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Extensive analysis of the molecular biomarkers excision repair cross complementing 1, ribonucleotide reductase M1, β-tubulin III, thymidylate synthetase, and topoisomerase IIα in breast cancer

Association with clinicopathological characteristics

Juncheng Li, MS[a,b], Peng Sun, MD[a], Tao Huang, MS[a], Shengdong He, MS[a], Lingfan Li, MS[a], Gang Xue, MD[a,b,*]

Abstract

Excision repair cross complementing 1 (*ERCC1*), ribonucleotide reductase M1 (*RRM1*), β-tubulin III (*TUBB3*), thymidylate synthetase (*TYMS*), and topoisomerase IIα (*TOP2A*) genes have been shown to be associated with the pathogenesis and prognosis of various types of carcinomas; however, their roles in breast cancer have not been fully validated. In this study, we evaluated the correlations among these biomarkers and the associations between their expression intensity and the clinicopathological characteristics to investigate whether the above genes are underlying biomarkers for patients with breast cancer.

Ninety-seven tissue specimens collected from breast cancer patients. The expression levels of these biomarkers were measured by the multiplex branched DNA liquidchip (MBL) technology and clinicopathological characteristics were collected simultaneously.

The expression levels of *ERCC1*, *TUBB3*, *TYMS*, and *TOP2A* were significantly associated with the characteristics of menopausal status, tumor size, lymph node metastasis, hormone receptor status, triple-negative status, Ki-67 index, and epidermal growth factor receptor. The expression intensity of *ERCC1* negatively associated with that of *TUBB3* and *TYMS*, and positively associated with that of *RRM1*. The expression intensity of *TOP2A* positively associated with that of *TYMS*. Hierarchical clustering analysis and difference test indicated that breast cancer with higher levels of *TUBB3*, *TYMS*, and *TOP2A*, as well as lower levels of *ERCC1* and *RRM1* tended to have higher histological grade and Ki-67 index.

Our studies showed that *ERCC1*, *TYMS*, *TUBB3*, and *TOP2A* may be potential biomarkers for prognosis and individualized chemotherapy guidance, while there may be interactions between *ERCC1* and *RRM1*, or *TUBB3*, or *TYMS*, as well as between *TOP2A* and *TYMS* in pathogenesis and development of breast cancer.

Abbreviations: AJCC = American Joint Committee on Cancer stage system, ASCO = American Society of Clinical Oncology, CCLE = cancer cell line encyclopedia, DFS = disease-free survival, EGFR = epidermal growth factor receptor, ER = estrogen receptors, *ERCC1* = excision repair cross complementing 1, FFPE = formalin-fixed paraffin-embedded, GTEx = The Genotype-tissue Expression Project, HER-2 = human epidermal growth factor receptor 2, ICGC = International Cancer Genome Consortium, IDC = invasive ductal carcinoma, ISH = in situ hybridization, MBL = multiplex branched DNA liquidchip, NER = nucleotide excision repair, NSCLC = non-small cell lung cancer, OS = overall survival, PR = progesterone receptors, *RRM1* = ribonucleotide reductase M1, RT-PCR = reverse transcription-polymerase chain reaction, TCGA = The Cancer Genome Atlas, TNBC = triple-negative breast cancer, *TOP2A* = topoisomerase IIα, *TUBB3* = β-tubulin III, *TYMS* = thymidylate synthetase.

Keywords: biomarker, breast cancer, clinicopathological characteristic, individualized chemotherapy

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The authors have no conflicts of interest to disclose.

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

[a] Department of Thyroid and Breast Surgery, the General Hospital of Western Theater Command of People's Liberation Army, Chengdu, [b] Department of Breast Surgery, the Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan Province, China.

[*] Correspondence: Gang Xue, Department of Thyroid and Breast Surgery, the General Hospital of Western Theater Command of People's Liberation Army, No.270 Rongdu avenue, Chengdu 610083, Sichuan Province, China (e-mail: kpardan@163.com).

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1. Introduction

Breast cancer is the most commonly diagnosed and the most life-threatening tumor in women, with an estimated 2.1 million newly diagnosed cases in 2018, representing 25% of all cancers in women.[1,2] Primary breast cancer is a highly heterogeneous disease, and a rational individualized treatment is needed. Based on personalized clinicopathological characteristics, radical surgery selectively followed by adjuvant chemotherapy, endocrine therapy, molecular targeted therapy, and radiotherapy are the mostly used strategy for management of breast cancer. To date, reliable biomarkers are available for endocrine therapy and molecular targeted therapy. The expressions of estrogen receptor (ER) and the progesterone receptor (PR) are used to identify patients who benefit from endocrine therapy[3]; while overexpression of human epidermal growth factor receptor 2 (HER-2) protein is used to identify patients who benefit from anti-HER-2 therapy.[4] Clinically, adjuvant chemotherapy plays crucial roles in prolonging disease-free survival (DFS) and overall survival (OS) in patients with early-stage breast cancer, but it is a lack of guidance of molecular biomarker and is still bind. The insensitivity and/or resistance to chemotherapy drugs may result in the subsequent recurrence and metastasis of cancer.[5] Consequently, here came the importance of searching for reliable biomarkers to formulate individualized chemotherapy.

