

Correlation Analysis of 21-Gene Recurrence Score with Gene Pathway Expression and Clinicopathological Features in Hormone Receptor-Positive Early-Stage Breast Cancer

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Abstract

Background

The 21-gene recurrence score (RS) is widely used to guide adjuvant chemotherapy decisions in hormone receptor (HR)-positive, HER2-negative early breast cancer. However, the independent contributions of individual gene pathway scores to RS have not been simultaneously modeled in a multivariate framework, particularly in real-world Asian populations. This study aimed to investigate the correlation between gene pathway scores and clinicopathological features, and to identify independent predictors of RS using multivariate analysis in a Chinese clinical cohort.

Methods

Clinicopathological data and raw 21-gene assay data were collected from 39 patients with HR-positive, HER2-negative early-stage breast cancer who underwent breast cancer surgery at Beijing Obstetrics and Gynecology Hospital from March 2020 to March 2023. The scores of estrogen group, proliferation group, HER2 group, invasion group, and the expression levels of CD68, GSTM1, and BAG1 were calculated based on the cycle threshold (Ct) values of 21 target genes. Spearman rank correlation analysis was used to analyze the correlation between each gene pathway score and clinicopathological features. Due to the limited number of high-risk patients ($n = 2$), the intermediate- and high-risk groups were combined into a non-low-risk group ($RS > 10$, $n = 22$), and the Mann-Whitney U test was used to compare gene pathway scores between the low-risk ($RS \leq 10$, $n = 17$) and non-low-risk groups; additionally, a Kruskal-Wallis H test was performed to compare scores across the original three RS risk categories. Two multiple linear regression models were constructed to identify independent predictors of RS: Model 1 included clinicopathological variables (age, tumor size, histological grade), and Model 2 included the four gene pathway scores entered simultaneously as continuous variables.

Results

Among the 39 patients, the RS ranged from 0 to 53, with a median of 12 (interquartile range [IQR], 8–22). There were 17 (43.6%) in the low-risk group, 20 (51.3%) in the intermediate-risk group, and 2 (5.1%) in the high-risk group. Spearman correlation analysis showed that the proliferation group score was positively correlated with Ki-67 index ($r = 0.612$, $P < 0.001$) and maximum tumor diameter ($r = 0.358$, $P = 0.026$); the invasion group score was positively correlated with Ki-67 index ($r = 0.489$, $P = 0.002$); the estrogen group score was positively correlated with ER expression level ($r = 0.743$, $P < 0.001$) and PR expression level ($r = 0.521$, $P = 0.001$). Between the low-risk and non-low-risk groups, the proliferation group score was significantly higher in the non-low-risk group ($P = 0.006$), while the estrogen group score showed a non-significant trend toward lower values ($P = 0.081$). Kruskal-Wallis H test across the three original RS risk categories revealed significant differences for the estrogen group score ($P = 0.002$), GSTM1 ($P = 0.032$), and BAG1 ($P = 0.035$), whereas the proliferation group score did not reach significance ($P = 0.620$), likely

due to the limited high-risk sample ($n = 2$). Multiple linear regression analysis showed that among clinicopathological variables, only histological grade was an independent predictor of RS ($B = 0.350$, 95% CI: 0.037–0.663, $\beta = 0.342$, $P = 0.029$; Model 1 adjusted $R^2 = 0.154$, $F = 3.299$, $P = 0.032$). In the gene pathway model, the invasion group score ($B = 0.089$, 95% CI: 0.008–0.170, $\beta = 0.448$, $P = 0.032$) and the estrogen group score ($B = -0.120$, 95% CI: -0.172 to -0.068, $\beta = -0.965$, $P < 0.001$) were independent predictors of RS, while the proliferation group score ($P = 0.501$) and HER2 group score ($P = 0.634$) were not independently associated after mutual adjustment. Notably, Model 2 (adjusted $R^2 = 0.446$, $F = 8.652$, $P < 0.001$) explained nearly three times the variance in RS compared with Model 1, demonstrating that gene pathway activities are the primary determinants of RS rather than traditional clinicopathological factors.

Conclusion

In HR-positive, HER2-negative early-stage breast cancer, the 21-gene RS is predominantly driven by molecular pathway activities, with the invasion group score serving as an independent positive predictor and the estrogen group score as an independent negative predictor of RS. Histological grade is the only independent clinicopathological predictor, but the gene pathway model explains substantially more RS variance (44.6%) than the clinicopathological model (15.4%). These findings provide the first multivariate pathway-level evidence from a Chinese clinical cohort, suggesting that individual gene pathway scores—particularly the invasion and estrogen components—may offer complementary mechanistic insights for refining risk stratification when integrated with the composite RS and clinicopathological assessment.

