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Development of recurrence risk score using 95-gene classifier and its application to formalin-fixed paraffin-embedded tissues in ER-positive, HER2-negative and node-negative breast cancer

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Abstract. We previously developed a 95-gene classifier (95GC) to classify ER-positive/HER2-negative/node-negative ($ER^+/HER2^-/N0$) breast cancer as high- and low-risk. The present study aimed to devise a 95GC recurrence score (^{95GC}RS) to estimate recurrence risk more precisely and, although the 95GC was originally developed using fresh-frozen (FF) tissues, this was applied to formalin-fixed paraffin-embedded (FFPE) tissues. ^{95GC}RS was calculated using between-group analysis and denominated as a value from 0 to 100. Correlation of ^{95GC}RS with distant recurrence rate and response to neoadjuvant chemotherapy (NAC) was evaluated in 257 patients with $ER^+/HER2^-/N0$ breast cancer treated with adjuvant hormonal therapy at Osaka University Hospital and in 425 patients with ER^+ breast cancer treated with NAC at Osaka University Hospital and the University of Texas MD Anderson Cancer Center (GSE25066 dataset). Correlation of ^{95GC}RS between FF and FFPE tissues was evaluated in paired tissues from 56 $ER^+/HER2^-/N0$ breast cancer types obtained from patients without NAC treatment. Distant recurrence rates were remarkably low in patients with ^{95GC}RS ≤50 and increased proportionally in patients with ^{95GC}RS >50. Pathological complete response (pCR) rates to NAC were increased in proportion to ^{95GC}RS, indicating a greater sensitivity of breast cancers with high ^{95GC}RS to chemotherapy. ^{95GC}RS was highly correlated (R=0.92) between FF and FFPE tissues, and the concordance rate (94.6%) of high- and low-risk groups was also considerably high. Overall, the present study developed a ^{95GC}RS that correlated with distant recurrence rate and pCR rate to NAC. The 95GC was applicable to FFPE tissues with a high concordance rate in FF tissues.

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Abbreviations: ER, estrogen receptor; FF, fresh-frozen; FFPE, formalin-fixed paraffin-embedded; HER2, human epidermal growth factor receptor 2; NAC, neoadjuvant chemotherapy; pCR, pathological complete response; ^{95GC}RS, 95-gene classifier recurrence score; P-FEC, paclitaxel followed by a combination of 5-fluorouracil, epirubicin and cyclophosphamide; P-[F]AC, taxane and fluorouracil, doxorubicin and cyclophosphamide

Key words: breast cancer, chemosensitivity, 95-gene classifier, microarray, recurrence score, formalin-fixed paraffin-embedded

Introduction

Predicting the prognosis of patients with estrogen receptor-positive/human epidermal growth factor receptor 2-negative/node-negative ($ER^+/HER2^-/N0$) breast cancer with a high accuracy is critical to decide the indication for adjuvant chemotherapy. Accordingly, several multigene assays (MGAs) that have been developed based on the expression of multiple genes in breast cancer tissues, such as Oncotype DX and MammaPrint (1-4), are widely used in clinical practice.

We previously developed Curebest 95GC Breast (Sysmex Co., Kobe, Japan), a 95-gene classifier (95GC) using DNA microarray (GeneChip Human Genome U 133 Plus 2.0 Array). The 95GC helps classify patients with ER^+/HER2-/N0 breast cancer types into high- and low-risk groups. In addition, intermediate risk breast cancer types [recurrence score (RS), 18-30] classified using 21GC (^{21GC}RS were calculated using Recurrence Online) can be further dichotomized using a 95GC into low- and high-risk groups (5). This dichotomization is expected to lead to a significant difference in disease prognosis, suggesting the use of 95GC in predicting the prognosis of intermediate-risk breast cancers.

Although 95GC dichotomizes breast cancer into high- and low-risk groups by using a defined RS cutoff, the recurrence risk is thought to increase in proportion to the RS, as was

Table I. Clinicopathological parameters of patients with estrogen receptor-positive/human epidermal growth factor receptor 2-negative/node-negative breast cancer included in the study for correlation between ^{95GC}RS and distant recurrence rate.

