

## ORIGINAL ARTICLE

# Diagnostic and prognostic value of thymidylate synthase expression in breast cancer

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## Abstract

Nucleotide metabolism is the driving force of cell proliferation, and thymidylate synthase (TYMS) catalyzes a rate-limiting step in the initial synthesis of nucleotides. Previous studies reported that TYMS activity significantly affected the proliferation of tumour cells. However, the diagnostic and prognostic significance of TYMS expression in breast cancer remains unclear. Here, we used the Breast Cancer Integrative Platform (BCIP) to investigate the relationship between progression and prognosis of breast cancer with TYMS expression, and then verified the database analysis using immunohistochemical staining. Our results indicated TYMS expression was greater in breast cancer than adjacent normal tissues and greater in triple-negative breast cancer (TNBC) than non-TNBC tissues. TYMS expression also had significant positive correlations with histological grade, tumour size, and ER negativity, and PR negativity. The increased copy number of the *TYMS* gene appears to be the reason for its upregulation in breast cancer. Breast cancer patients with higher TYMS expression had poorer prognosis. Our data suggest that TYMS has potential use as a diagnostic and prognostic marker for breast cancer patients.

## KEYWORDS

breast cancer, diagnosis, prognosis, thymidylate synthase

## 1 | INTRODUCTION

Many fields of medical science, including basic research, clinical research, and drug development,<sup>1,2</sup> use bioinformatics and biological databases. These resources have become important means to evaluate clinical diagnoses and treatments.<sup>3-5</sup> The large amount of bioinformatics data greatly facilitates the accurate diagnosis of diseases and identification of treatment targets. The general purpose of the

present study was to use bioinformatics data to identify potential diagnostic and prognostic indicators of breast cancer.

Biologists have classically considered that nucleotides are required for a wide variety of biological processes. Increased nucleotide synthesis is necessary for DNA replication to support protein synthesis at different stages of the cell cycle. Thymidylate synthase (TYMS) is a rate-limiting enzyme in thymidylate biosynthesis. It transfers methylene folic acid from CH<sub>2</sub>H<sub>4</sub> and catalyzes the

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reductive methylation of 2'-deoxyuridine-monophosphate (dUMP), thereby producing deoxypyrimidine 5'-monophosphate (dTMP). dTMP is further phosphorylated to form a triphosphate (dTTP), one of four precursors used to synthesize DNA.<sup>6</sup> Thus, TYMS plays a crucial role in DNA synthesis.

The sustained proliferation of cancer cells is an important factor leading to the development of cancer. Cancer cells are highly dependent on the synthesis of nucleotides to produce DNA that is needed to support their rapid growth and proliferation.<sup>7,8</sup> Absence or blockage of nucleotides synthesis prevents cell proliferation and leads to cell death.<sup>9</sup> At present, studies on TYMS mainly focus on the cell level. However, the diagnostic and prognostic significance of TYMS in breast cancer still remains unclear. In this study, we first used the Breast Cancer Integrative Platform (BCIP), Xena from the University of California Santa Cruz (UCSC), and the UALCAN integrative platform to analyze the relationships between TYMS expression and the pathological features and survival rate from breast cancer. We then used tissue chips to verify the database results. Finally, we examined the expression of TYMS in breast cancer tissues and adjacent noncancerous breast tissues using immunohistochemical (IHC) staining and evaluated the use of TYMS level as a diagnostic and prognostic marker for breast cancer.

