A Novel Signature Predicts Recurrence Risk In Breast Cancer Patients

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ABSTRACT Breast cancer is one of the most leading causes of death in females all over the world. Although, the incidence rate of breast cancer is low, the death proportion is relatively high. For breast cancer patients, the recurrence ratio is rare, yet it still happens. The highest risk of recurrence usually happens at the early time of treatment, and its severeness depends on the diagnosed stage and the size of the tumor. However, the recurrence prognostic of breast cancer still limited due to the poor prediction strength of input data. Therefore, in this study we used four independent data sets ($n = 793$) with the same chipsets (HG-U133A) of breast cancer patients to investigate a 67 gene signature used in further building the prognostic model for breast cancer patients. Our results finally confirmed that a 67 gene set was significantly associated with the recurrence (RFS) ($p = 1.66e^{-17}$), and overall survival (OS) ($p = 9.27e^{-06}$). Besides, the gene signature also correlated with survival outcomes, such as RFS ($p = 0.00167$), and distant metastasis-free survival (DMFS) ($p < 0.001$). Remarkably, our classification analysis results reveal that the breast cancer patients who were categorized into the low-risk group tend to have significantly associated with the longer recurrence time in both training and validation data sets. In conclusion, the 67 gene signature should be considered as potential candidates to be used in building up models or integrated into the current treatment regime for breast cancer.

KEYWORDS

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INTRODUCTION

Breast cancer can occur in both males and females, which is related to genetic and heterogeneity [1]; however, it is more familiar to females [2]. Even though the early diagnosis by biomarker detection and screening is improved, tumor relapse (local relapse or distant relapse) is the main event that causes death in breast cancer patients [3]. The recurrence of patients who had the first treatment of breast cancer is usually high, and the prognosis for this recurrence is still limited [4]. Therefore, it is necessary to identify biomarkers that could predict the relapse time of patients. DNA microarray was suggested to be a potential method that can identify gene signatures in breast cancer [5, 6, 7, 8, 9]. The gene signature from only a single data set gave a poor strength of prediction for patients in breast cancer. Thus, the difference from the single training data set, this study introduced testing in multiple data sets which can increase the accuracy of the prognostic

value of gene signature. In this work, we identified gene signature over the analysis of RNA expression profiles of 793 breast cancer patients from the Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) database. The comparisons between groups of patients who had relapse or no relapse and groups of patients who had normal breast or cancer breast established a 67 gene signature. Finally, the 67 gene signature was defined by a risk scoring method that was highly predictive of relapse outcomes of breast cancer patients.

MATERIALS AND METHODS

Patients and gene expression profiles

All clinical information and gene expression data were retrieved from the Gene Expression Omnibus database <http://www.ncbi.nlm.nih.gov/geo/> with the HG-U133A chipset. The follow-up infor-

Tumor vs. normal

Genes related to relapse

Figure 1 The 67-gene signature in both GSE1456 and GSE15852. 668 gene probes related to a survival of tumor patients from GSE1456, and 1180 gene probes what related to differential expression between tumor and normal patients from GSE15852. After filtering the overlap gene probes, we obtained a 67-gene signature.

mation which included relapse-free survival (RFS), overall survival (OS), and distant metastasis-free survival (DMFS) were selected. Besides, the unfollow-up dataset was excluded from the survival analysis. The gene expression data was normalized from using a robust multiarray averaging method [10, 11]. In this analysis, 793 breast cancer patients from four different datasets were used. The GSE1456 ($n = 318$, Karolinska Institute) [12] and GSE15852 ($n = 86$, Institute for Medical Research) [13] were chosen as main databases to find a set of signature genes in relapse patients. Furthermore, GSE11121 ($n = 200$, Bayer Technology Services GmbH) [14], GSE2990 ($n = 189$, Princess Margaret Cancer Centre) [15] were the validated datasets (Table 1).