Recently, studies have reported that the expression levels of several genes, such as excision repair cross complementing 1 (*ERCC1*), ribonucleotide reductase M1 (*RRM1*), thymidylate synthetase (*TYMS*), β-tubulin III (*TUBB3*), and topoisomerase IIα (*TOP2A*), are differential in tumor tissues and are closely associated with the clinicopathological characteristics of patients, which suggests that there is a potential role for them as predictors of chemoresistance and prognosis in cancer patients.[6–9] For example, the expression level of *ERCC1*, which is crucial in the repair of platinum-DNA adducts, was reported to negatively affect the effectiveness of platinum drugs and could be used as a major predictor of disease response to platinum-based chemotherapy.[10,11] Furthermore, a randomized prospective clinical study confirmed that customized cisplatin chemotherapy based on quantitative *ERCC1* mRNA expression improved the survival of patients with non-small-cell lung cancer.[12] These findings indicate that *ERCC1* mRNA expression assessment is feasible in a clinical setting and can predict the response to cisplatin-based treatment. The expression level of *RRM1*, which is the main target of gemcitabine, was reported to be negatively correlated with the efficacy of gemcitabine.[12,13]*TUBB3* is thought to be a marker of taxane resistance and high expression levels of *TUBB3* correlate with low response rates in patients treated with taxane-containing regimens.[14,15] The expression level of *TYMS*, which is a central enzyme in the folate metabolic pathway and is a major target for antifolate cytotoxic chemotherapy drugs, such as 5-fluorouracil and capecitabine, is negatively correlated with the efficacy of anti-metabolism drugs.[16,17]*TOP2A* is an essential nuclear enzyme that changes DNA topology and the primary molecular target of various cytotoxic agents including anthracyclines. The expression level of *TOP2A* was reported to be positively correlated with the efficacy of anthracyclines drugs.[18,19] More importantly, increasing studies also reveal their values in breast cancer. High expression of ERCC1 is associated with favorable prognostic parameters such as a positive ER expression status in breast cancer.[20] In vitro experiment, RRM1 gene silencing can reverse paclitaxel resistance in human breast cancer cell line MCF-7/R.[21] Elevated TYMS expression was a detrimental factor for pemetrexed treatment in advanced breast cancer, indicating that it may be a biomarker to choose chemotherapy regimens.[16] Hellenic Cooperative Oncology Group evaluated the prognostic and predictive utility of TUBB3 transcription in early breast cancer patients and confirmed that transcriptional activity of TUBB3 is an adverse prognostic factor for early breast cancer patients.[22] TOP2A protein showed a time dependent influence on prognosis in stage I–II luminal breast cancer, suggesting it might be a potential predictor of late recurrence for this group of patients.[23] However, information in the literature regarding a simultaneous evaluation of their expression in breast cancer like other cancers is very limited.

In the present study, the expression levels of *ERCC1*, *RRM1*, *TUBB3*, *TYMS*, and *TOP2A* were simultaneously detected in the tissue specimens of breast cancer in order to evaluate whether the genes *ERCC1*, *RRM1*, *TUBB3*, *TYMS*, and *TOP2A* are underlying biomarkers for patients with breast cancer, hoping to provide guidance for developing personalized chemotherapy.

2. Materials and methods

2.1. Ethics

This study was approved by the ethics committee of the General Hospital of Western Theater Command of People's Liberation Army (Chengdu, China) (Registration Number 2011ky020). All patients gave written informed consents for tissue samples retention, analysis for research, and paper publication.

2.2. Patients' clinicopathological characteristics

All 97 enrolled patients were cases of primary operable breast cancer from January 1, 2012 to December 31, 2013. All selected patients had complete clinical history data and no one received neoadjuvant therapy prior to the primary surgery. Formalin-fixed paraffin-embedded (FFPE) tissue samples were retrieved and were then marked as 1 to 97 according to the ID numbers of the patients. All of them were histologically confirmed as invasive breast cancer by 2 independent, experienced pathologists. The individuals in this study comprised 90 invasive ductal carcinoma (IDC), 2 invasive lobular carcinoma, and 5 invasive breast carcinomas of special histological types such as mixed carcinoma and medullary carcinoma. Multiple clinicopathological parameters were obtained, including age, menopausal status, histological type, histological grade, tumor size, lymph node status, ER, PR, HER-2, Ki-67 index, E-cadherin, epidermal growth factor receptor (EGFR), and P53.

2.3. Detection of mRNA expression levels

The mRNA expression levels of *ERCC1*, *RRM1*, *TUBB3*, *TYMS*, and *TOP2A* in breast cancer tissues were simultaneously measured by multiplex branched DNA liquidchip (MBL) technology as previously reported.[24,25] The tissue samples were processed by following steps. Firstly, the samples were lysed in buffer at 56 °C for 2 hours. Then the lysed product was added to each well of a 96-well plate which contains blocking reagent, target gene-specific probe sets, and capture beads. The plate was sealed, and then incubated for 18 hours at 54 °C on a shaker, followed by adding the hybridization mixture. Afterwards, the

unbound mRNA and other debris in each well were removed by washing 3 times with buffer. Signals for bound target mRNA were amplified with streptavidin-hycoerythrin solution at 50 °C for 30 minutes. Finally, the fluorescence value of each sample was recognized and analyzed by the Luminex 200 system (Luminex, Austin, TX) to represent the mRNA expression level of each gene. Compared with the cut-off value of each gene, the mRNA expression level was divided into low expression (<25%), low to medium (25–40%), medium expression (40–60%), medium to high expression (60–75%), and high expression (>75%).[26]

Additionally, RNA quality and quantity were assessed by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) on the ABI PRISMH 7900HT Sequence Detection System (Applied Biosystems, Carlsbad, CA). The qRT-PCR was conducted in 96-well optical reaction plates in a final reaction volume of 25 μL. Optimum reaction conditions were obtained with 15 μL of 2× SYBR Green PCR MasterMix (Applied Biosystems), 1 pmol specific forward primer and 1 pmol specific reverse primer. Finally, 25 ng template cDNA was added to the reaction mixture and amplifications were performed using the standard HT7900 program.