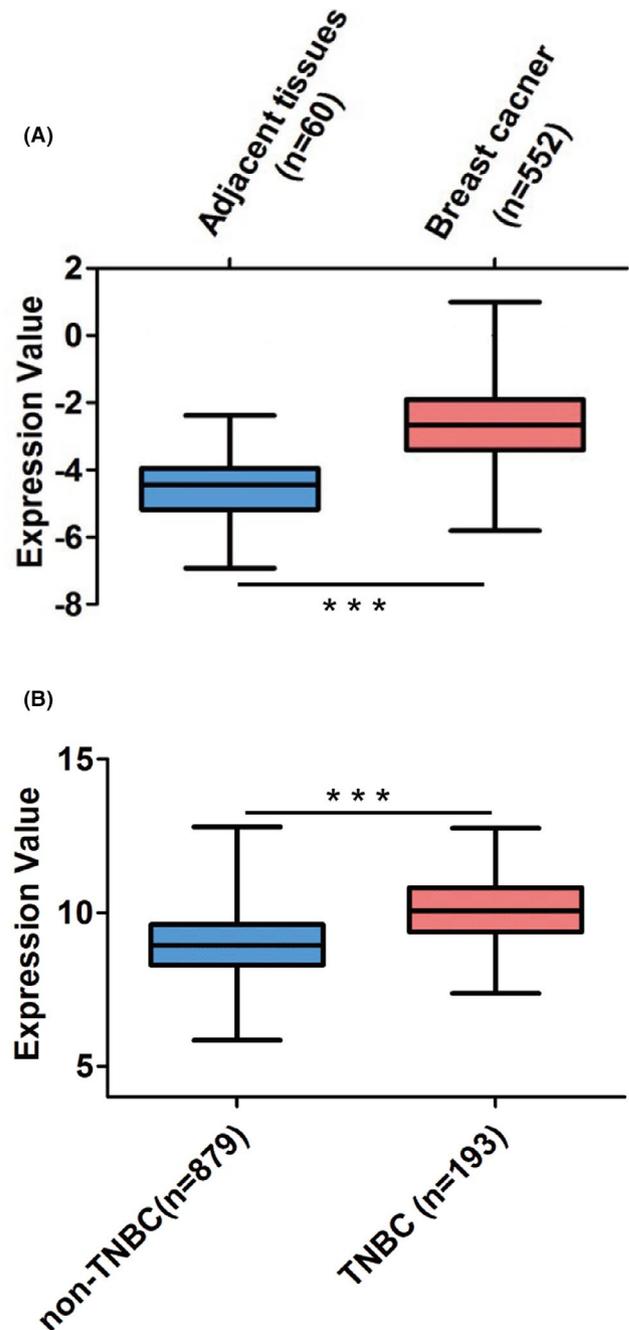
## 2 | RESULTS

### 2.1 | TYMS expression in breast cancer and triple-negative breast cancer

To determine the diagnostic role of TYMS in breast cancer, the expression of TYMS was analyzed by the large amount of bioinformatics data. We initially used the BCIP integrative platform to compare expression of TYMS mRNA in breast cancer ( $n = 552$ ) and adjacent normal tissues ( $n = 60$ ). The results indicated significantly higher levels of TYMS in breast cancer tissues ( $P < .001$ ) (Figure 1A). We then compared TYMS expression in triple-negative breast cancer (TNBC) and non-TNBC cancer tissues. The results indicated significantly higher TYMS expression in TNBC tissues ( $P < .001$ ) (Figure 1B).

### 2.2 | Association of TYMS expression with pathological features of breast cancer

To further investigate the relationship between TYMS expression and clinico-pathological parameters of breast cancer, we used the BCIP integrative platform to study the association of TYMS expression with the progression and pathological features of breast cancer. The results indicated significant positive correlations of TYMS expression with histological grade ( $P < .001$ ) and tumour size ( $P < .01$ ) (Figure 2A,B), but no association with metastasis (Figure 2C). Further analysis indicated that ER-negative and PR-negative breast cancer tissue had significantly higher TYMS expression than ER-positive