	95GC				
Variable	Low-risk (n=180)	High-risk (n=77)	OR	95% CI	P-value[a]
Menopause, n (%)				0.39-1.15	0.167
Premenopausal	69	37 (35)	1.00		
Postmenopausal	111	40 (26)	0.672		
cT, n				0.61-1.80	0.890
1	105	44	1.00		
2+3	75	33	1.05		
HG, n				1.38-9.30	<0.0001
1	93	16	1.00		
2	79	50	1.00		
3	8	11	3.58		
PR, n				0.22-0.93	0.045
Negative	19	16	1.00		
Positive	161	61	0.45		
Ki67, n[b]				0.81-4.00	0.197
<20%	94	34	1.00		
≥20%	20	13	1.80		

[a]Fisher's exact test. [b]Unknown data were not included in the analysis. CI, confidence interval; cT, clinical tumor size; OR, odds ratio; PR, progesterone receptor; ^{95GC}RS, 95GC recurrence score; HG, histological grade.

clearly shown by the correlation between recurrence rate and RS in Oncotype DX (1,2,6,7). Predicting the recurrence risk using an RS for each patient could enable better decision-making for adjuvant chemotherapy indication than using 95GC information of high or low-risk patients. The present study primarily aimed to develop a 95GC-based RS (^{95GC}RS) and demonstrate a correlation with recurrence rate in ER$^+$/HER2$^-$/N0 breast cancer types. In addition, the study aimed to apply 95GC, originally developed using fresh-frozen (FF) tissues, to FFPE tissues, because FFPE tissues are routinely prepared and are readily available. Although we previously reported the applicability of 72GC to FFPE tissues (8), the present study aimed to improve the accuracy of 95GC for FFPE tissues using the reference robust multiarray average (refRMA) method, optimized for FFPE tissues. Therefore, a ^{95GC}RS was first developed and then the accuracy of the newly developed 95GC algorithm for FFPE tissues was evaluated using the ^{95GC}RS.

Materials and methods

Breast cancer tissues

Development of ^{95GC}RS. A total of 257 patients with ER$^+$/HER2-/N0 breast cancer who underwent breast-conserving surgery or mastectomy at Osaka University Hospital (Suita, Japan) and were treated using only adjuvant hormonal therapy were retrospectively included in this study (Table I). FF tumor tissues were obtained from surgical specimens and stored at -80˚C until use. The median follow-up period was 86 months (range, 12-190 months). Of the 257 patients, 106 were treated postoperatively with goserelin (3.75 mg/4 weeks) plus tamoxifen (20 mg/day) and 151 were treated with anastrozole (1 mg/day). Tamoxifen and anastrozole were administered for 5 years or until recurrence, whichever occurred earlier, whereas goserelin was administered for 2 years. Informed consent to participate in the study was obtained from all patients before surgery. The present study was approved by the Ethics Committee of Osaka University Hospital.

Correlation between ^{95GC}RS and response to chemotherapy. A total of 126 patients with ER$^+$ breast cancer (stage II-III) who were treated with neoadjuvant chemotherapy (NAC) followed by mastectomy or breast-conserving surgery at Osaka University Hospital between 2004 and 2012 were retrospectively included in this study (Table II). NAC consisted of paclitaxel (80 mg/m^2) weekly for 12 cycles, followed by a combination of 5-fluorouracil (500 mg/m^2), epirubicin (75 mg/m^2) and cyclophosphamide (500 mg/m^2) every 3 weeks for four cycles (P-FEC). Before initiating NAC, all patients underwent tumor biopsy using a vacuum-assisted core-biopsy instrument (Mammotome 8G HH; Ethicon Endosurgery Inc.) under ultrasonographic guidance for histological examination and gene expression analysis. Tumor samples for histological examination were fixed in 10% buffered formaldehyde, and tumor samples for gene expression analysis were snap-frozen in liquid nitrogen and stored at -80˚C until use. Informed consent to participate in the study was obtained from all

Table II. Clinicopathological parameters of patients included in the study for correlation between ^{95GC}RS and pCR rate to neoadjuvant chemotherapy in patients (n=126) at Osaka University Hospital.