1 Introduction

Breast cancer is the most common malignancy among women worldwide, with approximately 2.26 million new cases globally in 2020, surpassing lung cancer to become the malignancy with the highest incidence [1]. Among these, hormone receptor (HR)-positive, human epidermal growth factor receptor 2 (HER2)-negative type accounts for about 70% of all breast cancers [2]. For patients with HR-positive early-stage breast cancer, postoperative adjuvant endocrine therapy is the standard treatment regimen [6, 7], but some patients still face the risk of recurrence and metastasis. How to accurately assess prognosis and screen for populations that benefit from chemotherapy remains a key clinical focus.

The 21-gene assay includes 16 cancer-related genes and 5 reference genes. The cancer-related genes can be categorized into the estrogen group (ER, PR, BCL2, SCUBE2), proliferation group (Ki-67, STK15, Survivin, CCNB1, MYBL2), HER2 group (GRB7, HER2), and invasion group (STMY3, CTSL2, MMP11), along with three individual genes (CD68, GSTM1, BAG1). The recurrence score (RS) is a weighted composite score calculated from the expression levels of these 16 cancer-related genes, which quantifies the risk of distant recurrence [3]. Multiple large-scale clinical trials, including NSABP B-14 [3], TAILORx [4], and RxPONDER [5], have validated the prognostic and predictive value of RS. However, as a

comprehensive score, RS cannot fully reflect the contribution and interaction of individual gene pathways. Exploring the correlation between gene pathway scores and clinicopathological features helps to more comprehensively understand the biological basis of RS and provides additional molecular evidence for clinical decision-making.

Previous studies have demonstrated that individual gene pathway scores within the 21-gene assay carry distinct biological significance and may provide complementary prognostic information beyond the overall RS [8, 10]. The proliferation group score has been shown to correlate with Ki-67 index and to differ significantly across RS risk strata [9, 12], while the estrogen group score reflects hormone-driven tumor biology [8]. However, several knowledge gaps remain. First, most existing studies have examined each pathway score in isolation using univariate methods, and few have simultaneously modeled all four pathway scores in a multivariate framework to disentangle their independent contributions to RS [11]. Second, the majority of evidence originates from Western populations enrolled in clinical trials such as NSABP B-14 and TAILORx, whereas real-world data from Chinese clinical cohorts—who may differ in tumor biology, healthcare access, and treatment patterns—remain scarce [12, 15]. Third, the independent predictive value of the invasion group score, comprising STMY3, CTSL2, and MMP11, has been relatively underexplored compared with the proliferation and estrogen pathways. To address these gaps, the present study utilized real-world data from a Chinese clinical cohort to systematically evaluate the correlation between individual gene pathway scores and clinicopathological features, compare pathway scores across RS risk groups, and—critically—employ multiple linear regression with all four pathway scores entered simultaneously to identify independent predictors of RS. This approach aims to provide pathway-level mechanistic insights into RS determination and to contribute Chinese real-world evidence for the clinical application of the 21-gene assay.

2 Materials and Methods

2.1 Study Subjects

The study included 39 female breast cancer patients who underwent surgical treatment and received 21-gene testing at the Department of Breast Surgery, Beijing Obstetrics and Gynecology Hospital, from March 2020 to March 2023. This study was approved by the Ethics Committee of Beijing Obstetrics and Gynecology Hospital, Capital Medical University(2025-KY-007-03) .Given the retrospective nature of the study and the use of anonymized data, the requirement for informed consent was waived by the Ethics Committee. This study was reported in accordance with the STROBE statement [23]. Inclusion criteria were as follows: (1) pathologically confirmed invasive breast cancer; (2) ER-positive and/or PR-positive, HER2-negative; (3) no neoadjuvant therapy prior to surgery; (4) available 21-gene assay results. Exclusion criteria: (1) distant metastasis at initial diagnosis; (2) concurrent malignancies of other systems; (3) incomplete clinicopathological data.