Development of the prognostic gene expression signature

From GSE1456 and GSE15852, 404 breast cancer patients were selected to build a set of signature gene. These genes were developed by BRB-Microarray Tools <https://brb.nci.nih.gov/BRB-ArrayTools/> which was an add-in tool in Microsoft Excel. The process was divided into five steps. Firstly, the normalized gene expression from the two datasets was imported into BRB-Array Tools. Secondly, gene probes associated with RFS were identified by the univariate Cox proportional hazard regression ($p < 0.001$) [16] in GSE1456. Thirdly, the gene probes that significantly expressed differently in tumor and normal patients were selected from GSE15852 data set ($p < 0.001$). Forth, the overlapped gene probes from the second and third steps were identified by the Venn diagram. Fifth, the common gene probes from the forth step were applied to the survival risk prediction analysis [17]. In this prediction analysis, a prognostic index was produced for each patient by the formula $\sum_i w_i x_i$ where w is weight and x is logged gene expression of the n^{th} gene, respectively. Patients were classified into high (greater than the median value of prognostic indices) and low (equivalent to or less than the median value of prognostic indices) risk group based on the value of prognostic indices (Fig. 1).

Validation of the prognostic signature

The validation datasets were from different sources and prediction model was developed using BRB-Array Tools [18]. Complete leave-one-out cross validation (LOOCV) that displayed the accuracy of the prediction was 99% for the compound covariate predictor (CCP) [19]. Kaplan-Meier Plots were performed the survival analysis of patients in two classified risk groups. Moreover, to evaluate the survival risk of two groups, Chi-square and t-tests were applied.

STRING analysis

Protein-protein interaction was achieved by a functional protein association network named Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) <https://string-db.org/>. This included text mining, experiments, databases, co-expression, neighborhood, gene fusion, and co-occurrence criteria [20].

Statistical methods of microarray data

Data analysis including microarray data and heat map were based on BRB-Microarray Tools. Kaplan-Meier Plot, Chi-square test, a two-sample t-test were performed by the R language environment www.r-project.org and the statistical package for social sciences (SPSS) (version 20, SPSS Inc., Chicago, IL, USA). A p-value less than 0.05 was considered significant.

RESULT

Identification of the genes related to relapse-free survival and significantly different gene expression between breast cancer and normal tissues

We developed a 67 gene signature which was associated with relapse-free survival from breast cancer patients and significantly different gene expression from breast cancer and normal patients (Fig. 1). In this study, GSE1456 was the training dataset with the relapse clinical outcome considered as the main follow up information. GSE15852 was used as independent data that could increase the significant accuracy of gene signature. After overlapping 668 RFS-associated gene probes in GSE1456 ($p < 0.001$), and 1180 gene probes that significant different expressed in GSE15852 ($p < 0.001$), 67 gene probes were identified (Table 2). In 67 gene set, there were 59 annotated genes, in there, 5 genes that had more than one probe, 4 probes had no annotated genes, and 50 genes had only one probe. A flow chart used to develop a gene set was presented in Fig. 2.

Develop a gene signature to predict a recurrence outcome

In GSE1456, patients were classified into high ($n = 79$) and low ($n = 80$) risk groups based on the gene expression of 67 gene probes and the recurrence information from each patient. The classification was following the median of prognostic index generated by Principal Component Analysis. These 67 gene set showed distinct clustering of genes in tumor patients (Fig. 3A). In addition, the survival differences of the predicted high risk and low risk groups were performed by Kaplan-Meier curves in RFS ($p = 1.66 \exp(-07)$, Fig. 3B), and OS ($p = 9.27 \exp(-06)$, Fig. 3C). The results showed that patients in low group had a longer recurrence and survival time. Taken together, the 67 gene signature was correlated to the recurrence and overall survival outcomes of breast cancer patients.

Validation of the 67 gene predictive signature

To evaluate the accuracy of the 67 gene signature, we validated it in two independent data sets of breast cancer. The procedure of

Figure 2 A flow chart of developing the gene signature. Databases from GSE1456 and GSE15852 were imported into BRB-Array Tools. Then, genes related to survival in GSE1456 overlapped with genes differentially expressed between tumor and normal patients in GSE15852. The Prediction model was built based on the 67-gene signature and validated from GSE11121 and GSE2990. The results were visualized by the heat map and Kaplan-Meier Plot.