2.4. Assessment of molecular parameters

ER, PR, HER-2, Ki-67 index, E-cadherin, EGFR, and P53 were detected via IHC staining assay. The FFPE were sectioned by using a microtome, and further re-evaluated by 2 independent, experienced pathologists. The criteria were as follows: any staining of 1% of cells or more is considered positive for ER and PR.[27] HER-2 protein expression was ranked between 0 and 3+. According to the American Society of Clinical Oncology (ASCO) guideline recommendations, 3+ staining (>30% intense and complete staining) was considered as HER-2 positivity and 0 or 1 + was considered negative and in situ hybridization (ISH) test would be performed if IHC is equivocal (2+ pattern).[4] The amount of all cancer cells was counted in 10 random high-powered field of each light microscope slices and the average proportion of cell with Ki-67 nuclei-immunoreactivity of all cancer cells was regarded as proliferation index. The cases with Ki-67 index ≥20% were considered of high "Ki-67 status." E-cadherin was evaluated as positive when present in at least 70% of examined cells.[28] For EGFR, 0, no membrane staining; 1+, faint, partial membrane staining; 2+, weak, complete membrane staining in >10% of tumor cells; 3+, intense complete membrane staining in >10% of tumor cells.[29] EGFR was defined as positivity when staining result is 1+, 2+, or 3+. Finally, for P53, intensity of nuclear staining was categorized into no staining (0), weak (1+), intermediate (2+), strong (3+). Intermediate to strong staining in >10% cancer cells was considered positive while no staining or weak staining in <10% cancer cells was taken as negative.[30]

2.5. Statistical analysis

Categorical data are presented as numbers and corresponding percentages, while continuous data are presented as mean ± standard deviation and range. The correlation of the 2 biomarkers was revealed by the Spearman rank correlation and the correlation intensity was described as the Spearman rank correlation coefficient (r_s). The Mann–Whitney U test or Kruskal–Wallis H test was used to assess the association between gene expression grades and each of the clinicopathological characteristics. Hierarchical cluster was used to classify the gene mRNA expression profile of all patients. Patients were grouped as group A and group B based on the clustering results. The clinicopathological differences between group A and group B were evaluated by the chi-square test or, when necessary, by Fisher exact test. For all analysis, a 2-tailed $P \leq .05$ was considered statistically significant. Hierarchical cluster was fulfilled by R software package (v1.1.453) and other statistical analyses were performed using SPSS version 17.0 statistical software (SPSS Inc., Chicago, IL).

Table 1

Clinicopathological characteristics of 97 breast cancer patients.

Parameter	Cases, n (%)
Age	
≤50 years	57 (58.8)
>50 years	40 (41.2)
Menopausal status	
Premenopausal	52 (53.6)
Postmenopausal	45 (46.4)
Pathological type	
IDC	90 (92.8)
Non-IDC	7 (7.2)
Histological grade	
G1	17 (17.5)
G2	46 (47.4)
G3	34 (35.1)
Tumor size	
T_1	29 (29.9)
T_2	68 (70.1)
Lymph node status	
Negative	52 (53.6)
Positive	45 (46.4)
ER	
Negative	33 (34.0)
Positive	64 (66.0)
PR	
Negative	47 (48.5)
Positive	50 (51.5)
HER-2	
Negative	77 (79.4)
Positive	20 (20.6)
TNBC	
No	73 (75.3)
Yes	24 (24.7)
Ki-67	
<20%	21 (21.6)
≥20%	76 (78.4)
E-cadherin	
Negative	5 (5.2)
Positive	87 (89.6)
Undetermined	5 (5.2)
EGFR	
Negative	31 (32.0)
Positive	49 (50.5)
Undetermined	17 (17.5)
P53	
Negative	37 (38.1)
Positive	58 (59.8)
Undetermined	2 (2.1)

EGFR = epidermal growth factor receptor, ER = estrogen receptors, HER-2 = human epidermal growth factor receptor 2, IDC = invasive ductal carcinoma, PR = progesterone receptors, TNBC = triple-negative breast cancer.