2.2 Collection of Clinicopathological Data

Clinicopathological data were collected, including: age, maximum tumor diameter, histological grade, estrogen receptor (ER) expression percentage, progesterone receptor (PR) expression percentage, Ki-67 proliferation index, HER2 expression level, and lymph node status. ER and PR positivity was defined as $\geq 1\%$ of tumor cell nuclei showing positive staining [18]. HER2 negativity was defined as immunohistochemistry (IHC) 0 or 1+, or IHC 2+ with negative in situ hybridization (ISH) confirmation. Ki-67 index was recorded as the percentage of positively stained tumor cells. Histological grading was performed according to the Nottingham modification of the Bloom-Richardson system.

2.3 21-Gene Testing and Pathway Score Calculation

Formalin-fixed paraffin-embedded breast cancer tissue samples were collected, and mRNA expression levels of 21 breast cancer-related genes were detected by Shanghai Yijian Medical Laboratory using RT-PCR technology. Based on the Ct values of each gene, the scores for the four gene groups were calculated according to the Oncotype DX algorithm: estrogen group score, proliferation group score, HER2 group score, and invasion group score, as well as the expression levels of CD68, GSTM1, and BAG1. The RS was calculated using the standard weighted formula provided by the assay.

Based on the RS grouping criteria from the TAILORx study [4], patients were divided into three groups: low-risk group ($RS \leq 10$), intermediate-risk group ($RS 11-25$), and high-risk group ($RS \geq 26$). Due to the limited number of patients in the high-risk group ($n = 2$), for certain comparative analyses, the intermediate-risk and high-risk groups were combined into a medium-high risk group ($RS > 10$).

2.4 Statistical Analysis

Statistical analyses were performed using SPSS 26.0 software. Categorical data were summarized using frequencies (n) and percentages (%), and comparisons between groups were made using the chi-square (χ^2) test; when the expected frequency in any cell was less than 5, Fisher's exact test was used instead. Continuous variables with non-normal distribution were expressed as median (interquartile range [IQR]). Correlations between gene pathway scores and clinicopathological features were assessed using Spearman rank correlation analysis, with correlation strength interpreted as follows: $|r| < 0.3$, weak; $0.3 \leq |r| < 0.7$, moderate; $|r| \geq 0.7$, strong. Comparisons of gene pathway scores between the low-risk and non-low-risk groups were performed using the Mann-Whitney U test. Additionally, a Kruskal-Wallis H test was used to compare gene pathway scores and individual gene expression levels across the original three RS risk categories (low, intermediate, and high). Multiple linear regression analysis was performed to identify independent predictors of RS. Clinicopathological variables with $P \leq 0.20$ in univariate analysis (chi-square test or Fisher's exact test for categorical variables; Spearman correlation for continuous variables) were entered into Model 1 using the enter method. Additionally, the four gene pathway scores (estrogen group, proliferation group, HER2 group, and invasion group) were simultaneously entered as continuous variables into Model 2 to evaluate their independent contributions to RS. The results were expressed as unstandardized regression coefficients (B) with 95% confidence intervals (CI) and standardized coefficients (β). Model assumptions, including multicollinearity (variance inflation factor

[VIF] < 5; condition index < 30) and normality of residuals (standardized residuals within ± 3), were verified. All tests were two-sided, and $P < 0.05$ was considered statistically significant.

3 Results

3.1 Clinicopathological Characteristics of Patients

All 39 patients were female, aged 24–72 years, with a median age of 55 years. The RS ranged from 0 to 53, with a median of 12 (IQR, 8–22). According to the TAILORx criteria, 17 patients (43.6%) were classified as low-risk ($RS \leq 10$), 20 (51.3%) as intermediate-risk ($RS 11–25$), and 2 (5.1%) as high-risk ($RS \geq 26$). The clinicopathological characteristics of the patients are summarized in Table 1.

Table 1
Comparison of clinicopathological characteristics between low-risk and medium-high risk breast cancer patients [n (%)]

Characteristic	Low-risk group (RS ≤ 10) n (%)	Medium-high risk group (RS > 10) n (%)	χ^2	P value
Age			2.464	0.117
≤ 50 years	5 (29.4)	12 (54.5)		
> 50 years	12 (70.6)	10 (45.5)		
Maximum tumor diameter			3.501	0.174
≤ 1 cm	10 (58.8)	7 (31.8)		
1–2 cm	5 (29.4)	13 (59.1)		
> 2 cm	2 (11.8)	2 (9.1)		
Histological grade			5.640	0.060
I	10 (58.8)	5 (22.7)		
II	7 (41.2)	16 (72.7)		
III	0 (0)	1 (4.5)		
Ki-67 index			0.022	0.883
≤ 14%	5 (29.4)	6 (27.3)		
> 14%	12 (70.6)	16 (72.7)		
HER2 expression			0.560	0.756
0	3 (17.6)	5 (22.7)		
1+	9 (52.9)	9 (40.9)		
2+	5 (29.4)	8 (36.4)		

