

Evaluation of 95-Gene Classifier of Formalin-Fixed Paraffin-Embedded Tissues in ER-Positive, HER2-Negative, and Node-Negative Breast Cancer

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Abstract

Background A subset of patients with estrogen receptor (ER)-positive, HER2-negative, and node-negative breast cancer experience recurrences. Predicting patients who will have recurrences within 5 years of surgery is essential so that patients can be selected to receive adjuvant chemotherapy. The 95-gene classifier (95-GC) has been validated as a method to differentiate patients into high and low-risk groups for early recurrence.

Methods In this study, we performed 95-GC analysis on 56 formalin-fixed paraffin-embedded (FFPE) tissue samples from patients who underwent surgery for ER-positive, HER2-negative, and node-negative breast cancer and did not receive adjuvant chemotherapy. We associated the obtained high- and low-risk groups with clinicopathological characteristics and recurrence-free survival (RFS).

Results We classified 12 out of 56 patients into the high-risk recurrence group. We found significantly higher KI67 scores in patients in the high-risk group. Other clinicopathological characteristics were not associated with the 95-GC risk groups. Patients in the 95-GC low-risk group had a significantly better prognosis than those in the high-risk group ($p = 0.0387$). The 5-year RFS rate was 97.6% in the low-risk group and 74.1% in the high-risk group, while the 10-year RFS rates were 90.1% and 74.1%, respectively.

Conclusions Our study shows that the 95-GC score can accurately predict RFS within 5 years of surgery for ER-positive, HER2-negative, and node-negative breast cancer using FFPE tissue samples. These prediction models could help assign patients to the most effective treatment regimen.

Background

Over the past decades, new strategies to complement therapies for early-stage breast cancer have improved disease outcomes.(1–3) For instance, within the first 5 years of diagnosis, chemotherapy in the adjuvant setting can reduce the recurrence risk.(3) Therefore, predicting the risk of early recurrence is vital to determine the appropriate type of chemotherapy treatment after surgery. Accurate diagnostic and prognostic prediction methods using clinicopathological characteristics are warranted to determine the best personalized treatment regimen for each patient.

In recent years, multi-gene assays, such as OncotypeDx® and MammaPrint®, have made it possible to predict the prognosis of breast cancers.(4–7) However, a disadvantage of some of these methods is that patients are often classified into a low, intermediate, or high-risk group for recurrences. Choices in clinical practice for post-surgery treatment are challenged by this grouping, as it is unclear whether the intermediate group would benefit from adjuvant chemotherapy.

The 95-gene classifier (Curebest™ 95GC Breast, Sysmex, Kobe, Japan) is a multi-gene assay that uses DNA microarray analysis to classify breast cancer patients as having a high or low risk of recurrence, based on the expression levels of 95 genes.(8) In this study, patients who were grouped into the low-risk group had a better outcome than those in the high-risk group. The use of the 95-gene classifier allows for

a clear division into high and low risk, without referring to intermediate groups. This distinction aids treatment strategies in clinical practice.

The 95-GC method was initially optimized to be used for frozen tissue samples but has since been shown to also be effective and consistent in formalin-fixed paraffin-embedded (FFPE) specimens.(9) However, it has not yet been established whether the results obtained from FFPE tissue are also predictive of outcome. We hypothesized that the 95-GC can be accurately used on FFPE material to not only determine high or low risk of recurrence but also predict the outcome. In this study, we performed the 95-GC analysis on FFPE tissue specimens of 56 patients with ER-positive, HER2-negative, and node-negative breast cancer who did not receive adjuvant chemotherapy and compared 95-GC signatures with the outcome.

Methods

Patients and cancer specimens

We recruited 56 women with stage I or II breast cancer for this study. All patients were treated between 2005 and 2012 at Hokkaido University Hospital (Table 1). The institutional review board approved the study protocol and our study met the guidelines of the Declaration of Helsinki (1996). All patients provided written informed consent before treatment for the collection of surgically resected tumor tissues. The samples were collected continuously from all patients with ER-positive and HER2-negative invasive ductal carcinoma of the breast. All patients underwent mastectomy or lumpectomy, during which tumor samples were obtained. Patients received adjuvant endocrine therapy (tamoxifen with or without luteinizing hormone-releasing hormone agonist for premenopausal women and aromatase inhibitors for postmenopausal women) after surgery. We excluded patients treated with adjuvant or neoadjuvant chemotherapy.