Figure 4 The validation of the 67 gene signature in GSE2290 and GSE11121. (A) A workflow of validation of 67-gene signature in both datasets. Our classification divided patients in GSE2290 into high- and low risk groups with the significant p-value associated with the (B) recurrence and (C) DMFS. (D) The DMFS in GSE11121 also significantly correlated with the predicted groups classified by the 67-gene signature. Besides, the gene signature distributed the patients into two clusters in validation datasets as well as the training dataset (E, F).

validation was provided in Fig. 4A. In the GSE2290 validation data set, the 67 gene signature predicted patients into high ($n = 94$) and low ($n = 93$) risk groups. Our classification was significant associated with the RFS ($p = 0.00176$, Fig. 4B), and DMFS ($p = 0.00667$, Fig. 3C). In the GSE11121 validation data set, 102 and 98 patients were classified into high risk and low risk groups, respectively. Accordingly, the DMFS significant correlated to our classification ($p = 0.00575$, Fig. 3D). Moreover, the 67 gene signature also categorized the patients into two clusters (Fig. 3E, 3F) in each validation data sets. Altogether, the 67 gene signature was successfully evaluated in both validation data sets.

Protein interaction network of 67 gene signature

The protein network of 59 annotated genes of 67 gene signature in breast cancer patients was generated by STRING. There were 41 of 59 genes that had a closed connection in a single network, 18 genes had no connection (Fig. 5).

DISCUSSION

[] The recurrence of breast cancer after treatment was an unwanted event that every patient has not expected. Many efforts have been made to enhance the clinical management of breast cancer; however, the mortality rate is still rising [21, 22]. Furthermore, a vast quantity of patients had to face breast cancer return within two decades. Due to the heterogeneity in breast cancer, traditional methods (such as tumor size, tumor type, stage) were not enough efficiency to predict the risk of recurrence in tumor patients [21]. The development of a DNA microarray was considered as a potential method of generating gene signatures and used to predict

Figure 3 The 67 gene signature in the training dataset (GSE1456). (A) Heatmap of 67 gene expression of tumor patients. (B) Kaplan-Meier Plot of recurrence survival of patients who had recurrence or recurrence-free status. The high-risk group of patients displayed a shorter recurrence time compared to patients with low risk. (C) Similarity as (B), the high-risk group showed the short-lived time than the low-risk one in tumor patients.

Figure 5 The connection of gene signature generated by STRING. There were 59 annotated genes of 67 gene probes. In the network, only 55 genes had a closed connection with each other.

recurring, survival, and metastasis risks in breast cancer. In this study, we developed a novel gene signature that could accurately predict the recurrence outcome of breast cancer patients. The robustness of the 67 gene signature was supported by the reproducibility through a significant association between the predicted outcome and patient prognosis in two independent validation data sets. The gene signature mainly contains many genes playing critical biology roles in breast cancer progression. Some of these relate to proliferation functions of cells, such as CAV1 [23], CCNA2 [24], CDC20 [25], CDK1 [26], CLDN5 [27], GPC3 [28], IGF1 [29], JUN [30], MELK [31], NTRK2 [32], PRC1 [33], SFN [34], and TXNIP [35]. Besides, some genes are involved in angiogenesis, migration, and inflammation in many cancers, such as CLDN5 [27], JAK1 [36], IGF1 [37], PLPP3 [38], and PBK [39]. Moreover, several genes are related to the Wnt signaling pathway, a hallmark of many cancers, including CAV1 [23], DIXDC1 [40], GPC3 [28], and PLPP3 [38]. Furthermore, the signature also had oxidoreductase-related genes, such as CDO1 [41], ETVFDH [42], MTHFD1 [43], MTHFD2 [44], and RRM2 [45]. Therefore, we argue that the many genes in this signature are involved in proliferation, angiogenesis, migration, and inflammation in breast cancer. In conclusion, we identified the 67 gene signature for prognostic breast cancer to predict recurrence outcomes. The results showed that 67 gene set significantly correlated with survival outcomes, such as recurrence, survival, and metastasis status. In addition, the predictive value and robustness were successfully validated in two independent cohorts of breast cancer patients. The high-risk group had a shorter time of recurrence, and metastasis in both the training, and validation datasets ($p < 0.001$, Fig. 3, 4). This study provides a potential tool for the prediction of recurrence in breast cancer in the age of personalized medicine.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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APPENDIX