Parameter	Value
Age, years	
Median	47
Range	24-76
Postmenopausal, n	57
cT, n	
T1	8
T2	89
T3	18
T4	11
cN, n	
N0	46
N1	80
Histological grade, n	
1	26
2	80
3	20
ER, n	
Positive	126
Negative	0
PR	
Positive	84
Negative	42
HER2	
Positive	25
Negative	101
Ki67[a]	
Positive	41
Negative	47

[a]Unknown data were not included in the analysis. ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.

patients before performing the tumor biopsy. In addition, 299 patients with ER$^+$ breast cancer who were treated with neoadjuvant sequential taxane and fluorouracil, doxorubicin and cyclophosphamide [P-(F)AC] were selected from the GSE25066 dataset (9) available in the public database GEO (https://www.ncbi.nlm.nih.gov/geo/).

Application of 95GC to FFPE tissues. Two adjacent tumor specimens were obtained from each of the 56 ER$^+$/HER2$^-$/N0 breast cancers: One specimen was stored at -80˚C as FF tissue and the other was fixed in 10% buffered formalin for FFPE tissue preparation (Table SI). Of these 56 breast cancer types, 25 samples were from patients treated at Osaka University Hospital without NAC and 31 were purchased from East West Biopharma LLC as paired FF/FFPE specimens (Table SI).

RNA extraction and DNA microarray assay in FF and FFPE tissues

FF tissues. RNA was extracted from FF tumor tissues using Qiagen RNeasy Lipid Tissue Mini kits (Qiagen GmbH). Approximately 100 ng RNA (RNA integrity number >7) was used to generate second-strand cDNA, and cRNA was amplified using the oligodeoxynucleotide ribosylthymine primers, then biotinylated and fragmented using the Gene Profiling Reagent kit (Affymetrix; Thermo Fisher Scientific, Inc.), followed by hybridization using U133 Plus 2.0 arrays overnight (17 h) according to the manufacturer's protocol. Finally, the hybridized DNA microarray was fluorescently stained with GeneChip Fluidics Station 450, and scanned using a GeneChip Scanner 3000.

FFPE tissues. RNA was extracted from four consecutive sections (10 μm) of each FFPE tissue using RNeasy FFPE kits (Qiagen GmbH). Second-strand cDNA was generated using 70-100 ng of RNA, and cDNA was amplified using the oligodeoxynucleotide ribosylthymine and random primers using an Ovation FFPE whole-transcriptome amplification system (NuGEN Technologies, Inc.) according to the manufacturer's protocol. The cDNA was then biotinylated and fragmented using the Encore Biotin Module (NuGEN Technologies, Inc.), followed by hybridization on U133 Plus 2.0 arrays overnight (17-20 h) according to the manufacturer's protocol. Finally, the hybridized DNA microarray was fluorescently stained using a GeneChip Fluidics Station 450, and scanned using a GeneChip Scanner 3000 (both Affymetrix; Thermo Fisher Scientific, Inc.).

Histological examination. Pathological response to NAC was evaluated using surgical specimens obtained during surgery. Specimens were cut into 5-mm slices, and hematoxylin and eosin-stained sections were prepared to determine the presence or absence of tumor cells. A complete absence of invasive tumor cells in the breast and lymph nodes was defined as pathological complete response (pCR), irrespective of the presence or absence of non-invasive breast cancer cells. ER, PR, and Ki67 levels in the tumor biopsy samples were immunohistochemically determined as previously described (10-12). The cut-off values were 10% for ER, 10% for PR and 20% for Ki67. HER2 amplification was determined using fluorescence *in situ* hybridization (FISH) using the PathVysion HER-2 DNA Probe kit (Vysis/Abbott Molecular Inc.) according to the manufacturer's instructions. Tumors were classified as HER2-amplified if the FISH ratio was ≥2.0.

Statistical analysis. Gene expression datasets obtained by DNA microarray were normalized using the refRMA procedure, followed by analysis using a between-group analysis (BGA) classifier model for 95GC as previously reported by our group (5) for classifying patients into low- and high-risk groups. All statistical analyses were performed using R statistical software (version 3.5.1; http://www.r-project.org/), apart from the comparison of ^{95GC}RS between FF and FFPE tissues, as shown in Fig. 3, which was performed in Microsoft Excel 2010 (Microsoft Corporation) using the CORREL function. Fisher's exact test was used to compare 2x2 groups. All statistical analyses were two-sided, and $P<0.05$ was considered to indicate a statistically significant difference.