FFPE tissues were stored at 4°C until the study was performed and four sections of 10 µm thickness of FFPE specimens were obtained for RNA extraction.

RNA extraction and DNA microarray analysis

RNA extraction was performed using the RNeasy FFPE Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). The extracted RNA was synthesized into cDNA using the Ovation FFPE WTA Systems (Affymetrix, Santa Clara, CA, USA) and the Encore Biotin Module (Affymetrix) kits according to the manufacturer's instructions. The cDNA (100 ng) was then used for gene expression analysis by DNA microarray using the Human Genome U133 Plus 2.0 Array according to the manufacturer's instructions (Affymetrix).

Immunohistochemistry

Tissue sections were stained for Ki67 using a mouse monoclonal anti-human Ki67 antibody (MIB-1; Dako, Glostrup, Denmark) at 1:200 dilution, and the Dako FLEX Envision system for visualization of antibody binding. We assessed the labeling index (LI), which we defined as the percentage of invasive

tumor cells (> 1,000) with nuclear staining.(10) All slides were scanned using NanoZoomer 2.0-HT (Hamamatsu Photonics, Hamamatsu, Japan) and Tissue Studio was used to perform the LI analysis (Definiens, Munich, Germany) automatically.

Statistical analysis

Risk scores for high and low recurrence groups were calculated based on the method by Naoi *et al.*(11) Recurrence-free survival (RFS) was defined as the time between surgery and recurrence, death, or end of follow-up. The Kaplan-Meier method was used to calculate RFS, and differences between groups were evaluated using the log-rank test. Spearman's rank correlation coefficient was used to evaluate the correlation between Ki67 LI and 95-GC. A univariate analysis was performed using the Cox proportional hazards model. The correlation between 95-GC and clinicopathological factors was evaluated using Fisher's exact test (for age, menopausal status, T stage, and grade) and the Mann-Whitney U Test (for ER, PgR, and Ki67 LI). $P < 0.05$ was considered statistically significant.

Results

Associations between 95-GC scores and clinicopathological factors

Patient characteristics are shown in Table 1. We included 56 patients with ER-positive, HER2-negative, and node-negative breast cancer, of whom 46 patients (82%) had stage I and 10 patients (18%) had stage II breast cancer. Using the 95-GC classifier, we classified all 56 patients into two groups according to risk of recurrence: a high-risk group ($n = 12$) and a low-risk group ($n = 44$). No correlations were observed between the groups with high and low risk based on age, menopausal status, T stage, grade, or ER and progesterone receptor (PgR) expression levels. In contrast, the KI67 (LI) was significantly higher in the high-risk group, with a median score of 20.6 as compared to 9.93 in the low-risk group ($p = 0.0033$). Additionally, we found a significant correlation between the 95-GC score and Ki67 LI (%) using Spearman's Rank correlation coefficient ($p < 0.0001$) (Fig. 1).

Table 1
Clinicopathological characteristics of included breast cancer patients

		95-Gene classifier			
Factors		Low (n = 44)	High (n = 134)	Total	<i>p</i> -value
Age	≤ 50 years	12 (71%)	5 (29%)	17	0.4797
	> 50 years	32 (82%)	7 (18%)	39	
Menopausal status	Premenopausal	11 (69%)	5 (31%)	16	0.2929
	Postmenopausal	33 (83%)	7 (17%)	40	
T stage	T1	38 (83%)	8 (17%)	46	0.3707
	T2	6 (60%)	4 (40%)	10	
ER (%)	Median	100	100	-	0.991
	Min to Max	50 to 100	30 to 100	-	
PgR (%)	Median	45	55	-	0.92
	Min to Max	0 to 100	0 to 100	-	
Grade	1	19 (86%)	3 (14%)	22	0.1816
	2	25 (76%)	8 (24%)	33	
	3	0	1 (100%)	1	
Ki67	Median	9.93	20.6	-	0.0033 *
	Min to Max	1.9 to 32.1	8.5 to 39.6	-	

Univariate analysis comparing 95-GC and other clinicopathological factors

We performed a univariate analysis for all clinical characteristics and the 95-GC scores to determine which factors affect the development of recurrences (Table 2). The only factor with a significant association with recurrences was the 95-GC score, which had a hazard ratio of 11.09 (95% CI: 1.15-106.87, $p = 0.0375$). None of the clinicopathological characteristics, except the 95-GC score, was significantly correlated with recurrences.