■ **Table 2** The first 67 genes are significant at the nominal 0.99 level of the univariate test

No.	FDR	Probe Set	Symbol	Name	Entrez ID
1	0.00011	209220_at	GPC3	Glypican 3	2719
2	0.00021	208022_s_at	CDC14B	Cell division cycle 14B	8555
3	0.00026	201377_at	UBAP2L	Ubiquitin associated protein 2 like	9898
4	0.0384	202712_s_at			

■ **Table 2** The first 67 genes are significant at the nominal 0.99 level of the univariate test

No.	FDR	Probe Set	Symbol	Name	Entrez ID
5	0.0502	33494_at	ETFDH	Electron transfer flavoprotein dehydrogenase	2110
6	0.0604	220392_at	EBF2	Early B cell factor 2	64641
7	0.0719	218834_s_at	TMEM132A	Transmembrane protein 132A	54972
8	0.326	201465_s_at	JUN	Jun proto-oncogene, AP-1 transcription factor subunit	3725
9	1.29E-07	200996_at	ACTR3	ARP3 actin related protein 3 homolog	10096
10	1.29E-07	200910_at	CCT3	Chaperonin containing TCP1 subunit 3	7203
11	<1e-07	202309_at	MTHFD1	Methylenetetrahydrofolate dehydrogenase, cyclohydrolase and formyltetrahydrofolate synthetase 1	4522
12	<1e-07	212560_at	SORL1	Sortilin related receptor 1	6653
13	<1e-07	204773_at	IL11RA	Interleukin 11 receptor subunit alpha	3590
14	<1e-07	219534_x_at	CDKN1C	Cyclin dependent kinase inhibitor 1C	1028
15	<1e-07	204964_s_at	SSPN	Sarcospan	8082
16	<1e-07	201008_s_at	TXNIP	Thioredoxin interacting protein	10628
17	<1e-07	221795_at	NTRK2	Neurotrophic receptor tyrosine kinase 2	4915
18	<1e-07	201761_at	MTHFD2	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	10797
19	<1e-07	35776_at	ITSN1	Intersectin 1	6453
20	<1e-07	209355_s_at	PLPP3	Phospholipid phosphatase 3	8613
21	<1e-07	218585_s_at	DTL	Denticleless E3 ubiquitin protein ligase homolog	51514
22	<1e-07	202551_s_at	CRIM1	Cysteine rich transmembrane BMP regulator 1	51232
23	<1e-07	213175_s_at	SNRNPB	Small nuclear ribonucleoprotein polypeptides B and B1	6628
24	<1e-07	221796_at	NTRK2	Neurotrophic receptor tyrosine kinase 2	4915
25	<1e-07	207165_at	HMMR	Hyaluronan mediated motility receptor	3161
26	<1e-07	222036_s_at	MCM4	Minichromosome maintenance complex component 4	4173
27	<1e-07	207828_s_at	CENPF	Centromere protein F	1063
28	<1e-07	208696_at	CCT5	Chaperonin containing TCP1 subunit 5	22948
29	<1e-07	204482_at	CLDN5	Claudin 5	7122
30	<1e-07	219148_at	PBK	PDZ binding kinase	55872
31	<1e-07	213348_at	CDKN1C	Cyclin dependent kinase inhibitor 1C	1028
32	<1e-07	213364_s_at	SNX1	Sorting nexin 1	6642
33	<1e-07	201009_s_at	TXNIP	Thioredoxin interacting protein	10628
34	<1e-07	203213_at	CDK1	Cyclin dependent kinase 1	983
35	<1e-07	221521_s_at	GINS2	GINS complex subunit 2	51659
36	<1e-07	214724_at	DIXDC1	DIX domain containing 1	85458
37	<1e-07	201890_at	RRM2	Ribonucleotide reductase regulatory subunit M2	6241
38	<1e-07	205559_s_at	PCSK5	Proprotein convertase subtilisin/kexin type 5	5125
39	<1e-07	204825_at	MELK	Maternal embryonic leucine zipper kinase	9833
40	<1e-07	203799_at			

