Clinical significances of *RPL15* gene expression in circulating tumor cells of patients with breast cancer

YING ZHUANG[1-3], KELI SU[4], SHUSHU LIU[1-3], WEI FAN[1-3], HUIJUAN LV[4] and WEI ZHONG[1-3]

[1]Breast Cancer Center, Hubei Cancer Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430079, P.R. China; [2]National Key Clinical Specialty Discipline Construction Program, Hubei Provincial Clinical Research Center for Breast Cancer, Wuhan, Hubei 430079, P.R. China; [3]Wuhan Clinical Research Center for Breast Cancer, Wuhan, Hubei 430079, P.R. China; [4]Department of Oncology, The Fourth People's Hospital of Jinan, Jinan, Shandong 250031, P.R. China

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Abstract. The preferred biomarkers for evaluating the outcomes of patients with breast cancer (BC) remain poorly understood. The present study aimed to investigate the predictive roles of circulating tumor cells (CTCs) and ribosomal protein L 15 (*RPL15)* expression in the prognosis of patients with BC. A total of 170 patients were included in the present study, all of whom were female. BC was diagnosed by combining clinical features, imaging and pathological findings. CanPatrol™ technology and triple color *in situ* RNA hybridization were used to detect CTC subtypes and *RPL15* gene expression levels. CTCs were classified into epithelial CTCs, mesenchymal CTCs (MCTCs), and hybrid CTCs (HCTCs) according to cellular surface markers. Risk factors for recurrence and metastasis were validated by a multivariate COX regression model. Kaplan-Meier survival curves were used to determine the progression-free survival (PFS) of patients. The results showed that patients with advanced tumor-node-metastasis stage and triple negative BC had high MCTCs, HCTCs and *RPL15* levels (P<0.05). Furthermore, the multivariate COX regression analysis revealed that MCTCs, HCTCs, HER2^{+} and positive *RPL15* gene expression were key factors for recurrence and metastasis of patients (P<0.05). The PFS of patients with >2 MCTCs/5 ml blood, >5 HCTCs/5 ml blood, and positive *RPL15* gene expression in CTCs were significantly shorter than that of patient with 2 MCTCs, 5 HCTCs, and negative *RPL15* gene expression in CTCs (P<0.05). By contrast, the PFS of patients with positive HER2 also was significantly shorter than that of patients with negative HER2. Overall, the present data indicated that the PFS of patients with BC with >2 MCTC or >5 HCTCs, and positive *RPL15* gene expression was shorter than that of those with 2 MCTCs or 5 HCTCs, and negative *RPL15* gene expression. Additionally, the prognosis of patients with BC with negative HER2 is more favorable than the prognosis of patients with positive HER2 expression.

Correspondence to: Dr Wei Zhong, Breast Cancer Center, Hubei Cancer Hospital, Tongji Medical College, Huazhong University of Science and Technology, 116 Zhuo Daoquan South Road, Wuhan, Hubei 430079, P.R. China
E-mail: bellway@163.com

Abbreviations: BC, breast cancer; CTCs, circulating tumor cells; EMT, epithelial-mesenchymal transition; eCTCs, epithelial CTCs; ER, estrogen receptor; HCTCs, hybrid CTCs; HER2, human epidermal growth factor receptor2; HR, hormone receptor; IHC, immunohistochemistry; MCTCs, mesenchymal CTCs; OS, overall survival; PFS, progression-free survival; PR, progesterone receptor; RNA-ISH, RNA *in situ* hybridization; RPL15, ribosomal protein L 15; TNBC, triple negative BC; TNM, tumor-node-metastasis

Key words: BC, CTC, *RPL15*, RNA-ISH, PFS

Introduction

The incidence of breast cancer (BC) in women always is increasing and rank first according to statistics in China and other countries, worldwide (1,2). The mortality rate of patients with BC ranks second after lung cancer, which account for ~30% of cancers in women (3,4). According to the World Health Organization (WHO) report, ~2 million new patients with BC are diagnosed per year, and this has been attributed to the increased life span and improved medical care worldwide (5-7). Recently, the mortality rate of patients with BC has decreased because of early screening with mammography and advanced therapeutic methods, such as targeted and immune therapies (8-13) or the combination of ferroptosis with photodynamic therapy (14). However, for those patients with advanced BC, 5-year survival rate is only 5-10% (15); therefore, a sensitive and reliable biomarker is critical for predicting the outcomes of patients with BC.

Emerging evidence indicates that the detection of circulating tumor cells (CTCs) in peripheral blood are markedly related to the recurrence, metastasis and the outcomes of numerous cancers (16-18). CTCs originate from primary tumor or metastatic tumors and enter peripheral circulation, potentially giving rise to tumor cell proliferation in other parts of the body undergoing epithelial-mesenchymal transition (EMT) (16,19-21). CTCs can be divided into epithelial CTCs (eCTCs), mesenchymal CTCs (MCTCs) and hybrid CTCs (HCTCs), which express EpCAM and ck8/18/19 as epithelial marker and vimentin combined twist as mesenchymal marker,

respectively (19,22). Initially, EpCAM was considered as a major marker for epithelial cell because it is a transmembrane glycoprotein and mediates cell-cell adhesion of epithelial tissues (23,24), but later cytokeratin 8/18/19CTCs combined EpCAM measurements to enhance epithelial cell specificity because cytokeratin 8/18/19 proteins are essential components of the cytoskeleton in epithelial cells (25,26). In addition, vimentin was identified as a mesenchymal cell marker and twist was a relevant transcription factor for mesenchymal cells (27). Therefore, EMT markers are more likely to reflect biological functions of CTCs. CTCs' detecting methods were varied dependent on available equipment and patients' status, including density-gradient centrifugation, filtration approaches, flow cytometry, immunohistochemistry (IHC), glycolysis-associated long non-coding RNAs (IncRNAs) (20), long non-coding RNA detection (28), and RNA *in situ* hybridization (RNA-ISH) (29-32). Among these techniques, RNA-ISH has numerous advantages over other methods, such as high sensitivity, less CTCs loss, detecting CTCs undergoing EMT (33).