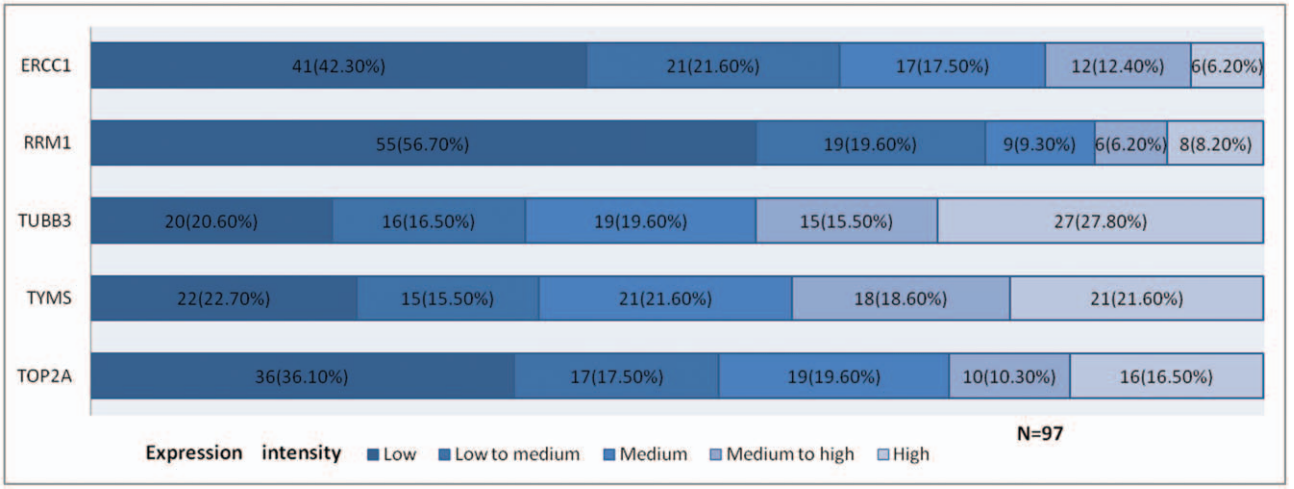

Figure 1. The case distribution by expression intensity for the 5 genes in all patients. The proportion of patients showing expression intensity of medium to high and high were 18.6% for ERCC1, 14.4% for *RRM1*, 43.3% for *TUBB3*, 40.2% for *TYMS*, 26.8% for *TOP2A*. *ERCC1* = excision repair cross complementing 1, *RRM1* = ribonucleotide reductase M1, *TOP2A* = topoisomerase IIα, *TUBB3* = β-tubulin III, *TYMS* = thymidylate synthetase.

3. Results

3.1. Clinicopathological characteristics

The patient characteristics are summarized in Table 1. All patients included in the study were women. The mean age at first confirmed diagnosis within the cohort of 97 patients was 50.53 ± 8.27 (range, 32–68) years. There were 52 (53.6%) premenopausal patients and 45 (46.4%) postmenopausal patients. Histologically, 90 (92.8%) were IDC and 7 (7.2%) were non-IDC. Additionally, a total of 17 (17.5%), 46 (47.4%), and 34 (35.1%) samples were histological grade 1, 2, and 3, respectively. With regard to tumor size, all lesions were classified into T_1 (n = 29, 29.9%) and T_2 (n = 68, 70.1%) tumor. Lymph node metastasis was present in 45 (46.4%) patients while distant metastasis was absent in all patients. Importantly, the re-evaluation of immunohistochemical characteristics was performed. It revealed that positive expression of HER-2 was present in 20 (20.6%) patients and approximately 66.0% (n = 64) and 51.5% (n = 50) of the patients was diagnosed with ER positive and PR positive, respectively. Comprehensively, there were 24 (24.7%) triple-negative breast cancer (TNBC) patients. Furthermore, the majority of breast cancer patients were Ki-67 ≥ 20% (76, 78.4%), E-cadherin positive (87, 89.6%), EGFR positive (49, 50.5%), and P53 positive (58, 59.8%).

3.2. Expression intensity and correlations between genes

Figure 1 shows the case distribution by expression intensity for the 5 genes in all patients. The proportion of patients showing expression intensity of medium to high and high were as follows: *ERCC1*, 18.6%; *RRM1*, 14.4%; *TUBB3*, 43.3%; *TYMS*, 40.2%; *TOP2A*, 26.8%. Correlations between genes were illustrated in Fig. 2. Spearman rank correlation analysis indicated that the expression intensity of *ERCC1* gene negatively associated with the expression intensity of *TUBB3* gene ($r_s = -0.293$, $P = .004$) and *TYMS* gene ($r_s = -0.205$, $P = .045$). And the expression intensity of *ERCC1* gene positively associated the expression intensity of *RRM1* gene ($r_s = 0.229$, $P = .024$) and the expression intensity of *TOP2A* gene positively associated with the expression intensity of *TYMS* gene ($r_s = 0.513$, $P < .001$).

3.3. Relationships between gene expression intensity and clinicopathological characteristics

With respect to *ERCC1* gene, the higher intensity was significantly related to T_1 tumor (mean rank: 64.79 > 42.26, $P < .001$), ER-positive (mean rank: 54.98 > 37.41, $P = .002$), PR-positive (mean rank: 58.35 > 39.05, $P < .001$) and Ki-67 <20% (mean rank: 66.00 > 44.30, $P = .001$). Furthermore, the *ERCC1* expression intensity of non-TNBC patients was higher than that of TNBC patients (mean rank: 52.06 > 39.69, $P = .050$). There were no significant associations between all clinicopathological parameters and *RRM1* gene. As for *TUBB3* gene, a significant correlation was found between higher expression level and EGFR-positive (mean rank: 47.99 > 28.66, $P < .001$). In terms of *TYMS* gene, patients with Ki-67 ≥20% exhibited higher expression level (mean rank: 52.76 > 35.40, $P = .011$). Besides, *TOP2A* gene closely related with menopausal status, lymph node status, and Ki-67 index. The expression intensity was higher in the premenopausal group (mean rank: 54.28 > 42.90, $P = .040$) and lymph node metastasis group (mean rank: 55.19 > 43.64, $P = .037$). Similar results were observed in Ki-67 ≥20% group (mean rank: 53.63 > 32.26, $P = .001$) (Table 2).

3.4. Hierarchical clustering classification

Hierarchical clustering of the 5 genes expression intensity of all patients yielded 2 patient subgroups, with 63 (64.9%) cases in group A and 34 (35.1%) cases in group B. Group A exhibited higher levels of *ERCC1* and *RRM1*, as well as lower levels of *TUBB3*, *TYMS*, and *TOP2A*, than group B (Fig. 3). Subsequently, the clinicopathological differences between group A and group B were evaluated by the chi-square test or Fisher exact test, which revealed that significant differences were identified between group A and B regarding histological grade ($P < .001$) and Ki-67 index ($P = .024$, Table 3). Group B included more cases of histological grade 3 (44.1% vs 3.2%) and Ki-67 ≥ 20% (91.2% vs 71.4%) than group A (Table 3).