■ **Table 2** The first 67 genes are significant at the nominal 0.99 level of the univariate test

No.	FDR	Probe Set	Symbol	Name	Entrez ID
41	<1e-07	213226_at	CCNA2	Cyclin A2	890
42	<1e-07	219563_at			
43	<1e-07	213247_at	SVEP1	Sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1	79987
44	<1e-07	218542_at	CEP55	Centrosomal protein 55	55165
45	<1e-07	202920_at	ANK2	Ankyrin 2	287
46	<1e-07	202746_at	ITM2A	Integral membrane protein 2A	9452
47	<1e-07	211577_s_at	IGF1	Insulin like growth factor 1	3479
48	<1e-07	218009_s_at	PRC1	Protein regulator of cytokinesis 1	9055
49	<1e-07	209894_at			
50	<1e-07	204154_at	CDO1	Cysteine dioxygenase type 1	1036
51	<1e-07	202870_s_at	CDC20	Cell division cycle 20	991
52	<1e-07	218087_s_at	SORBS1	Sorbin and SH3 domain containing 1	10580
53	<1e-07	212713_at	MFAP4	Microfibril associated protein 4	4239
54	<1e-07	218901_at	PLSCR4	Phospholipid scramblase 4	57088
55	<1e-07	212423_at	ZCCHC24	Zinc finger CCHC-type containing 24	219654
56	<1e-07	212097_at	CAV1	Caveolin 1	857
57	<1e-07	209542_x_at	IGF1	Insulin like growth factor 1	3479
58	<1e-07	212419_at	ZCCHC24	Zinc finger CCHC-type containing 24	219654
59	<1e-07	209540_at	IGF1	Insulin like growth factor 1	3479
60	<1e-07	209541_at	IGF1	Insulin like growth factor 1	3479
61	2.48E-07	216894_x_at	CDKN1C	Cyclin dependent kinase inhibitor 1C	1028
62	2.48E-07	201648_at	JAK1	Janus kinase 1	3716
63	3.65E-07	205047_s_at	ASNS	Asparagine synthetase (glutamine-hydrolyzing)	440
64	5.98E-07	203228_at	PAFAH1B3	Platelet activating factor acetylhydrolase 1b catalytic subunit 3	5050
65	6.58E-05	33322_i_at	SFN	Stratifin	2810
66	7.52E-06	218976_at	DNAJC12	Dnaj heat shock protein family (Hsp40) member C12	56521
67	9.31E-05	200708_at	GOT2	Glutamic-oxaloacetic transaminase 2	2806

■ **Table 1** Datasets used in this study.

Data accession no.	End point	Chip set	Year	Country	Patient no.	Patient no. used	Ref.
GSE1456	OS, RFS	HG-U133A	2006	Sweden	318	318	[12]
GSE11121	DMFS	HG-U133A	2008	Germany	200	200	[14]
GSE2990	RFS, DMFS	HG-U133A	2005	Canada	189	189	[15]
GSE15852		HG-U133A	2009	Malaysia	86	86	[13]

RFS, relapse-free survival; DMFS, distant metastasis-free survival; DFS, disease-free survival; DSS, disease specific survival; OS, overall survival; DRFS, distace relapse-free survival; MFS, metastasis-free survival.