Table 2

Investigation of prognostic factors using univariate analysis (Cox proportional hazards model)

Factors		Hazard ratio	Lower 95	Upper 95	p-value
Age	> 50 vs. \leq 50	0.45	0.06	3.22	0.4292
Menopausal status	Post vs. Pre	1.33	0.14	12.8	0.8043
T stage	\geq T2 v T1	N/A	-	-	-
ER (%)	Continuous variables	0.96	0.92	1.00	0.0613
PgR (%)	Continuous variables	0.98	0.94	1.01	0.1560
Grade	1 vs. 2 vs. 3	4.33	0.53	35.70	0.1734
Ki67 LI (%)	Continuous variables	1.09	1.00	1.18	0.0557
95-GC	High vs. Low	11.09	1.15	106.87	0.0375*

* p < 0.05 is considered significant

Prognostic value of 95-GC risk groups

Using the 95-GC classifier, we compared the prognosis of the 95-GC high and low-risk groups. Patients were followed up for 4-129 months, with a median of 87 months. We compared the RFS and found a significant prognostic value for the 95-GC risk groups. We found a significantly better prognosis in the 95-GC low-risk group than in the high-risk group (p = 0.0387). The 5-year RFS rate was 97.6% in the low-risk group and 74.1% in the high-risk group, while the 10-year RFS rates were 90.1% and 74.1%, respectively (Fig. 2).

Discussion

For clinical practice, it is essential to develop diagnostic tools to determine a patient's risk of developing recurrences to make the right therapeutic choices. While patients with ER-positive node-negative breast cancer have an overall good prognosis, 10–20% will develop recurrences.(12) This subset of patients would benefit from a combination treatment of endocrine therapy and chemotherapy to prevent relapse within 5 years of surgery, while patients not at risk of early recurrence require only adjuvant endocrine therapy.

Previous research (8) and indirect-comparative studies with OncotypeDx using Recurrence Online (11) have established that the 95-GC can be applied to frozen tumor tissue and is an effective early recurrence predictor for up to 5 years post-surgery. As such, it is used to identify cases in which chemotherapy

should also be used.(13) While it has been established that the 95-GC can also be applied to FFPE material, data obtained from such samples have not been associated with prognostic outcomes.(9)

In our study, we defined the 95-GC risk groups using FFPE specimens from patients with cancer who had received postoperative endocrine therapy without chemotherapy. All specimens were ER-positive and HER2-negative and had infiltrations of at least 5 mm that were indicated for postoperative drug treatment. When obtained from FFPE tissue, the 95-GC was demonstrated to be effective as an early recurrence predictor for up to 5 years post-surgery. Additionally, we found a strong correlation between the 95-GC score and Ki67, another reliable predictor of early recurrence.

Our investigation into prognostic factors using univariate analysis showed the 95-GC is a prognostic factor for early recurrence. However, in the present study, we found one case with a recurrence after the first 5-year observation period. This patient was categorized by the 95-GC into the low-risk group. This may suggest that the 95-GC from FFPE cannot be used to predict late-stage recurrence after 5 years. Indeed, based on risk factors for early and late recurrence, ER-positive breast cancers with early and late recurrences are considered biologically different. (14, 15) Additionally, Ki67 has been suggested not to be a prognostic factor for late-stage recurrences. Therefore, a different signature may need to be discovered for the prediction of late-stage recurrences.(16)

Our study has a few limitations. The sample size of this study is relatively low, with only 12 patients classified in the high-risk group. This limits the results from the long-term survival analysis. Second, we did not perform a comparison between results obtained from FFPE material with frozen tissue from the same tumor. However, a previous study has established that 95-GC scores obtained from both FFPE and frozen material provide a good correlation.(9)

Conclusions

We demonstrated that the 95-GC score can accurately predict recurrence within 5 years of surgery for ER-positive, HER2-negative, and node-negative breast cancer using FFPE tissue samples in patients who did not receive adjuvant chemotherapy. Future studies should focus on prediction models for late recurrences. These prediction models help assign patients to the most effective treatment regimen.