A comparison of clinicopathological characteristics between the low-risk and medium-high risk groups (RS > 10) is shown in Table 1. In terms of age distribution, patients > 50 years old accounted for 70.6% (12/17) in the low-risk group and 45.5% (10/22) in the medium-high risk group, with no statistically significant difference ($\chi^2 = 2.464$, $P = 0.117$). Regarding maximum tumor diameter, the low-risk group was predominantly ≤ 1 cm (58.8%, 10/17), while the medium-high risk group was predominantly 1–2 cm (59.1%, 13/22), with no significant difference between the two groups ($\chi^2 = 3.501$, $P = 0.174$). Histological grade showed a borderline difference between the two groups ($\chi^2 = 5.640$, $P = 0.060$). In the

low-risk group, grade I accounted for 58.8% (10/17), grade II for 41.2% (7/17), and no grade III cases were observed; in the medium-high risk group, grade II accounted for 72.7% (16/22), grade I for 22.7% (5/22), and grade III for 4.5% (1/22). Patients with Ki-67 proliferation index > 14% accounted for 70.6% (12/17) and 72.7% (16/22) in the low-risk and medium-high risk groups, respectively, with no statistically significant difference ($\chi^2 = 0.022$, $P = 0.883$). HER2 expression level was most commonly 1+, accounting for 52.9% (9/17) and 40.9% (9/22) in the two groups, respectively, with similar distributions for other expression levels (0, 2+), and no significant difference between groups ($\chi^2 = 0.560$, $P = 0.756$).

3.2 Correlation Between Gene Pathway Scores and Clinicopathological Characteristics

Spearman rank correlation analysis was performed to evaluate the associations between gene pathway scores and clinicopathological variables (Table 2). The proliferation group score demonstrated a moderate positive correlation with the Ki-67 index ($r = 0.612$, $P < 0.001$) and a weak positive correlation with maximum tumor diameter ($r = 0.358$, $P = 0.026$). The invasion group score showed a moderate positive correlation with the Ki-67 index ($r = 0.489$, $P = 0.002$) and a weak positive correlation with maximum tumor diameter ($r = 0.317$, $P = 0.026$). The estrogen group score exhibited a strong positive correlation with ER expression ($r = 0.743$, $P < 0.001$) and a moderate positive correlation with PR expression ($r = 0.521$, $P = 0.001$). Additionally, the RS showed a moderate positive correlation with the proliferation group score ($r = 0.487$, $P < 0.001$) and a weak positive correlation with the invasion group score ($r = 0.312$, $P < 0.05$). Notably, RS demonstrated a weak negative correlation with the estrogen group score ($r = -0.235$, $P > 0.05$), consistent with the inverse relationship inherent in the RS algorithm wherein lower estrogen pathway activity corresponds to higher recurrence risk. The HER2 group score and the individual genes CD68, GSTM1, and BAG1 showed no significant correlations with any of the clinicopathological characteristics (all $P > 0.05$).

Table 2
Spearman correlation analysis of gene pathway scores and clinicopathological characteristics (r value)

Variable	Estrogen group	Proliferation group	HER2 group	Invasion group	CD68	GSTM1	BAG1
Age	-0.154	0.089	-0.203	0.112	-0.098	0.105	-0.062
Tumor size	0.187	0.358*	0.156	0.317*	0.214	-0.128	0.165
Ki-67	-0.264	0.612**	0.187	0.489**	0.287	-0.074	-0.105
ER	0.743**	-0.198	-0.134	-0.162	-0.189	0.082	0.113
PR	0.521**	-0.156	-0.098	-0.187	-0.124	0.065	0.078
RS	-0.235	0.487**	0.102	0.312*	0.156	-0.089	-0.112
* $P < 0.05$; ** $P < 0.01$ (Spearman rank correlation).							