The relationship between the numbers of CTCs and the prognosis of patients with cancer were extensively investigated (34-37). Recent studies have revealed that the overall survival (OS) of patients with higher CTCs number was significantly shorter than that of patients with low CTCs number (38,39). Therefore, measurement of the CTCs number is very helpful for predicting the outcomes of patients with cancer and guiding treatment decisions.

Recently, Ebright *et al* (40) identified a ribosomal submit, ribosomal protein L15 (*RPL15)* gene, that plays a role in BC metastasis during an *in vivo* genome-wide CRISPR screening of CTCs from patients with hormone receptor positive (HR^+) BC. It was found that *RPL15* overexpression was strongly associated with the metastatic growth of BC in numerous organs of patients and a poor prognosis. *RPL15* gene encodes the 60S RPL15, which catalyzes protein synthesis (41). A previous study showed that *RPL15* also enhances drug resistance to chemotherapy in patients with gastric cancer (42); however, the exact mechanism of *RPL15* action in cancer development remains unclear. It was hypothesized that *RPL15* overexpression in CTCs promotes recurrence and metastasis of BC. To address this hypothesis, the CanPatrol technique was used to detect *RPL15* gene expression in CTCs of patients with BC and evaluate the relationship between *RPL15* expression and patient outcomes. Therefore, patients with BC were recruited to measure their CTCs and *RPL15* expression before treatment. The results provide insights into the metastatic mechanisms of BC and can be helpful for guiding clinical treatment decision.

Materials and methods

Study design and subjects. The present study recruited 170 patients with BC to evaluate the outcomes of patients with various CTCs levels. Female patients who were 27-83 years-old and admitted to the Hubei Cancer Hospital from November 2007 to July 2022 were included in the present study. The criteria for enrollment were as follows: i) age >18 years; ii) BC diagnosis confirmed by two independent clinical pathologists in tumor biopsy or fine-needle aspiration samples and combined computerized tomography (CT) scan images; iii) tumor-node-metastasis (TNM) stages I-III determined according to proposal criteria in AACR-8th edition (43); iv) with estrogen receptor (ER), progesterone receptor (PR) and epidermal growth factor receptor 2 (HER2) expression detected via IHC before surgery; and v) complete medical data and follow-up record. The following exclusion criteria were applied to the present study: i) incomplete clinical data; ii) lost to follow-up; and iii) no previous therapies, such as surgery, chemotherapy, or radiotherapy before the study. In addition, 10 patients with breast benign nodule were also recruited as a control. The present study was reviewed and approved (approval no. 2024-264) by the Ethical Committee of the Hubei Cancer Hospital (Wuhan, China). Written informed consent was obtained from all patients before the study. The present study was conducted in accordance with the principles of Declaration of Helsinki (2013 version).

CTCs enrichment and analysis. During EMT transition of CTCs, eCTCs, MCTCs and HCTCs subtypes can be differentiated using *EpCAM* and *CK8/18/19* for eCTCs biomarker, *Vimentin* and *Twist* for mesenchymal biomarker measurement. In the present study, CanPatrol™ technology was utilized to detect these biomarker-branched DNA (bDNA) (24). Briefly, CTCs were enriched via a filter-based process, then different CTCs subtypes were identified using an RNA-ISH technique.

CTCs enrichment was performed as previously described (19), and none of the patients received any therapy before the present study. To enrich CTCs in the peripheral blood of patients with BC, 5 ml of whole blood was collected from enrolled patients and was transferred into an EDTA-coated tube one day just before treatment. Blood samples was immediately processed or stored at 4˚C for <4 h before the next step. To obtain white blood cells, erythrocytes were mixed with 15 ml red blood lysis buffer and incubated for 30 min at room temperature (RT). After centrifugation for 5 min with 300 x g at RT, the supernatant was discarded. The cells were then washed twice with phosphate-buffered saline (PBS). The collected cells were transferred into a filter tube with 8-μM pore size membrane and connected with the vacuum filtration system at 0.08 MPa. The filtered cells were fixed in 4% paraformaldehyde (PFA) for 1 h at RT.