Abbreviations

ER: Estrogen receptor

FFPE: formalin-fixed paraffin-embedded

LI: labeling index

PgR: progesterone receptor

RFS: recurrence-free survival

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from the Ethics Committee of Hokkaido University Hospital (No. 017-0415) and Sysmex Corporation (IRB No. 2017-57), and the study was conducted as per the current relevant laws of Japan. A comprehensive informed consent was obtained for each patient before surgery.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Authors' contributions

All authors contributed to the study conception and design, material preparation, and data collection. Analysis was conducted by YS, KY, and KH. The first draft of the manuscript was written by HY, YH, and KH. HY and YH commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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References

1. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Aromatase inhibitors versus tamoxifen in early breast cancer: patient-level meta-analysis of the randomised trials. *Lancet Lond Engl*. 2015 Oct 3;386(10001):1341–52.
2. Early Breast Cancer Trialists' Collaborative Group (EBCTCG), Davies C, Godwin J, Gray R, Clarke M, Cutter D, et al. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. *Lancet Lond Engl*. 2011 27;378(9793):771–84.

3. Palmieri C, Jones A. The 2011 EBCTCG polychemotherapy overview. *Lancet Lond Engl*. 2012 Feb 4;379(9814):390–2.
4. Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. *N Engl J Med*. 2004 Dec 30;351(27):2817–26.
5. Paik S, Tang G, Shak S, Kim C, Baker J, Kim W, et al. Gene expression and benefit of chemotherapy in women with node-negative, estrogen receptor-positive breast cancer. *J Clin Oncol Off J Am Soc Clin Oncol*. 2006 Aug 10;24(23):3726–34.
6. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AAM, Mao M, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature*. 2002 Jan 31;415(6871):530–6.
7. van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AAM, Voskuil DW, et al. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med*. 2002 Dec 19;347(25):1999–2009.
8. Naoi Y, Kishi K, Tanei T, Tsunashima R, Tominaga N, Baba Y, et al. Development of 95-gene classifier as a powerful predictor of recurrences in node-negative and ER-positive breast cancer patients. *Breast Cancer Res Treat*. 2011 Aug;128(3):633–41.
9. Naoi Y, Saito Y, Kishi K, Shimoda M, Kagara N, Miyake T, et al. 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. *Oncol Rep*. 2019 Dec;42(6):2680–5.
10. Yoshioka T, Hosoda M, Yamamoto M, Taguchi K, Hatanaka KC, Takakuwa E, et al. Prognostic significance of pathologic complete response and Ki67 expression after neoadjuvant chemotherapy in breast cancer. *Breast Cancer Tokyo Jpn*. 2015 Mar;22(2):185–91.
11. Naoi Y, Kishi K, Tsunashima R, Shimazu K, Shimomura A, Maruyama N, et al. Comparison of efficacy of 95-gene and 21-gene classifier (Oncotype DX) for prediction of recurrence in ER-positive and node-negative breast cancer patients. *Breast Cancer Res Treat*. 2013 Jul;140(2):299–306.
12. Park S, Koo JS, Kim MS, Park HS, Lee JS, Lee JS, et al. Characteristics and outcomes according to molecular subtypes of breast cancer as classified by a panel of four biomarkers using immunohistochemistry. *The Breast*. 2012 Feb;21(1):50–7.
13. Tsunashima R, Naoi Y, Kishi K, Baba Y, Shimomura A, Maruyama N, et al. Estrogen receptor positive breast cancer identified by 95-gene classifier as at high risk for relapse shows better response to neoadjuvant chemotherapy. *Cancer Lett*. 2012 Nov 1;324(1):42–7.
14. Ogiya A, Yamazaki K, Horii R, Shien T, Horimoto Y, Masuda N, et al. Post-relapse survival in patients with the early and late distant recurrence in estrogen receptor-positive HER2-negative breast cancer. *Breast Cancer Tokyo Jpn*. 2017 May;24(3):473–82.
15. Yamashita H, Ogiya A, Shien T, Horimoto Y, Masuda N, Inao T, et al. Clinicopathological factors predicting early and late distant recurrence in estrogen receptor-positive, HER2-negative breast cancer. *Breast Cancer Tokyo Jpn*. 2016 Nov;23(6):830–43.
16. Tsunashima R, Naoi Y, Shimazu K, Kagara N, Shimoda M, Tanei T, et al. Construction of a novel multi-gene assay (42-gene classifier) for prediction of late recurrence in ER-positive breast cancer patients. *Breast Cancer Res Treat*. 2018 Aug;171(1):33–41.