3.3 Comparison of Pathway Scores Between Low-Risk and Non-Low-Risk Groups

Since only two patients fell into the high-risk category ($RS \geq 26$), we combined the intermediate- and high-risk groups into a single non-low-risk group ($RS > 10$, $n = 22$) for all subsequent comparisons to ensure statistical robustness. As shown in Table 3, the proliferation group score was significantly higher in the non-low-risk group compared to the low-risk group [median (IQR): 21.8 (18.3–29.5) vs. 18.5 (14.0–22.2), $P = 0.006$, Mann-Whitney U test]. The estrogen group score tended to be lower in the non-low-risk group but this difference did not reach statistical significance [33.4 (30.2–36.0) vs. 35.8 (32.1–37.9), $P = 0.081$]. No significant differences were observed for HER2 group, invasion group, CD68, GSTM1, or BAG1 expression levels between the two groups (all $P > 0.05$).

Table 3

Comparison of gene pathway scores between low-risk and non-low-risk groups [median (IQR)]

Variable	Low-risk group ($RS \leq 10$) $n = 17$	Non-low-risk group ($RS > 10$) $n = 22$	Z value	P value
Estrogen group score	35.8 (32.1, 37.9)	33.4 (30.2, 36.0)	-1.745	0.081
Proliferation group score	18.5 (14.0, 22.2)	21.8 (18.3, 29.5)	-2.743	0.006**
HER2 group score	14.8 (13.7, 16.7)	14.2 (13.0, 16.4)	-0.512	0.609
Invasion group score	11.1 (9.8, 12.7)	11.7 (10.2, 13.8)	-1.087	0.277
CD68	7.5 (7.0, 8.3)	7.5 (7.0, 8.2)	-0.201	0.841
GSTM1	7.5 (6.8, 8.2)	7.2 (6.1, 8.1)	-0.769	0.442
BAG1	7.2 (6.8, 7.9)	7.0 (5.9, 7.6)	-0.821	0.412
**$P < 0.01$ (Mann-Whitney U test). The non-low-risk group combines intermediate-risk ($RS 11–25$, $n = 20$) and high-risk ($RS \geq 26$, $n = 2$) patients due to the limited sample size in the high-risk category.				

To further evaluate the distribution of gene pathway scores and individual gene expression levels across the original three RS risk categories (low: $RS \leq 10$, $n = 17$; intermediate: $RS 11–25$, $n = 20$; high: $RS \geq 26$, $n = 2$), a Kruskal-Wallis H test was performed (Table 4). The estrogen group score differed significantly across the three risk groups ($P = 0.002$), with both the median test ($P = 0.002$) and the Kruskal-Wallis H test confirming this finding. Notably, GSTM1 expression ($P = 0.032$) and BAG1 expression ($P = 0.035$) also showed significant differences across the three RS risk categories, suggesting that these single-gene components may carry discriminative information for risk classification. In contrast, the proliferation group score ($P = 0.620$), HER2 group score ($P = 0.145$), invasion group score ($P = 0.580$), and CD68 ($P = 0.809$) did not differ significantly across the three groups. The discrepancy between the two-group ($P = 0.006$) and three-group ($P = 0.620$) analyses for the proliferation group score is likely

attributable to the extremely limited sample size in the high-risk group ($n = 2$), which reduces the statistical power of the three-group comparison and may produce unstable rank-based estimates.

Table 4
Kruskal-Wallis H test for gene pathway scores and individual gene expression across three RS risk categories

Variable	Median test P value	Kruskal-Wallis H P value	Significance
Estrogen group score	0.002**	0.002**	Yes
Proliferation group score	0.140	0.620	No
HER2 group score	0.079	0.145	No
Invasion group score	0.408	0.580	No
CD68	0.458	0.809	No
GSTM1	0.079	0.032*	Yes (K-W)
BAG1	0.301	0.035*	Yes (K-W)
* $P < 0.05$; ** $P < 0.01$. K-W, Kruskal-Wallis. Three RS risk groups: low ($RS \leq 10$, $n = 17$), intermediate ($RS 11-25$, $n = 20$), high ($RS \geq 26$, $n = 2$).			

3.4 Multiple Linear Regression Analysis of Independent Predictors of RS

To identify independent predictors of RS, two separate multiple linear regression models were constructed (Tables 5 and 6).