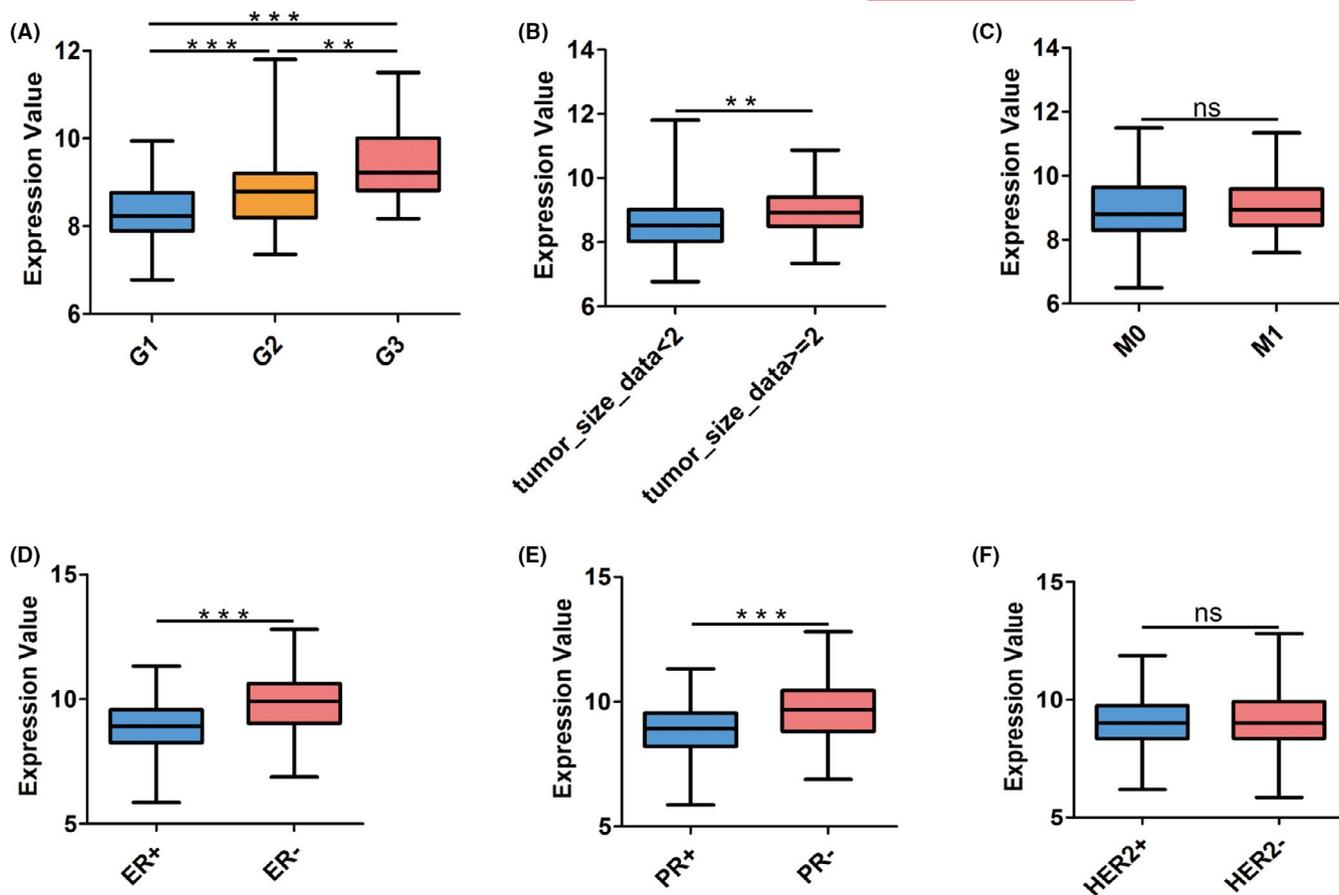


**FIGURE 1** A, Expression of thymidylate synthase (TYMS) mRNA in breast cancer and adjacent normal breast tissues ( $P < .0001$ ). B, Expression of TYMS mRNA in triple-negative breast cancer (TNBC) and non-TNBC tissues ( $P < .0001$ )

and PR-positive tissues (Figure 2D,E). However, TYMS expression was not significantly different in HER2-positive and HER2-negative tissues (Figure 2F).