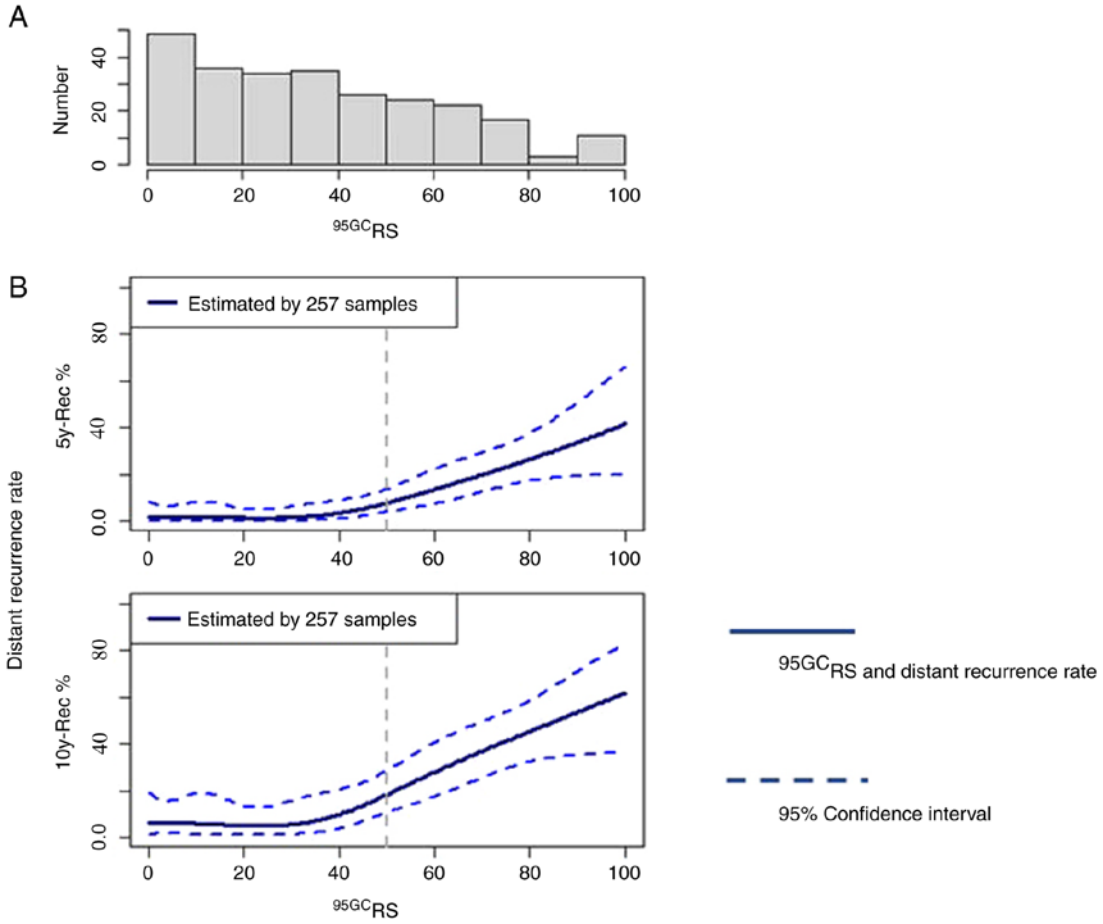


Figure 1. Association between 95GCRS and distant recurrence rate. (A) Histogram of patients according to 95GCRS. (B) 5 and 10-year distant recurrence rates are shown in proportion to 95GCRS in 257 patients with estrogen receptor-positive/human epidermal growth factor receptor 2-negative/node-negative breast cancer treated with adjuvant hormonal therapy and without chemotherapy. 95GCRS, 95-gene classifier recurrence score.