CTCs and RPL15 gene detection using mRNA probes in RNA-ISH. To evaluate total CTCs and subtypes as well as *RPL15* gene expression in the peripheral blood of patients with BC, the RNA-ISH technique was employed to detect specific genes based on the bDNA signal amplification technique because this technique is more powerful for rare gene expression. The bDNA signal amplification employs specific capture probes to bind the target gene sequences and combine bDNA signal amplification probes, including preamplifier sequence, the amplifier sequences and the label probes (24,44). The pre-amplifier sequence was designed to recognize the capture and bDNA amplifier sequences. The label probes were conjugated with fluorescent dye and counted under fluorescence scanning microscope (24). Briefly, previous fixed samples were digested with 0.1 mg/ml proteinase K for 30 min at 4˚C to enhance the cell membrane permeability. After rinsing twice with PBS solution, the following capture probes were added to the hybridization solution and incubated for 2 h at 40˚C:

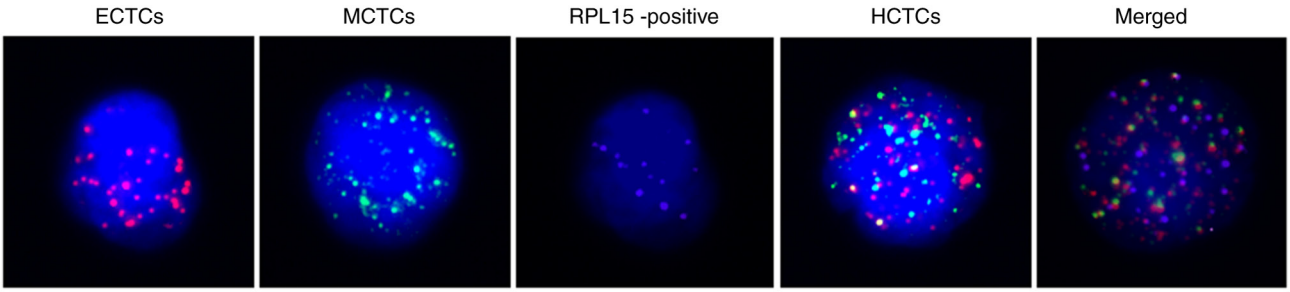

Figure 1. CTCs and *RPL15* mRNA expression images. Fluorescence microscope images of different subtypes of CTCs and *RPL15* mRNA on TCTCs. Red fluorescence: Epithelial marker expression signal spots (eCTCs); Green fluorescence, mesenchymal cell marker expression signal spots CTCs (MCTCs); Purple fluorescence *RPL15* expression signal spots on CTCs; Hybrid type CTCs (HCTCs); Merge: All three-color mixed dots on CTCs; Blue fluorescence: positive, DAPI-stained nucleus. eCTCs and MCTCs show individual picture. HCTCs show red dot and green merger picture. *RPL15* expression shows purple dots and green dots merger picture. CTCs, circulating tumor cells; RPL15, ribosomal protein L 15; eCTCs, epithelial CTCs; MCTCs, mesenchymal CTCs; HCTCs, hybrid CTCs.

EpCAM and *CK8/18/19* for epithelial biomarker; *Vimentin* and *Twist* for mesenchymal biomarker; *RPL15* bDNA probes. These bDNA specific probes were custom-made and purchased from Invitrogen; Thermo Fisher Scientific; Inc. based on their gene sequences. To deduce background signals, the cells were washed twice with 0.1X SSC eluent (MilliporeSigma). Cells were then added pre-amplification and amplification solution and incubated at 40˚C for 90 min to obtain a strong signal after adding Alexa Fluor (AF) 594 conjugated EpCAM and CK8/18/19 detection probe, AF488 conjugated Vimentin and Twist probe, and AF750 conjugated *RPL15* probe for 60 min incubation. Finally, DAPI was added into samples for cellular nucleus staining. Images were obtained using fluorescence scanning microscope at x100 magnification (Olympus BX53; Olympus Corporation).

Criteria for CTCs and RPL15 gene expression positivity. Positive eCTCs, MCTCs, HCTCs, and *RPL15* cells were determined and counted under a fluorescence microscope, following the manufacturer's instructions (SurExam; http://www.surexam.com). Only red or green fluorescence signal spots on cell surface represent eCTCs and MCTCs, respectively. If there were both red and green spots on cell surface, these cells were classified as HCTCs. The purple fluorescence signal dots represent *RPL15* gene on cellular surface. The positive *RPL15* gene was counted with ≥1 purple dots/5 ml peripheral blood on CTC, MCTCs, or HCTCs. No any purple dots on CTCs were observed and defined as *RPL15* negative. Images of 5 microscopy fields were randomly captured and average number of CTCs in each field was counted. These cell-specific images and characteristics are presented in Fig. 1 and Table I.

HR detection using IHC and fluorescence in-situ hybridization (FISH). HR expression levels, including ER, PR and HER2, were detected via IHC for ER and PR or FISH for HER2. Tumor samples were prepared as 4-μM wide sections and mounted into slides. The primary antibodies at 1:1,000 dilution were incubated overnight at 4˚C and the secondary antibody at 1:1,000 dilution were incubated at RT for 1 h according to the manufacturer's instructions (Roche Diagnostics). ER- and PR-positive cells were identified by at least three certified pathologists. Positive HER2 cells were quantified using FISH method (Sinomdgene; https://www.sinogenepets.com). Briefly, the deparaffinized sections of formalin-fixed paraffin-embedded tumor tissue were incubated with a serial of reagents. HER2 specific DNA probe labeled with spectrum orange and chromosome enumeration probe (CEP17) labeled with spectrum green were added to slides for staining. The cells were observed and counted under a fluorescence microscope following the 2018 ASCO/CAP criteria (45).

Table I. CTCs and *RPL15* classification features.

Subtypes	Spot color	DAPI
Epithelial CTCs	Red	+
Mesenchymal CTCs	Green	+
Hybrid CTCs	Red and green	+
RPL15+,	Purple	+

CTCs, circulating tumor cells.