### 2.3 | Association of TYMS expression with survival

To investigate the prognostic value of TYMS in breast cancer, we examined the relationship between TYMS expression with patient



**FIGURE 2** Association of thymidylate synthase (TYMS) mRNA expression with (A) breast cancer histological grade ( $P < .001$ ), (B) tumour size ( $P < .01$ ), (C) metastasis (NS), (D) ER status ( $P < .01$ ), (E) PR status ( $P < .01$ ), and (F) HER2 status (NS)

prognosis using the BCIP platform. The results showed that *TYMS* expression had a significantly negative correlation with overall survival (OS) (Figure 3A), disease-free survival (DFS) (Figure 3B), and recurrence-free survival (RFS) (Figure 3C) ( $P < .01$  for all). The results indicated that higher *TYMS* expression predicts poor prognosis of the breast cancer patient.

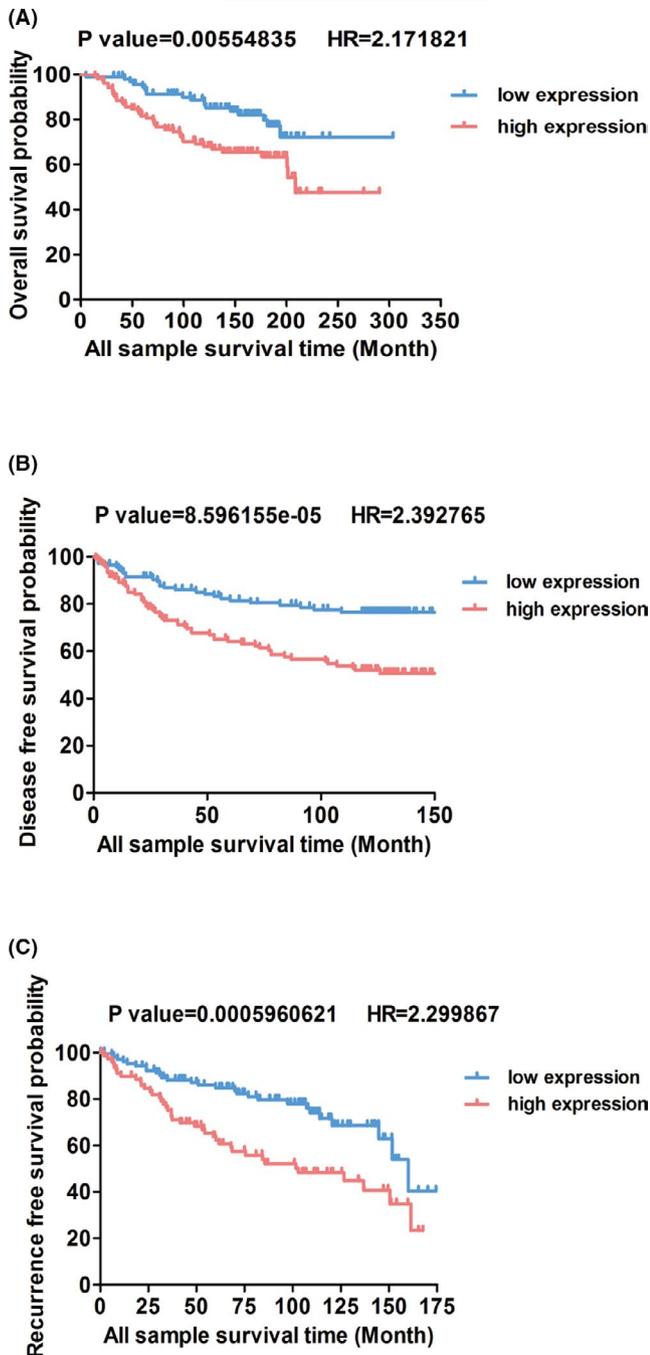
## 2.4 | Tissue microarrays and IHC of TYMS