Figures

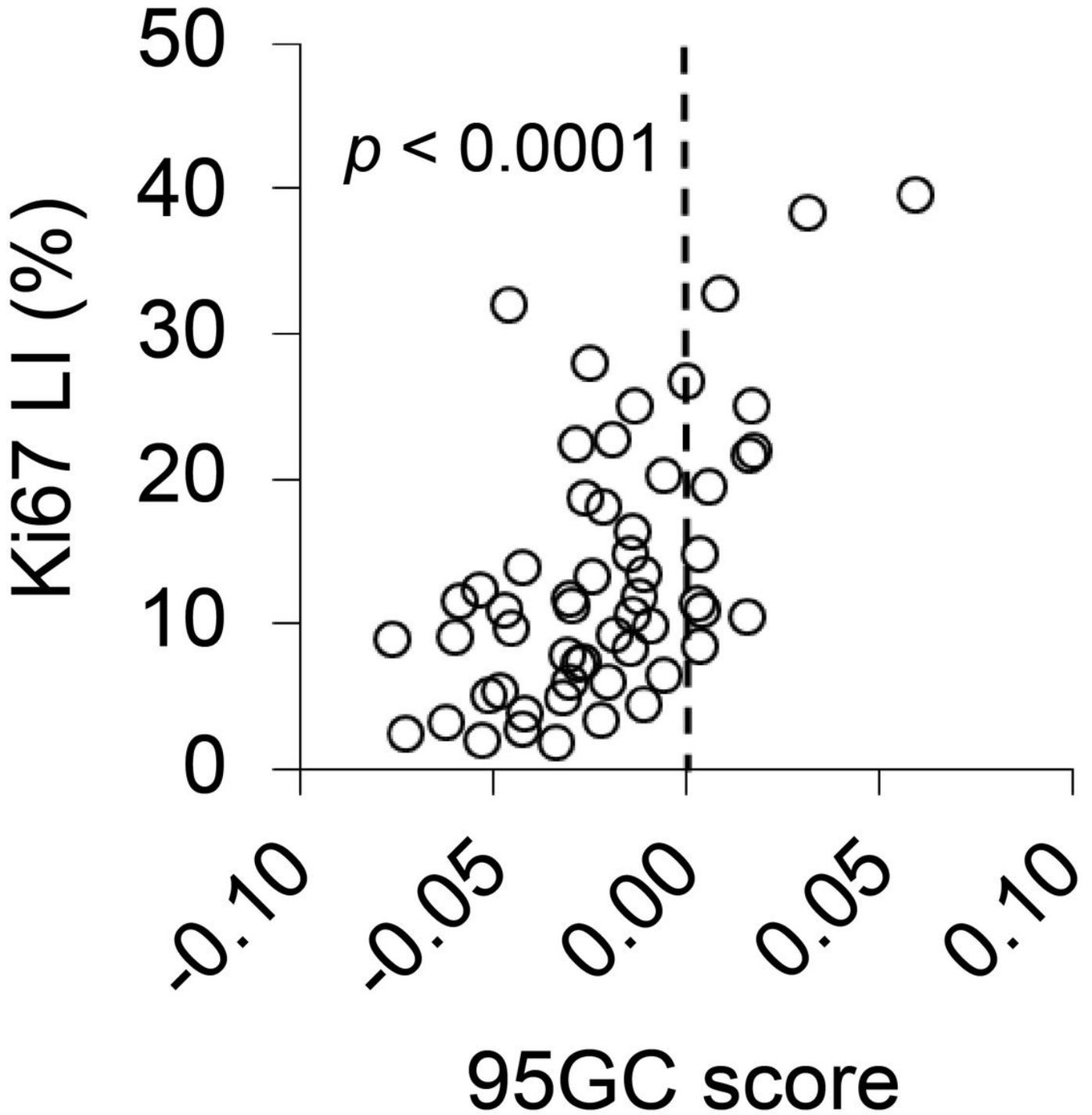


Figure 1

Spearman's rank correlation between 95-gene classifier (95-GC) score and Ki67 labeling index (LI)