Results

Development of 95GCRS. BGA was used to separate low- and high-risk groups in 95GC (5). BGA assigns a value to each tumor, where valueS >0 are considered high-risk tumors and valueS ≤0 are considered low-risk tumors. These values were then converted to 95GCRS 0-100 using the following formula:

95GC score: *round* {(1000/3) x original value+50}

95GCRS was calculated for 257 patients with ER^+/$HER2^-$/N0 breast cancer receiving adjuvant hormonal therapy alone as an independent validation set. The histogram of patients according to 95GCRS is shown in Fig. 1A and the correlation between 95GCRS and distant recurrence rates at 5 and 10 postoperative years is shown in Fig. 1B. Distant recurrence rates were significantly low in patients with 95GCRS ≤50 (low-risk) and increased in proportion to 95GCRS in patients with 95GCRS >50 (high-risk).

95GCRS and response to NAC. The correlation between 95GC*RS* and response (pCR) to NAC was examined in 425 patients with ER^+ breast cancer treated with NAC [P-FEC or P-(F)AC]. As shown in Fig. 2, pCR rates increased in proportion to 95GCRS, indicating the increased sensitivity of breast cancers with high 95GCRS to chemotherapy.

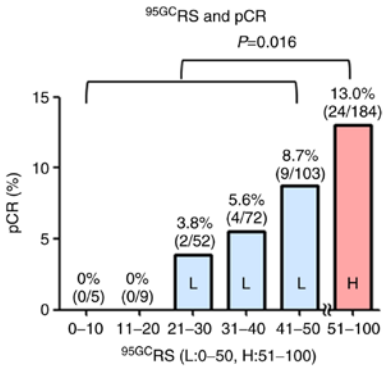
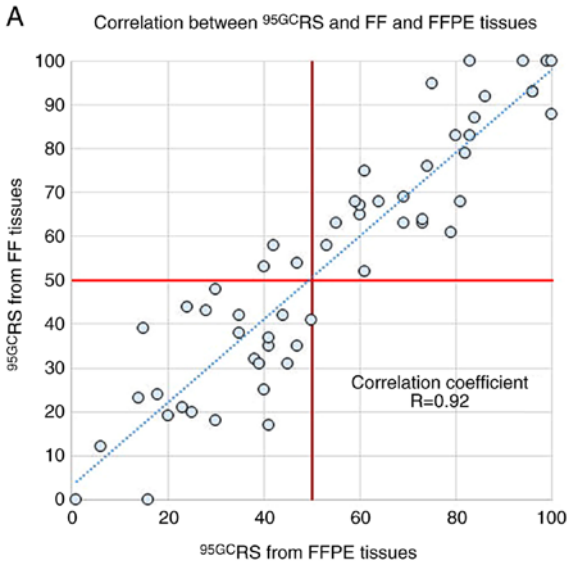


Figure 2. Association between 95GCRS and pCR rate. The pCR rate is shown in proportion to the 95GCRS in 425 patients with estrogen receptor-positive breast cancer treated with neoadjuvant chemotherapy [P-FEC or P-(F) AC]. The 95GC low-risk group (L) is shown as a blue bar and the high-risk group (H) as a red bar. In the x-axis, a cut was made between the low-risk group and the high-risk group. 95GCRS, 95-gene classifier recurrence score; pCR, pathological complete response.

Application of 95GCRS to FFPE tissues. When 95GC was calculated for FF tissues, gene expression data were normalized using refRMA constructed for FF tissues (5). Since gene expression in FFPE tissues is significantly affected by mRNA degradation during FFPE tissue preparation, it is necessary to construct a refRMA specific to FFPE tissues. A refRMA was constructed



B

Concordance of 95GC results between FF and FFPE

	95GC for FFPE	
95GC for FF	Low	High
Low	26	0
High	3	27

Concordance rate=94.6%

Figure 3. (A) Comparison of ^{95GC}RS between FF and FFPE tissues. ^{95GC}RS was compared between paired samples (n=56) of FF and FFPE tissues from the same tumor (CORREL test). (B) Concordance of 95GC high/low results between FF and FFPE. FF, fresh-frozen; FFPE, formalin-fixed paraffin-embedded; ^{95GC}RS, 95-gene classifier recurrence.

for FFPE tissues using the GSE47109 (13) and GSE51450 (14) datasets available in the GEO public database (https://www.ncbi.nlm.nih.gov/geo/) (comprising gene expression data from FFPE breast cancer tissues) to optimize the concordance of 95GC results between FF and FFPE tissues (Fig. S1). Subsequently, 56 pairs of FF and FFPE breast cancer tissues were subjected to 95GC assay and ^{95GC}RS values were calculated (FF tissues were analyzed using refRMA for FF tissues and FFPE tissues were analyzed using refRMA for FFPE tissues) (Fig. 3). The correlation coefficient was significantly high (R=0.92) between ^{95GC}RS obtained from the FF and FFPE tissues, and the concordance rate (94.6%) of the high- and low-risk groups was also notably high between the tissues (Fig. 3).