Bioinformatics database analyses demonstrated that *TYMS* expression is associated with breast cancer progression and prognosis. Thus, we next used tissue microarray experiments with IHC to verify these results. The results confirmed that *TYMS* protein expression was significantly higher in breast cancer than adjacent normal tissues (Figure 4A) and also higher in TNBC than non-TNBC (Figure 4B). Moreover, *TYMS* expression had significantly negative correlations with ER positivity and PR positivity (Figure 4C,D). However, the expression of *TYMS* was unrelated to HER2 status (Figure 4F). Ki67 is a non-histone nuclear cortex protein, involved in the early steps of ribosomal RNA synthesis. IHC detection of the Ki67 has been used for many years to assess cancer proliferation. Therefore, we

investigated the relationships of *TYMS* to Ki67 in clinical breast cancer tissues. *TYMS* expression had significantly positive correlation with Ki67 expression (Figure 4E). Further analysis indicated that *TYMS* expression had a significantly negative association with OS (Figure 4G).

## 2.5 | Knockdown of TYMS inhibits the proliferation of breast cancer cells

To investigate the biological function of *TYMS* in breast cancer, we used shRNAs to knockdown the expression of *TYMS* in MDA-MB-231 cells and BT549 cells. As indicated in Figure 5A, the expression of *TYMS* protein levels was significantly reduced in MDA-MB-231 cells and BT549 cells after transfection of shTS. We examined the effect of shTS on the cell proliferation using the MTT assays. As shown in Figure 5B, as compared with the shCon cells, knockdown of *TYMS* significantly suppressed the proliferation of MDA-MB-231 and BT549 cells. Similarly, the results of clone formation assays also showed that knockdown of *TYMS* inhibited the proliferation of MDA-MB-231 and BT549 cells (Figure 5C).



**FIGURE 3** Association of thymidylate synthase (TYMS) mRNA expression with (A) overall survival (OS) of breast cancer patients, (B) disease-free survival (DFS) of breast cancer patients and (C) recurrence-free survival (RFS) of breast cancer patients

## 2.6 | Association of high TYMS expression with gene copy number in breast cancer

Both database and immunohistochemical results showed that TYMS expression was significantly higher in breast cancer than adjacent

normal tissues. Thus, we next investigated the possible cause of the elevated TYMS expression in breast cancer using the UCSC Xena and UALCAN. The heat map analysis indicated that breast cancer cell lines with high TYMS expression also had more copies of the TYMS gene (Figure 6A) and there was a significant positive correlation between TYMS expression and gene copy number in 46 different breast cancer lines (Figure 6B) ( $r = .6024$ ). However, TYMS expression was not associated with the extent of gene promoter methylation (Figure 6C).

## 3 | DISCUSSION

Thymidylate synthase is a key enzyme in DNA synthesis.<sup>10</sup> More specifically, TYMS promotes the synthesis of (dTTP) and plays an essential role in DNA replication and repair.<sup>11</sup> Accordingly, this reaction is a rate-limiting step for cell proliferation in numerous cancers, and overexpression of TYMS promotes the transformation of immortalized mammalian cells into malignant tumour cells.<sup>8,12,13</sup> However, the diagnostic and prognostic value of TYMS expression in breast cancer is unknown.