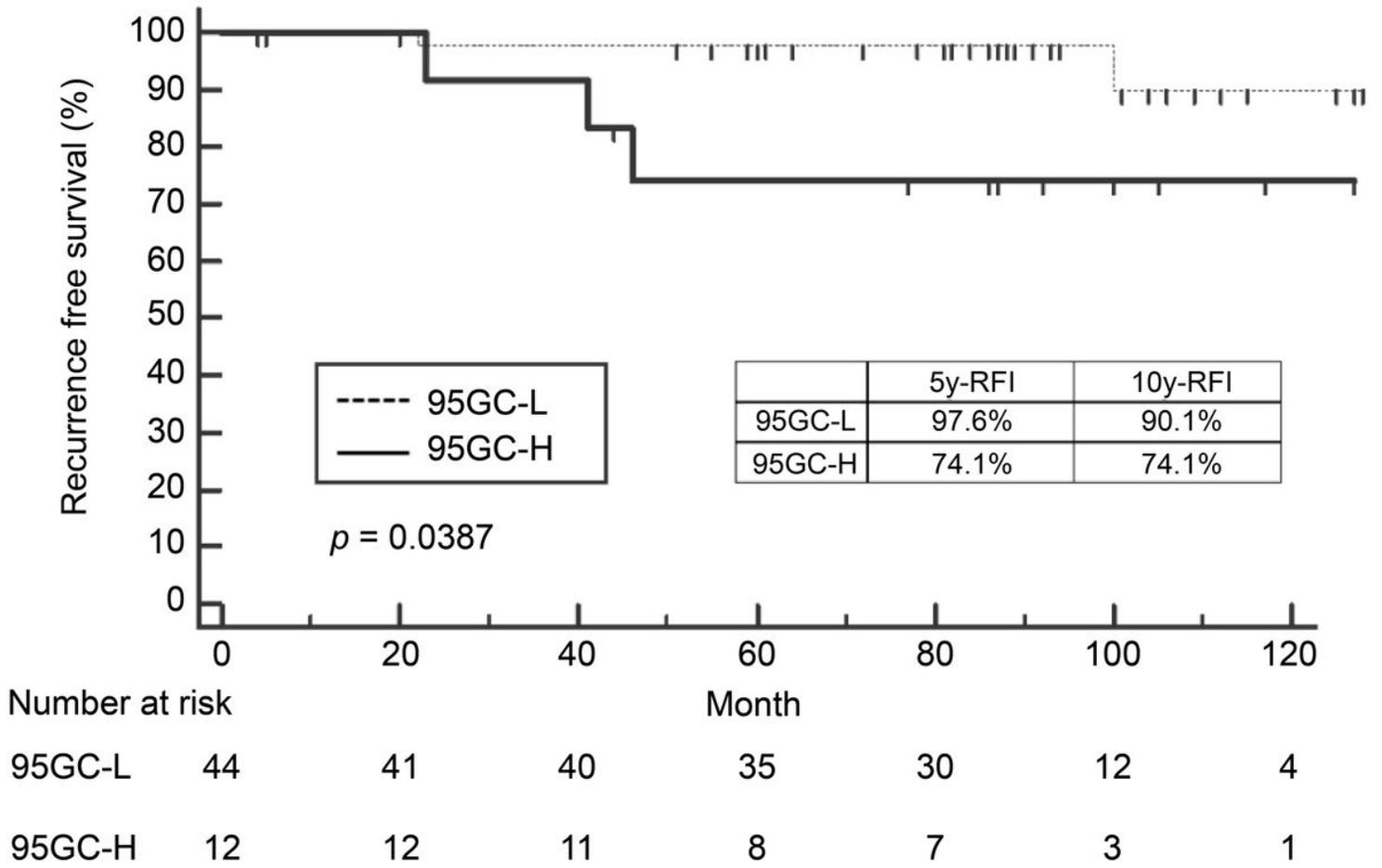


Figure 2

: Kaplan-Meier analysis of recurrence-free survival based on 95-gene classifier (95-GC) high (solid line) and low risk (dot line) groups (Log-rank test)