4. Discussion

Gene expression differences contribute to chemotherapeutic response variability between individuals. A rational personalized

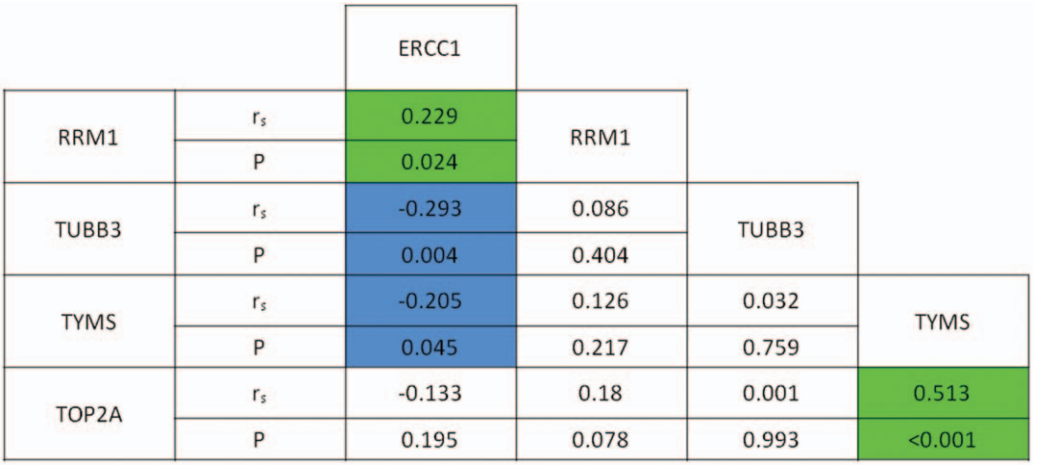

Figure 2. Correlations between genes. Positive correlation was shown as a green color, and negative correlation as a blue color. *ERCC1* = excision repair cross complementing 1, *RRM1* = ribonucleotide reductase M1, r_s = Spearman's rank correlation coefficient, *TOP2A* = topoisomerase IIα, *TUBB3* = β-tubulin III, *TYMS* = thymidylate synthetase.

Table 2

Relationships between genes expression intensity and clinicopathological characteristics.

	ERCC1		RRM1		TUBB3		TYMS		TOP2A	
Parameter	**Intensity**	***P***	**Intensity**	***P***	**Intensity**	***P***	**Intensity**	***P***	**Intensity**	***P***
Age										
≤50 years	50.54	.498	50.65	.444	50.11	.634	46.54	.295	49.39	.868
>50 years	46.80		46.65		47.41		52.50		48.45	
Menopausal status										
Premenopausal	46.29	.284	52.38	.157	51.90	.263	48.51	.851	54.28	**.040**
Postmenopausal	52.13		45.09		45.64		49.57		42.90	
Pathological type										
IDC	48.93	.994	51.14	.816	54.21	.602	48.71	.977	50.79	.857
Non-IDC	49.01		48.83		48.59		49.02		48.86	
Grade										
G1	47.21	.223	53.00	.068	50.00	.607	55.35	.523	51.03	.743
G2	44.99		42.78		51.39		48.84		50.38	
G3	55.32		55.41		45.26		46.04		46.12	
Tumor size										
T_1	64.79	**<.001**	54.98	.128	47.16	.666	45.55	.421	42.52	.125
T_2	42.26		46.45		49.79		50.47		51.76	
Lymph node status										
Negative	50.04	.682	48.30	.769	48.06	.717	48.81	.941	43.64	**.037**
Positive	47.80		49.81		50.09		49.22		55.19	
ER										
Negative	37.41	**.002**	44.00	.162	55.64	.088	53.42	.256	48.95	.991
Positive	54.98		51.58		45.58		46.72		49.02	
PR										
Negative	39.05	**<.001**	45.62	.202	54.05	.079	52.43	.235	48.28	.800
Positive	58.35		52.18		44.25		45.78		49.68	
HER-2										
Negative	51.51	.070	48.36	.627	48.51	.729	50.23	.389	49.19	.890
Positive	39.33		51.45		50.90		44.28		48.25	
Triple-negative										
No	52.06	**.050**	50.38	.348	48.23	.629	46.18	.078	47.56	.364
Yes	39.69		44.79		51.35		57.58		53.38	
Ki-67										
<20%	66.00	**.001**	48.24	.876	43.10	.266	35.40	**.011**	32.26	**.001**
≥20%	44.30		49.21		50.63		52.76		53.63	
E-cadherin										
Negative	64.00	.113	42.10	.669	27.40	.092	43.30	.778	39.10	.509
Positive	45.49		46.75		47.60		46.68		46.93	
EGFR										
Negative	40.71	.946	40.85	.903	28.66	**<.001**	42.45	.542	44.23	.238
Positive	40.37		40.28		47.99		39.27		38.14	
P53										
Negative	51.85	.252	47.22	.804	42.64	.121	51.80	.273	46.07	.573
Positive	45.54		48.50		51.42		45.58		49.23	

EGFR = epidermal growth factor receptor, ER = estrogen receptors, *ERCC1* = excision repair cross complementing 1, HER-2 = human epidermal growth factor receptor 2, IDC = invasive ductal carcinoma, PR = progesterone receptors, *RRM1* = ribonucleotide reductase M1, *TOP2A* = topoisomerase IIα, *TUBB3* = β-tubulin III, *TYMS* = thymidylate synthetase.
Bold indicates $P < .05$