Disease status follow-up. All patients were followed up to 24 months by visitor phone call by every three months in the first half year, then every six months thereafter. Follow-up data included disease symptoms, chest computed tomography (CT), skull magnetic resonance imaging, whole-body bone scan, abdominal color ultrasound, and positron emission tomography. Signs of recurrence and metastasis were determined by image data showing space-occupying lesions in the chest and other organs. Progression-free survival (PFS) was defined as time from treatment to recurrence.

Statistical analysis. All data analysis was performed using GraphPad Prism 9.0 version (Dotmatics). A comparison of the relationship between continuous or categorical variables and patient demographics was performed using the paired t-test and χ^2 tests. Cox proportional hazard regression model were used to investigate the factors (age, MCTCs, HCTCs, *RPL15*, ER, PR and HER2) impacting patients' outcomes. In this model, age, MCTCs, HCTCs, *RPL15*, ER, PR and HER2 were as variables and PFS was as events at 24 months

Table II. Baseline clinical characteristics of 170 patients with breast cancer.

Characteristics	Median	Range	Case number	Percentage (%)
Age, years				
≥60	65	61-83	29	17.1
<60	49	27-60	141	82.9
Histologic type				
Invasive ductal carcinoma	NA	NA	152	89.4
Invasive lobular carcinoma	NA	NA	3	1.8
DCIS	NA	NA	8	4.8
Papillary carcinoma	NA	NA	3	1.8
Metaplastic carcinoma	NA	NA	2	1.1
Mucinous carcinoma	NA	NA	2	1.1
Tumor-node-metastasis stage				
DCIS	NA	NA	8	4.8
I	NA	NA	70	41.2
II	NA	NA	72	42.3
III	NA	NA	20	11.7
Hormone receptor positive				
Estrogen receptor	NA	NA	114	67.1
Progesterone receptor	NA	NA	105	61.8
Epidermal growth factor receptor 2	NA	NA	88	51.8
Triple negative breast cancer	NA	NA	25	14.7
Therapies				
Surgery	NA	NA	170	100
Surgery + chemotherapy	NA	NA	162	95.3
Surgery + immunotherapy	NA	NA	90	52.9

NA, not applicable; DCIS, ductal carcinoma *in situ*.

cut-off. Hazard ratio (HR), 95% confidence intervals (CI), and P-values were compared in varous groups. The assessment of Schoenfeld's residuals was used to ensure the assumptions have not been violated. The PFS curve of patients was plotted by the Kaplan-Meier method after the log-rank test. A power calculation was used to determine differences in PFS of the subgroups. P<0.05 was considered to indicate a statistically significant difference.

Results

Patient clinico-pathological charateristics. All patients were female, and ages ranged from 27-83 years-old (median, 59 years-old; mean ± SD, 50.95±10.76 years-old). A total of 29 patients were ≥65 years-old (range 61-63) (29/170, 17.1%) and 141 patients were <60 years-old (range 27-60, 141/170, 82.9%). A total of 152 patients had invasive ductal carcinoma (IDC) (89.4%), and the rest of the study cohort included 3 patients with invasive lobular carcinoma (ILC, 1.8%), 8 with ductal carcinoma *in situ* (DCIS, 4.8%), 2 with mucinous carcinoma (MC, 1.1%), 2 with metaplastic carcinoma (MeC, 1.1%) and 3 with papillary carcinoma (PC, 1.8%), r espectively. Regarding patient TNM stages, 70 were stage I (70/170, 41.2%), 72 were stage II (72/170, 42.3%), 20 were stage III (20/170, 11.7%), and 8 patients had DCIS (8/170,4.8%) based on criteria recommended by AACR-8th edition (43). ER, PR and HER2 were measured using IHC and FISH methods. Regarding HR^+ status, 114 were ER^+ (114/170, 67.1%), 105 were PR^+ (105/170, 61.8%), 88 were $HER2^+$ (88/170, 51.8%), and 12 triple-negative BC (TNBC, 25/170, 14.7%). All patients performed surgery, 162 cases (95.3%) of which added chemotherapy. Additionally, 90 patients (52.9%) experienced immunotherapy using anti-PD1 administration (Table II).

CTCs and RPL15 expression in patients with BC. CTCs and *RPL15* gene expression are strongly associated with tumorigenesis and the outcome of patients with BC. CTCs, CTCs' subtypes and *RPL15* expression level in patients with BC were detected in the present study using CanPatrol technology combined triple color *in situ* RNA hybridization (Table I and Fig. 1). These specific biomarkers possess unique cellular surface molecules that can be labeled with differentiated fluorochromes and counted under a fluorescence microscope. For positive *RPL15* expression, CTC, MCTCs, or HCTCs were firstly counted, then *RPL15* gene expression was calculated on these CTCs. As can be observed in Table III, no significant differences were found in total CTCs, eCTCs, MCTCs, HCTCs and *RPL15* on TCTCs between patients aged ≥60 years and those <60 years. Furthermore, the CTCs in patients with different pathological subtypes, including IDC, ILC, DCIS,