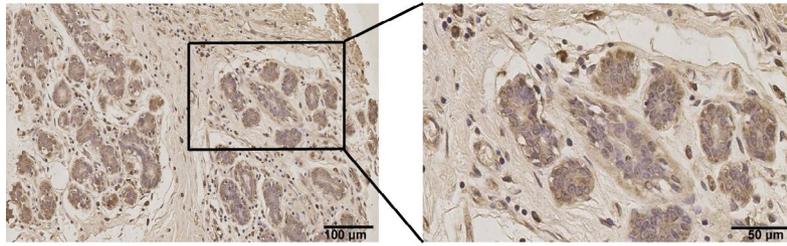
In the present work, our use of the BCIP integrative platform and our IHC analysis of tissue microarrays indicated higher TYMS expression in breast cancer than adjacent normal tissues, and higher expression in the TNBC subtype than the non-TNBC subtype. In addition, TYMS expression had positive correlations with ER negativity, PR negativity, advanced histological grade, tumour size, and Ki67 positivity. These results suggested that TYMS may act as a potential risk factor for disease breast progression. However, Yu-Hui Zhou and co-workers identified that the expression of TYMS was not significantly correlated with the pathological type of breast cancer ( $P = .095$ ).<sup>14</sup> In gastric cancer, TYMS expression was independent of clinical characteristics including differentiation degree, growth patterns, metastasis and TNM staging.<sup>15</sup>

Our further investigation of the prognostic value of TYMS in breast cancer using pooled database information indicated that higher TYMS expression in breast cancer correlated with worse OS, DFS, and RFS. Previous studies have reported that high expression of TYMS was an indicator of poor prognosis in prostate cancer,<sup>8,16,17</sup> non-small cell lung cancer,<sup>18-20</sup> and esophageal squamous cell carcinoma.<sup>21</sup> In contrast, high TYMS mRNA expression was associated with a significantly lower risk for relapse and death in patients with colorectal cancer.<sup>22</sup>

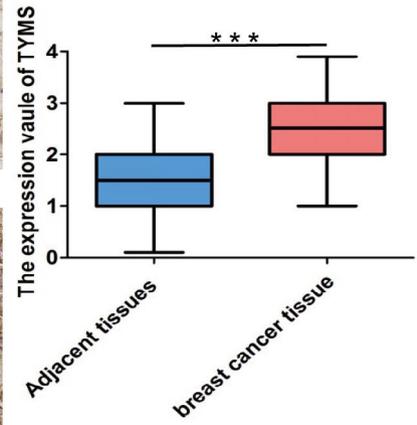
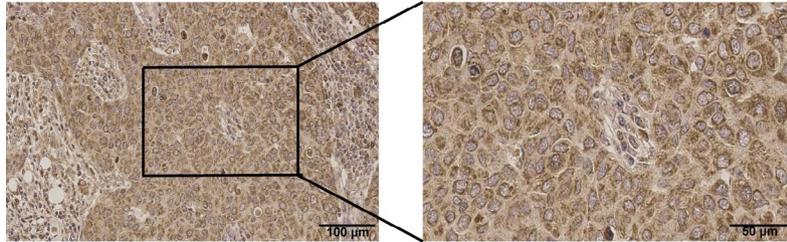
In conclusion, we found significant relationships of TYMS expression with clinical parameters and survival of breast cancer patients, thus suggesting that TYMS functions in the onset and progression of breast cancer. We suggest that TYMS might be useful as a diagnostic and prognostic indicator for breast cancer.

**FIGURE 4** Immunohistochemical analysis of thymidylate synthase (TYMS) protein expression in (A) breast cancer and adjacent normal breast tissues, (B) triple-negative breast cancer (TNBC) and non-TNBC breast cancer tissues. Association of TYMS protein expression with (C) ER status, (D) PR status, (E) Ki67 status and (F) HER2 status. (G) Association of TYMS protein expression with OS of breast cancer patients