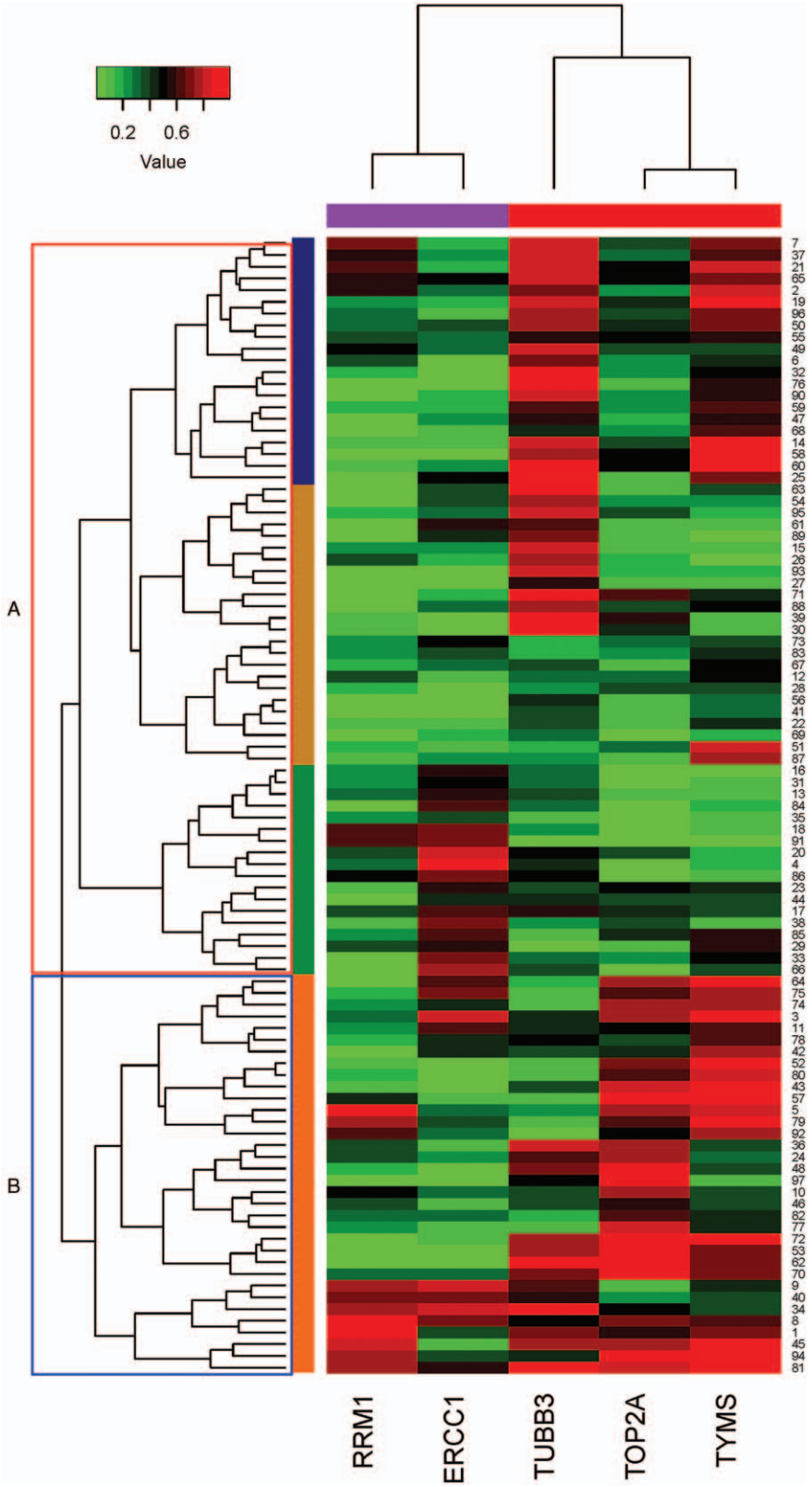

Figure 3. Expression patterns of *ERCC1*, *RRM1*, *TUBB3*, *TYMS*, and *TOP2A* in the 97 breast cancer patients. 1–97 is the sample ID number. As determined by clustering analysis of the gene expression intensity, patients were classified into 2 groups, with 63 (64.9%) cases in group A and 34 (35.1%) cases in group B. Group A exhibited higher levels of *ERCC1* and *RRM1*, as well as lower levels of *TUBB3*, *TYMS*, and *TOP2A*, than group B. *ERCC1* = excision repair cross complementing 1, *RRM1* = ribonucleotide reductase M1, *TOP2A* = topoisomerase IIα, *TUBB3* = β-tubulin III, *TYMS* = thymidylate synthetase.

Table 3

Distribution of clinicopathological characteristics of patients presented in subgroups (A and B).

Parameter	Group, n (%)		P
	A	B	
Pathological type			.693
IDC	59 (97.3)	31 (91.2)	
Non-IDC	4 (6.3)	3 (8.8)	
Histological grade			**<.001**
G1–2	61 (96.8)	29 (55.9)	
G3	2 (3.2)	15 (44.1)	
Tumor size			.939
T_1	19 (30.2)	10 (29.4)	
T_2	44 (69.8)	24 (70.6)	
Lymph node status			.342
Negative	36 (57.1)	16 (47.1)	
Positive	27 (42.9)	18 (52.9)	
ER			.482
Negative	23 (36.5)	10 (29.4)	
Positive	40 (63.5)	24 (70.6)	
PR			.292
Negative	33 (52.4)	14 (41.2)	
Positive	30 (47.6)	20 (58.8)	
HER-2			.595
Negative	49 (77.8)	28 (82.4)	
Positive	14 (22.2)	6 (17.6)	
Triple-negative			.434
No	49 (77.8)	24 (70.6)	
Yes	14 (22.2)	10 (29.4)	
Ki-67			**.024**
<20%	18 (28.6)	3 (8.8)	
≥20%	45 (71.4)	31 (91.2)	
E-cadherin			1
Negative	3 (5.0)	2 (6.3)	
Positive	57 (95.0)	30 (93.8)	
EGFR			.580
Negative	19 (36.5)	12 (42.9)	
Positive	33 (63.5)	16 (57.1)	
P53			.207
Negative	27 (43.5)	10 (30.3)	
Positive	35 (56.5)	23 (69.7)	