Discussion

In the present study, ^{95GC}RS ranging from 0 to 100 was first developed, which correlated well with distant recurrence. Breast cancer with ^{95GC}RS ≤50 had a significantly low recurrence rate, whereas that with ^{95GC}RS >50 had a high recurrence rate. In addition, the recurrence rate increased in proportion to ^{95GC}RS. Similar results were previously reported for ^{21GC}RS (1,2,6,7). Information on recurrence risk using ^{95GC}RS for individual patients could enable better decision-making in a clinical setting for adjuvant chemotherapy indication than binary results (high- or low-risk groups).

We previously reported a correlation between risk groups determined by 95GC and response to NAC (5,15). Breast cancers in the 95GC high-risk group exhibited a significantly higher response rate to NAC than those in the low-risk group. Similar reports have also been reported following the use of Oncotype DX and MammaPrint, which have shown the increased sensitivity of high-risk breast cancers to NAC (16-20). In the present study, breast cancers were further categorized using ^{95GC}RS and demonstrated the gradual increase of pCR in proportion to ^{95GC}RS, indicating greater chemosensitivity in breast cancers with high ^{95GC}RS. Altogether, the results suggest the remarkable ability of 95GC to categorize patients at high-risk for relapse who would likely benefit from adjuvant chemotherapy.

The 95GC was originally developed using gene expression data from FF tissues; however, this needs to be applicable to FFPE tissues prepared in routine practice to enhance its clinical use. Thus, the present study attempted to modify and apply 95GC to FFPE tissues. As preparation of FFPE tissues leads to mRNA degradation, refRMA was first developed for FFPE tissues and then ^{95GC}RS was calculated. A significantly high correlation coefficient (R=0.92) was demonstrated between ^{95GC}RS and FF and FFPE tissues, as well as a markedly high concordance rate (94.6%) between the high- and low-risk groups, demonstrating the potential use of 95GC for FFPE tissues.

In conclusion, in the present study, a ^{95GC}RS was developed that correlated well with recurrence rate, and it was demonstrated that 95GC is applicable to FFPE tissues. These preliminary results need to be confirmed in future studies.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to the patients' consent for provision to other facilities, but are available from the corresponding author on reasonable request. Datasets GSE25066, GSE47109 and GSE51450 are available from the GEO public database.

Authors' contributions

SN and YN conceived and designed the study, drafted and revised the paper critically for important intellectual content, and approved the final version of the manuscript. SN, YN, YS, KK, MS, NK, TM, TT, KS and SJK were responsible for the acquisition, analysis or interpretation of data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study complied with the current relevant laws and guidelines for Japan. Informed consent to participate in the study was obtained from all patients before surgery. Approval was obtained from the Ethics Committee of Osaka University Hospital. Patient consent was obtained from all patients before surgery.

Patient consent for publication

Not applicable.

Competing interests

SN has been an advisor for Taiho, AstraZeneca and Novartis, and received research funding for other studies from Sysmex, AstraZeneca, Novartis, Chugai, Daiichi-Sankyo, Kyowa-Kirin, Takeda, Pfizer, Ono, Taiho, and Eisai, and honoraria from AstraZeneca, Novartis, Pfizer, Chugai, Takeda, Sysmex, Nippon Kayaku. YN received research funding from Sysmex and AstraZeneca. NK received honoraria from AstraZeneca and Novartis. MS received research funding from Novartis and AstraZeneca, and honoraria from Chugai, Eisai, Novartis, and Takeda. KS received honoraria from AstraZeneca, Chugai, and Sysmex. SJK received honoraria from AstraZeneca, Chugai, Eisai, Kyowa-Kirin, Novartis, Pfizer, Shimadzu, Taiho, and Takeda. YS and KK are the agents of Sysmex Corporation. SN and YN are patent holders about 95GC.

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