Table 5
Multiple linear regression analysis of clinicopathological variables predicting the recurrence score (Model 1)

Variable	B	Std. Error	β	t	P value	VIF
(Constant)	1.231	0.444	—	2.772	.009	—
Maximum tumor diameter	0.107	0.125	0.129	.854	.399	1.025
Histological grade (G)	0.350	0.154	0.342	2.273	.029*	1.019
Age (≤ 50 vs > 50 years)	-0.251	0.165	-0.230	-1.522	.137	1.027
B, unstandardized regression coefficient; β , standardized regression coefficient; Std. Error, standard error of B; VIF, variance inflation factor; t, t-statistic. Dependent variable: recurrence score (RS). Variables entered simultaneously (enter method). Overall model: $F = 3.299$, $df = 3/35$, $P = 0.032$, $R^2 = 0.220$, adjusted $R^2 = 0.154$. Maximum condition index = 12.454. * $P < 0.05$.						

Table 6

Multiple linear regression analysis of gene pathway scores predicting the recurrence score (Model 2)

Variable	B	95% CI for B	β	t	P value	VIF
(Constant)	4.005	2.953 to 5.058	—	7.734	< .001***	—
Estrogen group score	-0.120	-0.172 to -0.068	-0.965	-4.698	< .001***	2.895
Proliferation group score	0.112	-0.023 to 0.247	0.134	0.681	.501	2.868
HER2 group score	0.019	-0.063 to 0.102	0.099	0.480	.634	2.995
Invasion group score	0.089	0.008 to 0.170	0.448	2.237	.032*	2.752
<i>B, unstandardized regression coefficient; β, standardized regression coefficient; CI, confidence interval; VIF, variance inflation factor; t, t-statistic. Dependent variable: recurrence score (RS). All four gene pathway scores were entered simultaneously as continuous variables (enter method). No significant multicollinearity detected (all VIF < 3.0). Overall model: $F = 8.652$, $df = 4/34$, $P < 0.001$, $R^2 = 0.504$, adjusted $R^2 = 0.446$. Standard error of estimate = 0.408. All standardized residuals within ± 3. *$P < 0.05$; ***$P < 0.001$.</i>						

Model 1 included clinicopathological variables (age, maximum tumor diameter, and histological grade) as predictors (Table 5). Histological grade was identified as the only independent predictor of RS ($B = 0.350$, 95% CI: 0.037 to 0.663, $\beta = 0.342$, $t = 2.273$, $P = 0.029$). Each one-grade increase in histological grade was associated with an increase of 0.350 points in RS after adjusting for age and tumor diameter. Age showed a trend toward inverse association but did not reach statistical significance ($B = -0.251$, $\beta = -0.230$, $t = -1.522$, $P = 0.137$). Maximum tumor diameter was not independently associated with RS ($B = 0.107$, $\beta = 0.129$, $t = 0.854$, $P = 0.399$). The overall model was statistically significant ($F = 3.299$, $df = 3/35$, $P = 0.032$), with an adjusted R^2 of 0.154, indicating that approximately 15.4% of RS variability was explained by the clinicopathological variables. No significant multicollinearity was detected (all VIF < 1.03; condition indices < 13).

Model 2 included the four gene pathway scores (estrogen group, proliferation group, HER2 group, and invasion group) entered simultaneously as continuous predictors of RS (Table 6). This model demonstrated substantially better fit compared to Model 1 ($F = 8.652$, $df = 4/34$, $P < 0.001$, adjusted $R^2 = 0.446$). Two pathway scores emerged as independent predictors of RS: the invasion group score showed a positive association ($B = 0.089$, 95% CI: 0.008 to 0.170, $\beta = 0.448$, $t = 2.237$, $P = 0.032$), and the estrogen group score showed a strong negative association ($B = -0.120$, 95% CI: -0.172 to -0.068, $\beta = -0.965$, $t = -4.698$, $P < 0.001$). The negative coefficient for the estrogen group score reflects the inverse relationship inherent in the RS algorithm, whereby lower expression of estrogen-responsive genes (indicative of less hormone-driven biology) corresponds to higher RS values. Neither the proliferation group score ($B = 0.112$, $\beta = 0.134$, $t = 0.681$, $P = 0.501$) nor the HER2 group score ($B = 0.019$, $\beta = 0.099$, $t = 0.480$, $P = 0.634$)

reached statistical significance in the multivariate model. No significant multicollinearity was detected among the four pathway scores (all VIF < 3.0). Residual diagnostics confirmed model assumptions were met (all standardized residuals within ± 3 ; mean residual ≈ 0).

The marked contrast in explanatory power between Model 2 (adjusted $R^2 = 44.6\%$) and Model 1 (adjusted $R^2 = 15.4\%$) underscores that RS is predominantly driven by molecular pathway activities rather than traditional clinicopathological factors alone. The identification of both the invasion group score (positive driver) and the estrogen group score (negative suppressor) as independent contributors provides mechanistic insight into the composite architecture of the RS algorithm.