EGFR = epidermal growth factor receptor, ER = estrogen receptors, HER-2 = human epidermal growth factor receptor 2, IDC = invasive ductal carcinoma, PR = progesterone receptors.
Bold indicates $P < .05$

chemotherapy according to reliable biomarker will improve efficacy and reduce adverse reaction. Previous research and experiments interchangeably confirm the significant role played by *ERCC1*, *RRM1*, *TUBB3*, *TYMS*, and *TOP2A* gene in the therapy and prognosis evaluation of various types of carcinomas.[6–23] However, to our knowledge, there is no study investigating simultaneously whether the above genes are underlying biomarkers for patients with breast cancer. The present findings demonstrated that the expression levels of *ERCC1*, *TUBB3*, *TYMS*, and *TOP2A* were significantly associated with the characteristics of menopausal status, tumor size, lymph node metastasis, hormone receptor status, triple-negative status, Ki-67 index, and EGFR status, which are important parameters in guiding breast cancer treatment and evaluating prognosis.

ERCC1 is a key nuclease regulating nucleotide excision repair (NER) pathway, which plays an essential role in DNA damage caused by platinum compounds such as carboplatin.[31] As expected, the expression intensity of *ERCC1* has been regarded as a predictive factor for resistance to platinum-based chemotherapy.[32] Certain studies have reported that resistance to platinum-based chemotherapy was associated with high expression levels of *ERCC1* in advanced cancer, including colorectal cancer,[33] urinary tract cancer[11] and non-small cell lung cancer (NSCLC).[34] In this study, it has been found that *ERCC1* was relatively higher expressed in the patients with T_1 tumor ($P < .001$), positivity for ER ($P = .020$) and PR ($P < .001$), non-TNBC ($P = .050$), and Ki-67 index <20% ($P = .001$), which were favorable prognostic factors for patients with breast cancer. Therefore, the above results implied that the patients with *ERCC1* overexpression may have a good prognosis and should not be recommended taking platinum-based chemotherapy in order to reduce resistance and improve survival. Our findings about *ERCC1* seemed not to be fully in accordance with the previous studies in breast cancer. Similarly, Kim et al[20] reported that high expression of *ERCC1* was statistically associated with lower T stage, lower tumor size, no lymph node metastasis, positive ER and PR expression status, non-TNBC, and no lymphovascular invasion. By contrast, there was no association between *ERCC1* expression and clinicopathological parameters, including age, histology, tumor stage at diagnosis, hormonal receptors (ER, PR) status, HER-2 status, presence of visceral disease, and pretreatment of metastatic disease, which has been studied by Metro et al[35] who analyzed *ERCC1* expression using an automated and quantitative immunofluorescence technique. The above differences may be caused by different detection or assessment methods. Unlike previous testing technology such as RT-PCR, MBL used in this study is a non-PCR-based technology at the molecular level. We believe our results is reliable because MBL is suitable for various sample types and is widely used in clinical diagnosis.[7,24,25]

TUBB3 is one of the major components of microtubules (a basic constructive unit of spindle and cytoskeleton) controlling in mitosis and cellular motility, possessing a significant role in malignant transformation and cancer aggressiveness.[36] Clinically, axanes are anti-microtubule chemotherapeutic agents that disrupt the dynamic equilibrium of microtubule polymerization and cause mitotic arrest and apoptosis of malignant cells. It has been reported that amplification of *TUBB3* expression may destabilize microtubules and counteract the effects of taxanes,[37] which have been confirmed in various cancer types, including breast,[8,38] lung, ovarian, prostate, stomach, and pancreatic tumors.[39] In breast tumors, although overexpression of *TUBB3* have been linked to high histological grade,[22,38] advanced tumor stage,[22,40] lymph nodes,[40] ER negativity,[22] PR negativity,[22,40] HER-2 positivity,[22,40,41] and a triple-negative phenotype,[41] these results were unable to prove in our study and alternative studies,[8,38,40,41] which may be attributed to technical issues and the variety of methods, scoring systems, and cutoffs used by particular groups. More importantly, our study revealed that the high expression level of *TUBB3* gene was strongly associated with EGFR positivity ($P < .001$). Given that the EGFR is known to be frequently involved in driving the proliferation and survival of tumor cells,[42] it is possible that EGFR signaling pathways may be involved in regulating *TUBB3* expression, suggesting taxanes may be an inappropriate treatment for breast cancer with EGFR positivity.

TYMS is a central enzyme in the folate metabolic pathway and is a major target for antifolate cytotoxic chemotherapy drugs, such as 5-fluorouracil and capecitabine. To date, analyzed in pancreatic cancer,[43] colorectal cancer,[44] and prostate can-

cer,[45] but the clinicopathological significance of *TYMS* in breast cancer still remains unclear. Shan et al[16] found increased *TYMS* expression was related with higher histological grade and lymph node metastasis. Besides, in the present study, elevated *TYMS* expression is significantly correlated with Ki-67 ≥20% ($P = .011$), which is consistent with previous report.[46] It is well recognized that Ki-67 is a nuclear protein associated with cellular proliferation and up-regulated Ki-67 generally indicate a poor outcome.[47] It may be hypothesized that *TYMS* expression may provide prognostic information in breast cancer. In a long-term follow-up study, Lee et al[48] have reported that breast cancer with higher *TYMS* expression showed poor prognosis.

