89	0.4986			
PR	Positive vs Negative	0.46	0.14–1.53	0.2055			
Ki-67	≥20% vs < 20%	3.69	1.16–11.67	0.0265	1.73	0.49–6.14	0.3982
PAM50	Others vs luminal A	5.74	0.74–44.56	0.0945			
95GC	95GC-H vs 95GC-L	6.26	1.69–23.27	0.0061	5.14	1.24–21.37	0.0242

Fig. 4 Relapse-free survival curves. **a** Relapse-free survival curves by histologic grade. **b** Relapse-free survival curves by 95GC result for histologic grade 2. Log-rank test was used to evaluate the statistical difference

With regard to the association with histologic grade, when 95GC was used to divide the patients with grade 2 tumors into two groups (95GC-L and 95GC-H), the 10-year relapse-free survival rate for 95GC-L was 96.3%, which was significantly better than that for 95GC-H, and the relapse rate was extremely low. Therefore, chemotherapy can be considered omissible in patients with a histologic grade of 2 if they are 95GC-L.

By performing a multivariate analysis of 95GC and clinicopathological factors utilized in previous studies to assess breast cancer recurrence risk, such as age, tumor diameter, tumor grade, Ki-67, and PAM50, we were able to confirm that 95GC is a significant independent prognostic factor that influences the recurrence risk. In our cohort we did not include patients that have received neoadjuvant therapy. Because neoadjuvant chemotherapy is often added in tumors ≥3 cm, the T2 cohort included only cases with a size close to T1 stage, and as a result, there was no difference in prognosis between T1 and T2 stages.

The 95GC assay could provide extremely useful information for the selection of therapeutic strategies, such as adjuvant chemotherapy. The 95GC-L group had a better prognosis in response to adjuvant endocrine therapy than the 95GC-H group. Further prospective studies are needed to evaluate whether 95GC assays could be used to determine the necessity of chemotherapy in patients with ER-positive, node-negative breast cancer.

The Oncotype DX®, a breast cancer multigene assay, analyzes 21 genes using reverse transcription polymerase chain reaction, and classifies patients by recurrence score (RS) (0–100) into low-, mid-, and high-RS groups. It is a useful prognostic factor in hormone receptor-positive, HER2-negative, node-negative breast cancer and a factor for predicting the effectiveness of adjuvant chemotherapy in the high-RS group [5, 6]. Naoi et al. reported that in an indirect comparison with Oncotype DX® using Recurrence Online, 95GC could be used to classify 81 patients as having a mid-range risk (95GC-L group of 38 patients and 95GC-H group of 43 patients), with a significantly better recurrence-free survival rate in the 95GC-L group than in the 95GC-H group [13].

In everyday clinical practice, if a multigene assay is not available, Ki-67 is often used as a prognostic factor for ER-positive, HER2-negative breast cancer. In our study, Ki-67 measurement was performed through a central pathology review, but it was not a significant prognostic factor. It is difficult to establish a standardized method for evaluating Ki-67; therefore, it is difficult to accurately assess recurrence risk based on this factor. In the future, multigene assays for breast cancer should be incorporated more widely in everyday clinical practice.

With regard to the usefulness of 95GC as a predictive factor for the effectiveness of chemotherapy,

Tsunashima et al. examined 72 patients from their facility and 287 public database patients who underwent neoadjuvant chemotherapy. They reported a significantly greater tumor-shrinking effect in the 95GC-H group than in the 95GC-L group [16]. Even though further prospective studies are needed, in the future, 95GC might be used not only as a prognostic factor but also as a predictive factor for the effectiveness of chemotherapy.

The main limitation of this study was its small sample size. Additionally, the retrospective nature of the study resulted in a selection bias. Moreover, because of the archived samples, the quality of which was not controlled owing to the surgical practices used in the earlier days, 26.5% (27/102) of the samples had poor RNA quality. However, in recent times, the strict rules enforced for the handling of post-extraction specimens have helped to preserve RNA quality and, thus, guarantee a high rate of sample recuperation in everyday clinical practice. Finally, all study participants were Japanese, and the performance when targeting other races in other countries is unknown. However, a recently published study evaluated the recurrence stratification of patients from both Japan and the US, with comparable results between populations [17].

Currently, 95GC has been available in Japan since 2013 for research purposes. Hence, further validation of the benefits of 95GC through a prospective study using a larger sample size is warranted to confirm the validity of incorporating 95GC in everyday clinical practice in Japan.

Conclusions

This retrospective study suggests that 95GC is a long-term prognostic factor for ER-positive, node-negative breast cancer. To more optimally treat breast cancer patients, a system must be established to accurately assess the patient's prognosis and determine whether the addition of chemotherapy will likely provide beneficial effects.

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Availability of data and materials
The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Ethical approval was obtained from institutional review board of Japan Community Health care Organization Osaka Hospital, institutional review board of Hiroshima University Hospital, institutional review board of National Hospital Organization Shikoku Cancer Center, the Medical Ethics Committee on Clinical Investigation of Shinshu University, National Cancer Center Hospital Certified Review Board and institutional review board of Sysmex Corporation (IRB No. 2014–46), and the study was conducted in accordance with the current laws of Japan. Comprehensive written informed consent was obtained from each patient before the surgery. For patients who have difficulty receiving informed consent with regard to the use of existing samples and information, an opportunity to refuse was given to them by posting information about the research, including the purpose of the research, on the homepage of each facility. This procedure has been approved by the institutional review board of each institution.

Consent for publication
Not Applicable.

Competing interests
KI received research funding from Eisai Co., Ltd. KK and KY, who are employees of the Sysmex Corporation, participated in the analysis.

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