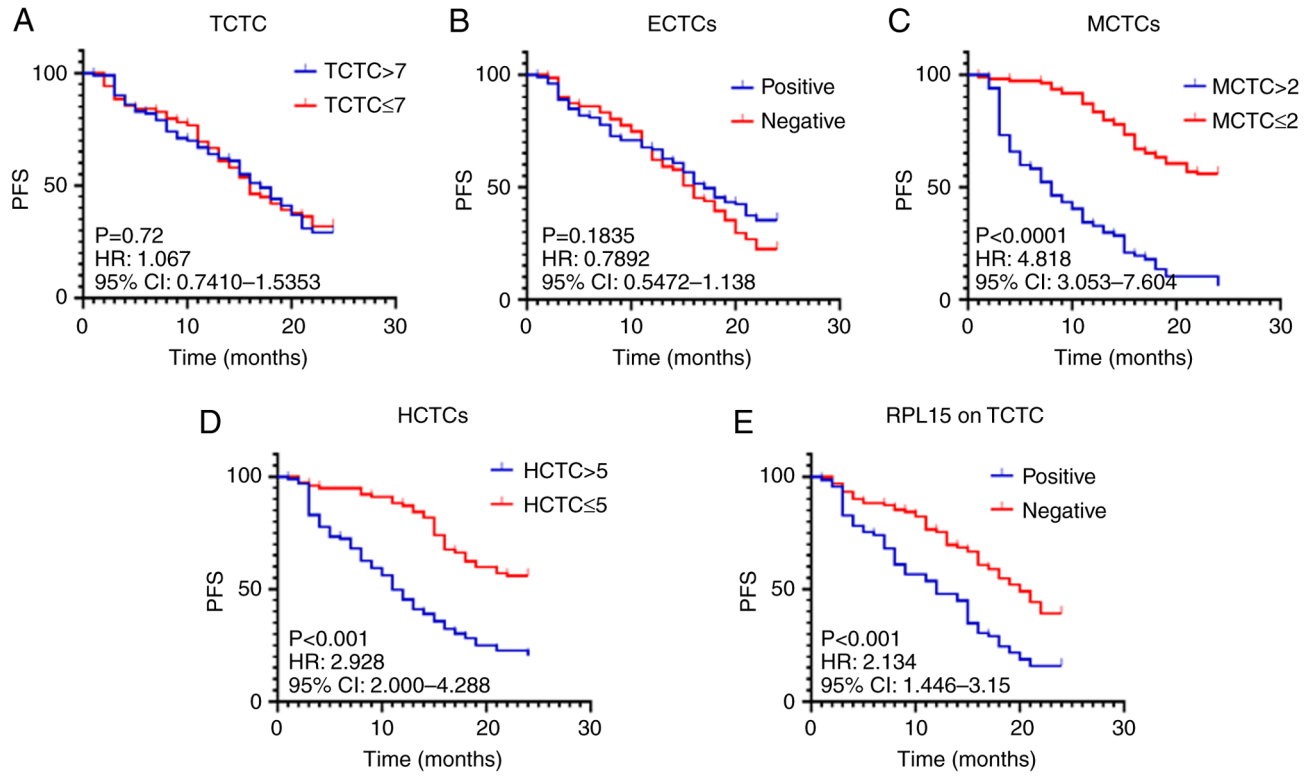

Figure 2. Survival curve of patients with CTCs and *RPL15* RNA. (A) PFS comparison between patients with >7 TCTCs and 7 TCTCs. (B) PFS comparison of patients with positive eCTCs and negative eCTCs. (C) PFS comparison of patients with >2 MCTCs and ≤2 MCTCs. (D) PFS comparison of patients with >5 HCTCs and ≤5 HCTCs. (E) PFS comparison of patients with positive *RPL15* and negative *RPL15* on TCTC. CTCs, circulating tumor cells; RPL15, ribosomal protein L 15; PFS, progression-free survival; TCTCs, total CTCs; eCTCs, epithelial CTCs; MCTCs, mesenchymal CTCs; HCTCs, hybrid CTCs; HR, hazard ratio; CI, confidence interval.

PC, MeC and MC, did not significantly differ. Interestingly, CTCs and RPL15 on the total CTCs of patients with BC were strongly associated with TNM stage (P=0.021) and HR levels (P=0.13). Positive CTCs and RPL15 on CTC rates were significantly increased in advanced TNM. Furthermore, patients with TNBC had significantly higher CTCs and *PRL15* expression than those with other HR types. Recent data have shown that CTCs and *RPL15* expression are significantly associated with TNM stages and HR expression levels in patients with BC.

The prediction of recurrence and metastaisis in patients with BCusing multivariate COX regression analysis. To further evaluate the outcomes of patients with different clinical characteristics, recurrence and metastasis in patients were predicted via multivariate COX proportional hazard regression analysis using age, MCTCs, HCTCs, *RPL15* on TCTCs, ER, PR and HER2 as co-variates. The follow-up time was variable, and PFS as well as relapse were defined as events. Age, CTC cut-off values, RPL15, ER, PR, HER2 positive or negative expression were used as the other variable, and Graphpad Prism softare was used for statitical analysis. A total of 24 months follow-up was set as cut-off duration for recurrence,metastasis and PFS time (Table IV). These cut-off values of biomarker expressive levels in patients with BC were determined according to *RPL15* CTCs and levels of 10 patients with breast benign nodule, which can distinguish benign and malignant breast mass with >80% sensitivity and specificity at these cut-off values. The patients with ≥/<60 years of age, $ER^{+/-}$ and $PR^{+/-}$ expression were not determining factors for recurrenece and metastasis. By contrast, high MCTCs, HCTCS, positive *RPL15* on CTCs had 2.73, 2.38, 1.83 and 2.76 HR for recurrence and metastasis, which mean that relapse and metastasis chances of patients with high MCTCs (>2), HCTCs (>5), and positive *RPL15* were 2.73,2.38 and 2,76-fold than that of patients with low MCTCs (≤2), HCTCs (≤5) and negative *RPL15*, respectively. The PFS of patients with low MCTCs, low HCTCs, negative RPL15 gene expression and negative HER2 expression was significantlylonger than that of the patients with positive expression levels, respectively (P<0.05).