TOP2A is an essential nuclear enzyme that changes DNA topology and the primary molecular target of various cytotoxic agents including anthracyclines.[49] As such, it has been widely investigated for potential applications in breast cancer detection and management. Qiao et al[50] have reported that positive rate of *TOP2A* expression showed significant correlations with ER, Ki-67, and HER-2. An et al[23] have found that *TOP2A* overexpression was associated with a higher tumor grade and Ki-67 index. In consistence with previous studies,[23,50,51] the significant finding in current study was the identification of correlation of *TOP2A* amplification with nodal involvement ($P = .037$) and Ki-67 ≥20% ($P = .001$), suggesting that tumors with high level of *TOP2A* expression were more aggressive. Additionally, we found the *TOP2A* expression levels of premenopausal woman was higher than that of postmenopausal woman ($P = .040$). Like Ki-67, *TOP2A* is regarded as a proliferation marker which is strongly expressed in proliferating cells.[51] Despite extensive research, the biological background underlying this association between *TOP2A* and Ki-67 is currently unknown. Proliferation is a salient feature for the survival and spread of malignancy, and thus the patients with amplified *TOP2A* should be employed with anthracyclines in clinical practice.

Notably, gene regulation is a dynamic and complex process involved multiple genes and other factor and increasing evidence exists that the aberrant regulation of multigene is associated with the abnormal proliferation of cancer cells. To avoid neglecting gene interactions, we also performed the bivariate correlation and hierarchical clustering classification among the 5 genes, which, to our knowledge, has not been studied in breast cancer. The results showed that *ERCC1* was negatively correlated with *TUBB3* ($r_s = -0.293$, $P = .004$) and *TYMS* ($r_s = -0.205$, $P = .045$), and positively correlated with *RRM1* ($r_s = 0.229$, $P = .024$). Moreover, there was a positive correlation between *TOP2A* and *TYMS* ($r_s = 0.513$, $P < .001$). From the results of cluster classification, the above-mentioned associations can also be confirmed indirectly. According to the cluster classification, all 97 patients were divided into 2 subgroups. Group B included more cases of histological grade 3 (44.1% vs 3.2%, $P < .001$) and Ki-67 ≥20% (91.2% vs 71.4%, $P = .024$) than group A. In terms of gene expression, we found group B patients exhibited higher levels of *TUBB3*, *TYMS*, and *TOP2A*, as well as lower levels of *ERCC1* and *RRM1*, than group A patients. As such, it could be assumed that there was a synergistic role in breast cancer aggressiveness among *TUBB3*, *TYMS*, and *TOP2A*. Their interactions deserve further study. Besides, as mentioned before, ERCC1 can be used to predict the sensitivity of platinum drugs; RRM1 can be used to predict the sensitivity of gemcitabine; TUBB3 can be used to predict the sensitivity of taxanes drugs; TYMS can be used to predict the sensitivity of antifolate cytotoxic chemotherapy drugs; TOP2A can be used to predict the sensitivity of anthracyclines drugs. In view of the important roles of the 5 genes in evaluating chemoresistance, this classification may provide useful information about formulating chemotherapy scheme.

Admittedly, this study has several limitations. Firstly, this was a study using a nonrandomized patient cohort and sample size was not enough large, which may bring about different conclusions with previous studies. Secondly, the gene expression was detected using the MBL technology, but not confirmed by other method with normal breast tissues or tissue adjacent to tumors as control. Nevertheless, the results of this study are reliable, because MBL technology is a mature gene detection technology which has been widely applied in clinical diagnosis and individualized treatment.[7,24,25] Thirdly, coexpression and negative correlation of those genes are not verified by the public bulk RNA-seq data for breast cancer cohorts or even cell lines. The bulk RNA-seq data of breast cancer cohorts and cell lines will be downloaded from the website portals: The Cancer Genome Atlas (TCGA), The Genotype-tissue Expression Project (GTEx), Cancer Cell Line Encyclopedia (CCLE), and International Cancer Genome Consortium (ICGC), to further confirm genes interactions and clinical practicality in the subsequent study. Fourthly, long-term follow up results had not been collected completely, but we have been performing the task to further evaluate the prognostic significance.

In summary, results of the analysis in the presented study indicate *ERCC1*, *TYMS*, *TUBB3*, and *TOP2A* may be potential biomarkers for prognosis and individualized chemotherapy guidance, while there may be interactions between *ERCC1* and *RRM1*, or *TUBB3*, or *TYMS*, as well as between *TOP2A* and *TYMS* in pathogenesis and development of breast cancer. Still, further large-scaled, prospective studies with multivariate prognostic analysis, addition of control samples, and standardized method confirmation are crucial to clarifying the utility of those biomarkers in breast cancer.

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Author contributions

Conceptualization: Juncheng Li, Gang Xue.
Data curation: Juncheng Li, Gang Xue.
Formal analysis: Tao Huang, Shengdong He.
Investigation: Juncheng Li, Lingfan Li.
Methodology: Juncheng Li, Peng Sun.
Project administration: Tao Huang, Gang Xue.
Resources: Peng Sun, Tao Huang.
Software: Shengdong He, Lingfan Li.
Supervision: Gang Xue.
Validation: Peng Sun, Tao Huang, Gang Xue.
Visualization: Peng Sun, Tao Huang.
Writing – original draft: Juncheng Li, Shengdong He, Lingfan Li.
Writing – review & editing: Peng Sun, Gang Xue.

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