4 Discussion

The 21-gene assay has become an important tool for prognosis evaluation in HR-positive, HER2-negative early breast cancer, but previous studies have mostly focused on the clinical application of the RS as a composite score [3, 4, 5], with relatively less attention paid to the individual gene pathway scores that constitute the RS. This study analyzed the correlation between gene pathway scores and clinicopathological features, compared the differences in pathway scores across different RS risk groups, and employed multiple linear regression to identify the independent contributions of each gene pathway to RS in a Chinese clinical cohort, aiming to elucidate the biological basis underlying the RS and to explore the complementary value of pathway scores in risk stratification.

The moderate positive correlation between the proliferation group score and the Ki-67 index ($r = 0.612$, $P < 0.001$) is consistent with the findings of Aghebati et al. [9], who demonstrated that Ki-67 can serve as a surrogate marker for the proliferation component of the 21-gene assay. Similarly, Bayram et al. [12] reported a significant association between Ki-67 and RS in a real-world cohort, further supporting the role of proliferation as a key driver of RS elevation. Notably, Lee et al. [21] demonstrated in a large Korean cohort that Ki-67 was independently associated with the 21-gene RS and endocrine resistance, providing survival-level evidence that the proliferation pathway reflects not only recurrence risk but also therapeutic vulnerability. The proliferation group score was also the only pathway score that differed significantly between the non-low-risk and low-risk groups ($P = 0.006$; Table 3), reinforcing its role as the primary discriminative pathway across RS risk strata. Interestingly, however, the proliferation group score did not retain independent significance in the multivariate model ($P = 0.501$). This apparent paradox may be explained by substantial covariance among the pathway scores: the proliferation pathway's contribution to RS appears to be partially mediated through its correlations with the invasion and estrogen pathways. When all four pathways are considered simultaneously, the unique variance explained by proliferation beyond the other pathways becomes limited. Notably, the Kruskal-Wallis H test across the three original RS risk groups did not show a significant difference for the proliferation group score ($P = 0.620$; Table 4), likely due to the extremely limited sample size in the high-risk group ($n = 2$) and the distribution characteristics of the intermediate group, which may attenuate the three-group comparison despite a significant two-group difference.

The most novel finding of this study is the identification of the invasion group score as an independent positive predictor of RS ($\beta = 0.448$, $P = 0.032$; Table 6). The invasion group comprises STMY3 (stathmin-like 3), CTSL2 (cathepsin L2), and MMP11 (matrix metalloproteinase 11)—genes involved in extracellular matrix remodeling, proteolytic degradation, and tumor cell invasion/metastasis. Our finding suggests that beyond proliferation, invasive potential constitutes a distinct and independent molecular axis driving RS elevation. This aligns with the biological role of MMP11 and CTSL2 in promoting tumor dissemination and supports their weighting in the RS algorithm. Notably, Molière et al. [19] recently demonstrated that MMP-11 expression in early luminal breast cancer was associated with higher tumor grade and shorter disease-free survival, and that combined MMP-11 and Ki-67 assessment provided additive prognostic value—providing independent clinical evidence that the invasion pathway captures recurrence risk beyond proliferation alone. Sughayer et al. [11] previously reported limited predictive value for invasion parameters in simplified RS prediction models; however, our multivariate approach, which explicitly models all four pathways simultaneously, reveals the invasion group's independent contribution that may be obscured in simpler analytic frameworks.

The strong negative association between the estrogen group score and RS ($\beta = -0.965$, $P < 0.001$) represents a critical validation of the RS algorithm's biological architecture. In the Oncotype DX system, higher expression of estrogen-responsive genes (ER, PR, BCL2, SCUBE2) indicates a more hormone-dependent, better-differentiated tumor phenotype with inherently lower recurrence risk—hence the negative relationship with RS. The strong univariate correlation of the estrogen group score with ER ($r = 0.743$) and PR ($r = 0.521$) further confirms its validity as a hormone pathway indicator. In the two-group comparison, the estrogen group score showed a trend toward lower values in the non-low-risk group ($P = 0.081$; Table 3), which did not reach statistical significance, likely reflecting the relatively uniform HR-positive status of our cohort that restricts the range of estrogen pathway variation. However, the Kruskal-Wallis H test across the three original RS risk groups demonstrated a highly significant difference for the estrogen group score ($P = 0.002$; Table 4), suggesting that when the full spectrum of risk stratification is considered, the estrogen pathway exhibits a more pronounced gradient across risk categories. Zhang et al. [8] recently demonstrated in a large cohort that the ER module contributed maximally to RS variance in ER-high tumors, whereas the proliferation module dominated in ER-low tumors—a nuanced pattern that our smaller cohort could not fully resolve but is consistent with the overall direction of our findings.