Survival comparison of patients with various CTCs, RPL15 and HR expression by Kaplan-Meier analysis. The multivariate COX regress analysis showed that MCTCs, HCTCs, *RPL15* and positive HER2 were critical risk factors for recurrence and metastasis of patients with BC;a survival analysis of patient with different CTCs and *RPL15* levels was further carried out via a Kaplan-Meier survival analysis (Fig. 2). Different cut-off values for total CTCs and subtypes were set to compare PFS duration. The results demonstrated that PFS of patients with >7/≤7 TCTCs (Fig. 2A), positive/negative eCTCs (Fig. 2B) did not significatly differ (P>0.05). By contrast, the PFS of patients with low MCTCs (Fig. 2C), HCTCs (Fig. 2D) and *RPL15* on TCTCs (Fig. 2E) was significantly longer than that of patients with high MCTCs, HCTCs, and positive *RPL15* expression. The associated HRs, 95% CIs, and P-value were: HR, 4.818; 95% CI, 3.053-7.604; P<0.0001 for MCTCs; HR, 2.928; 95% CI, 2-4.288; P<0.001 for HCTCs; HR, 2.134; 95% CI, 1.446-3.15; P<0.001 for *RPL15* (Fig. 2 and Table V). These

Table III. CTCs and RPL15 prevalence of patients with breast cancer (170 cases).

Characteristics	Total CTCs (>7/≤7, n %)	Epithelial CTCs (P/N, n %)	Mesenchymal CTCs (>2/≤2, n %)	Hybrid CTCs (>5/≤5, n %)	RPL15 on total CTCs (P/N, n %)	P-value
Age, years						0.763
≥60	15 (60.0)	2 (8)	14 (56)	18 (72)	7 (46.7)	
<60	70 (49.6)	40 (28.3)	50 (35.5)	75 (53.2)	40 (57.1)	
Histologic type						0.384
Invasive ductal carcinoma	50 (32.9)	60 (39.5)	70 (46)	55 (36.2)	40 (26.3)	
Invasive lobular carcinoma	1 (33.3)	2 (66.7)	1 (33.3)	1 (33.3)	1 (33.3)	
Ductal carcinoma *in situ*	2 (25)	1 (12.5)	1 (12.5)	1 (12.5)	0 (0)	
Papillary carcinoma	1 (33.3)	0 (0)	1 (33.3)	0 (0)	1 (33.3)	
Metaplastic carcinoma	1 (50)	0 (0)	0 (0)	1 (50)	1 (50)	
Mucinous carcinoma	1 (50)	0 (0)	0 (0)	1 (50)	1 (50)	
Tumor-node-metastasis stages						0.021
I	30 (42.9)	3 (4.3)	16 (22.9)	11 (15.7)	10 (33.3)	
II	50 (69.4)	5 (6.9)	26 (36.1)	19 (26.3)	35 (48.6)	
III	15 (75)	7 (35)	13 (65)	12 (60)	15 (75)	
Hormone receptor						0.013
Estrogen receptor	20 (18)	17 (15.3)	15 (13.5)	17 (15.3)	25 (22.5)	
Progesterone receptor	23 (22.3)	20 (19)	23 (21.9)	25 (23.8)	30 (28.7)	
Epidermal growth factor receptor 2	13 (14.8)	21 (23.9)	31 (35.2)	29 (32.9)	35 (39.7)	
Triple-negative breast cancer	15 (60)	5 (20)	16 (64)	20 (80)	19 (76)	

CTCs, circulating tumor cells; RPL15, ribosomal protein L 15.

Table IV. Multivariate analysis of risk factors for recurrence and metastasis in patients with breast cancer.

Variable	Hazard ratio	95% confidence interval	P-value
Age	1.004	0.978-1.031	0.505
Mesenchymal circulating tumor cells	2.73	1.25-116.9	0.014
Hybrid circulating tumor cells	2.38	2.14-3.27	0.027
Ribosomal protein L 15 on circulating tumor cells	1.83	1.87-2.79	0.0032
Estrogen receptor-positive	1.097	0.5456-2.3	0.801
Progesterone receptor-positive	1.09	0.558-2.27	0.807
Human epithelial growth factor receptor 2-positive	2.76	2.83-3.57	0.013

Table V. Survival comparison of CTCs and *RPL15* expression in patients with breast cancer.

Variables	Hazard ratio	95% confidence interval	P-value
TCTCs	1.067	0.741-1.535	0.72
Epithelial CTCs	0.7892	0.5472-1.138	0.1835
Mesenchymal CTCs	4.818	3.053-7.604	<0.0001
Hybrid CTCs	2.928	2-4.288	<0.0001
RPL15 on TCTCs	2.134	1.446-3.15	<0.0001

CTCs, circulating tumor cells; TCTCs, total CTCs; RPL15, ribosomal protein L 15.

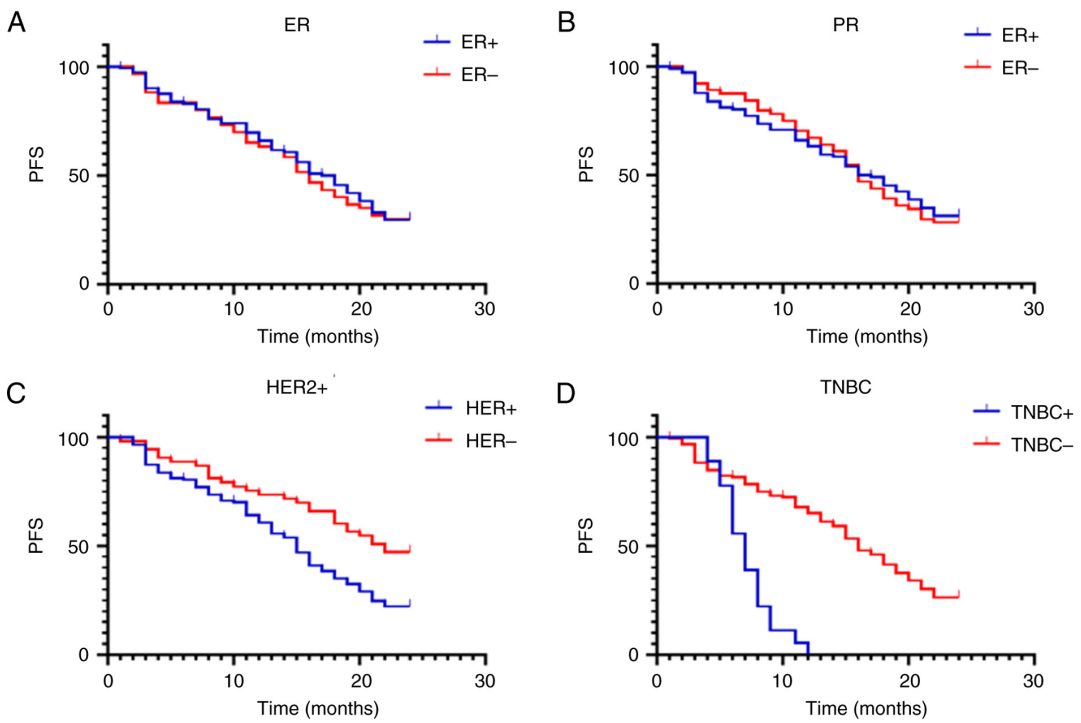

Figure 3. Survival curve of patient with breast cancer with different hormone receptor statuses. (A) PFS comparison of patients with positive and negative ER. (B) PFS comparison of patients with positive and negative PR. (C) PFS comparison of patients with positive and negative HER2. (D) PFS comparison of patients with and without triple-negative breast cancer. PFS, progression-free survival; ER, estrogen receptor; PR, progesterone receptor; HER2, human epithelial growth factor receptor2.

data revealed that high MCTCs, HCTCs, and positive *RPL15* expression on CTCs were critical risk factors for prognosis of patients with BC.

The PFS durations of patients with different HR expression levels was also compared (Fig. 3 and Table VI). The results identified that PFS duration of patients with positive

Table VI. Survival comparison of hormone receptor expression in patients with breast cancer.

Variables		95% confidence interval	P-value
Estrogen receptor-positive	0.9575	0.6558-1.398	0.8159
Progesterone receptor-positive	0.9578	0.6611-1.388	0.814
Human epithelial growth factor receptor 2-positive	1.929	1.33-2.797	0.0014
Triple-negative breast cancer	3.734	1.573-8.862	<0.0001

and negative ER (Fig. 3A) or PR (Fig. 3B) was not significant differences (P>0.05). By contrast, the PFS of patients with positive HER2 expression was significantly shorter than that of patients with negative HER2 (Fig. 3C; HR, 1.929; 95% CI, 1.33-2.797; P=0.0014). The PFS of patients with and without TNBC were also compared (Fig. 3D), and the following results were observed: HR, 3.734; 95% CI, 1.573-8.862; P<0.001 (Table VI).

Discussion

Numerous prior studies have indicated that CTCs are critical biomarkers for predicting tumor recurrence and metastasis. Notably, the results of the present study showed that patients with high MCTCs, HCTCs, *RPL15* positivity, and HER2 positivity had significantly poorer survival times than those without. In addition, the PFS of patients with TNBC was significanlty shorter than that of patients without TNBC.

BC is an exceedingly prevalent disease in women and can frequently recur (46,47). Chemotherapy is a critical therapeutic option for patients with advanced-stage cancers; however, the pathologic types of BC are very heterogeneous and occur in various locations (48). Most patients eventually experience relapse owing to drug resistance; therefore, there is an urgent need to identify sensitive and reliable biomarkers for predicting patient prognosis. Recently, CTCs have received increasing attention because they have the same features at the primary and metastatic sites (49), and CTC detection has been extensively used to predict the outcomes of numerous types of cancers (19,22,49,50). CanPatrol combined with RNA-ISH has been utilized to detect CTCs levels in patients with non-small cell lung cancer (NSCLC) and achieved 81.6% sensitivity and 86.8% specificity at 0.5 CTCs/5 ml cut-off (19). This technique has also been used to determine CTCs and programmed cell death-ligand 1 (PD-L1) expression in patients with NSCLC (50). The results indicated high levels of TCTCs, MCTCs and PD-L1 (+) CTCs levels in patients correlate with poor survival. The present data are consistent with these findings and indicate that patients with high levels of MCTCs and HCTCs had a shorter PFS. Prior results also suggest that the EMT is really involved in BC tumorigenesis, because MCTCs exhibit greater invasive ability (51).