The identification of histological grade as an independent predictor of RS in the clinicopathological model ($B = 0.350$, $P = 0.029$; Table 5) provides valuable evidence supporting the integration of traditional pathological markers with genomic assays. Each increment in histological grade corresponded to a 0.35-point increase in RS after controlling for age and tumor size. This finding corroborates the rationale proposed by Li et al. [13] and Al-Zawi et al. [14] for combining histological grade with genomic biomarkers to enhance prognostic accuracy. Furthermore, the recent RSClinN+ tool developed by Pusztai et al. [17] integrates multidimensional information including RS, tumor size, pathological grade, and lymph node status, providing refined prognostic assessment for node-positive patients. Song et al. [20] recently developed a clinicopathological nomogram for predicting Oncotype DX RS in a Chinese cohort of 703 patients, demonstrating that integrating histological grade, tumor size, and biomarker status

achieved superior predictive accuracy compared with models derived from Western populations. Similarly, Shibata et al. [22] developed a nomogram for Japanese patients and highlighted population-specific differences in RS predictors—collectively, these Asian studies underscore the importance of population-specific validation and support the relevance of our findings in a Chinese clinical context. Notably, the substantially higher adjusted R^2 of the gene pathway model (44.6%) compared to the clinicopathological model (15.4%) underscores that molecular information captured by the 21-gene assay provides prognostic insights beyond what traditional pathology alone can offer, reinforcing the clinical utility of genomic testing in this population [16].

Several limitations should be acknowledged. First, the sample size is modest ($n = 39$), with only 2 cases in the high-risk group, which limited the number of variables that could be included in the multivariate models and precluded separate analysis of the high-risk subgroup. Second, this is a single-center retrospective study with inherent selection bias. Third, the lack of long-term follow-up data prevents direct assessment of the relationship between pathway scores and survival outcomes. Nevertheless, the consistently strong correlations observed—such as the proliferation group score with Ki-67 ($r = 0.612$, $P < 0.001$) and the estrogen group score with ER expression ($r = 0.743$, $P < 0.001$)—support the biological coherence of these pathway scores and provide a foundation for larger prospective validation in Asian populations.

In conclusion, this study provides the first multivariate evidence from a Chinese clinical cohort that the 21-gene RS is predominantly driven by molecular pathway activities, with the invasion group score emerging as an independent positive predictor and the estrogen group score as a strong independent negative predictor—validating and extending the algorithm's biological architecture at the pathway level. The gene pathway model explained nearly three times the variance in RS compared with the clinicopathological model (44.6% vs. 15.4%), demonstrating that molecular information captured by the 21-gene assay provides prognostic resolution far beyond what traditional clinicopathological assessment can offer. These findings suggest that individual pathway scores—particularly the invasion and estrogen components—may provide complementary mechanistic insights for refining risk stratification when integrated with the composite RS. Prospective validation in larger, multicenter Asian cohorts with survival endpoints is warranted to determine whether incorporating invasion pathway assessment into clinical practice can further individualize treatment decisions for HR-positive, HER2-negative early breast cancer.

Abbreviations

RS
Recurrence Score
HR
Hormone Receptor
ER
Estrogen Receptor

PR
Progesterone Receptor
HER2
Human Epidermal Growth Factor Receptor 2
IHC
Immunohistochemistry
ISH
In Situ Hybridization
IQR
Interquartile Range
K-W
Kruskal-Wallis
M-W
Mann-Whitney
VIF
Variance Inflation Factor
CI
Confidence Interval
RT-PCR
Reverse Transcription Polymerase Chain Reaction
Ct
Cycle Threshold
APC
Article Processing Charge.

Declarations

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of Beijing Obstetrics and Gynecology Hospital, Capital Medical University and was conducted in accordance with the 1964 Helsinki Declaration and its later amendments.

Clinical Trial Number

2025-V1.0

Data Availability Statement

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Consent for Publication

Not applicable. No individual person's data in any form (including individual details, images, or videos) are included in this manuscript.

Competing Interests

The authors declare no conflicts of interest.

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