Prior studies revealed that CTCs levels were positively correlated with cancer stage. Dong *et al* (50) revealed that OS of patients with NSCLC at stage III was significantly shorter that those at stage I-II. Wang *et al* (52) indicated that positive CTCs' detection was most likely to occur in patients with NSCLC at stage IV. Actually, the Food and Drug Administration (FDA) approved CellSearch® system use in clinical practice in 2004, which measured expression of CTCs in patients with monitor metastatic BC based on the results of Cristofanilli *et al* (23). The present data showed that CTCs and positive *RPL15* of patients at stage III were significantly higher than those at stage I-II, indicating that CTCs and *RPL15* arereally associated with BC stage. It was also identified that PFS of patients with high MCTCs (>2) and HCTCs (>5) was significantly shorter than those with low MCTCs (≤2) and HCTCs (≤5) (P<0.001), suggesting that MCTCs may play more crucial roles in EMT transition in patients with BC. In addition, some positive CTCs and *RPL15* were also found at early stage of BC, illustrating early CTCs and *RPL15* detection have great clinical significances. These results drive physicians to measure CTCs and *RPL15* expression and guide optimal treatments before BC treatments.

RPL15 is a subunit of the large ribosomal complex and is involved in rRNA processing (40). Previous studies showed that high *RPL15* levels are associated with a poor prognosis in esophageal cancer (53), skin squamous cell carcinoma (54) and pancreatic cancer (55). Furthermore, Shi and Liu (56) reported that elevated *RPL15* expression in patients with hepatocellular carcinoma was associated with shorter survival times. The present study showed that the PFS of patients with positive *RPL15* on CTCs was shorter (2.134-fold) than that of patients with negative *RPL15* expression on CTCs. This result further confirms that *RPL15* expression in CTCs is also a key biomarker for predicting prognosis of patients with BC. Other studies also revealed that *RPL15* is involved in antitumor immune activation (57,58), indicating that *RPL15* plays crucial roles in occurrence, progression and prognosis of cancer. Therefore, *RPL15* detection may have great benefits for guiding treatment of patients with BC, which encourages clinicians to routinely perform these tests to improve evaluation of the status of patients and predict their prognosis. If *RPL15* of patients is positive on CTCs, chemotherapy, immune therapy and hormone therapy should be performed as soon as possible after surgery because these patients have high relapse chances.

The relationship between HR expression and outcome in patients with BC has been extensively investigated (49,59,60), and HR expression has been found to strongly associated with the therapeutic methods used by patients (61,62). For example, $HER2^+$ patients with Enhertu treatment have significantly longer survival times than those treated with regular chemotherapy (61). The outcomes of BC with $HER2^+$ are poor because HER2 amplification is closely associated with HER2 protein overexpression, indicating breast cancer with $HER2^+$ had more invasive ability than those with $HER2^-$ (63). The present data indicate that $HER2^+$ expression before treatment strongly associates with the prognosis of patients with BC,

although ER^+ and PR^+ were not risk factors for recurrence and metastasis. The PFS of patients with $HER2^+$ was significantly shorter than those with HER2-, indicating that targeting HER2 has significant benefits on the outcomes of patients with BC. Therefore, if HER2 in BC will be detected before treatment, immediate Enhertu administration may prolong survival time of patients. In the present study, $HER2^+$ expression was detected before treatment, and targeting $HER2^+$ therapy was not adjusted for survival analysis. This may give rise to bias based on targeting $HER2^+$ treatment. Further analysis will compare patient survival rates in the patients with and without targeting $HER2^+$ therapy.

The present findings are noteworthy; however, the following observations and limitations should be noted when interpreting our results: i) CTC number was found to drive BC tumorigenesis; ii) *RPL15* expression was associated with the prognosis of patients with BC; iii) the sample size patient subtypes is small, which may have resulted in a selection bias; iv) the lack of validation of cellular or clinical samples; and v) sub-analysis of *RPL15* on TCTC was unclear. To overcome these limitations, the authors plan to recruit more patients and perform additional biological, molecular biology and biochemical experiments to explore CTC biology in patients with BC. The prognosis of patients with *RPL15*-combined MCTCs or HCTCs will be explored in the future to further understand biological significance of *RPL15*. The clinical significances of sub-analysis of *RPL15* on TCTC will undoubtfully have great benefits for BC survival.

In conclusion, the PFS of patients with >2 MCTC, >5HCTCs, and positive *RPL15*expression was shorter than that of those with ≤2 MCTCs, ≤5 HCTCs, and negative *RPL15* expression, and the prognosis of BC patients with negative HER2 expression was favorable, compared with that of patients with positive HER2 expression.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YZ and WZ contributed to manuscript drafting and the design of the study. YZ, SSL and WF carried out experiments. YZ, KS, SSL and HL contributed to the acquisition and analysis of the data. KS and WF confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was reviewed and approved (approval no. 2024-264) by the Ethical Committee of Hubei Cancer Hospital (Wuhan, China). Written informed consent was obtained from all patients before the study. The present study was conducted in accordance with the principles of Declaration of Helsinki (2013 version).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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