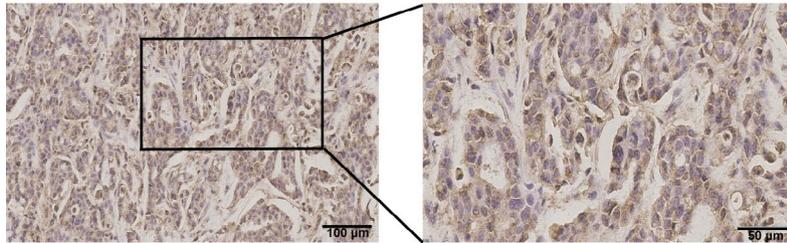
(A) Adjacent tissues of breast cancer



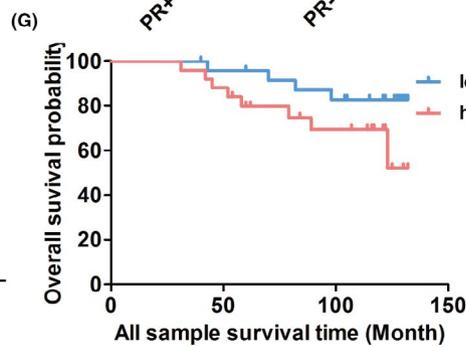
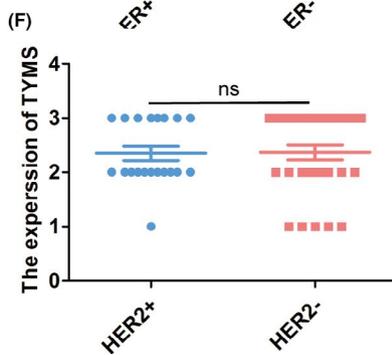
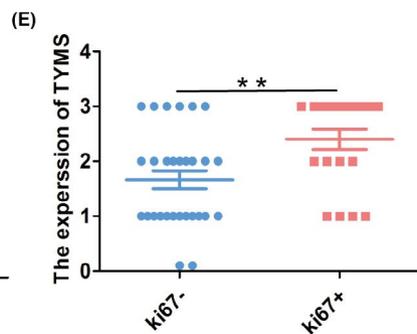
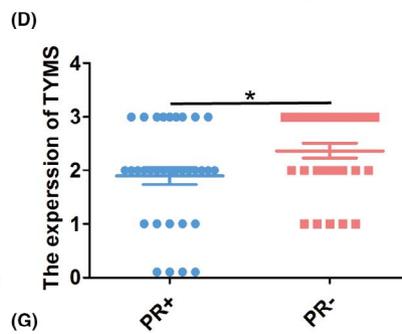
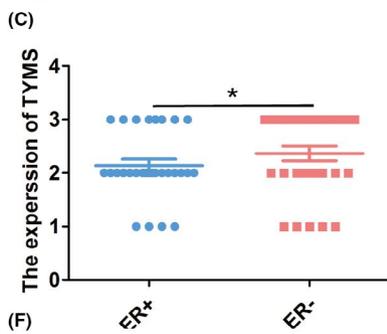
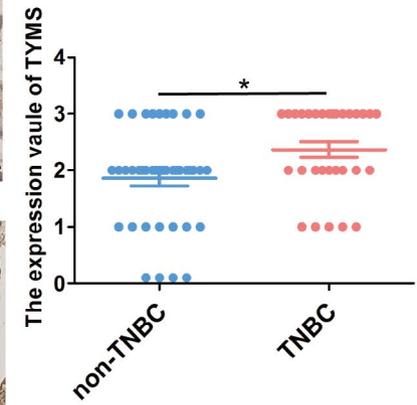
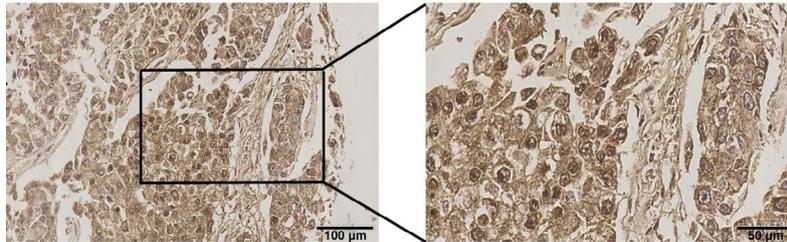
breast cancer tissue

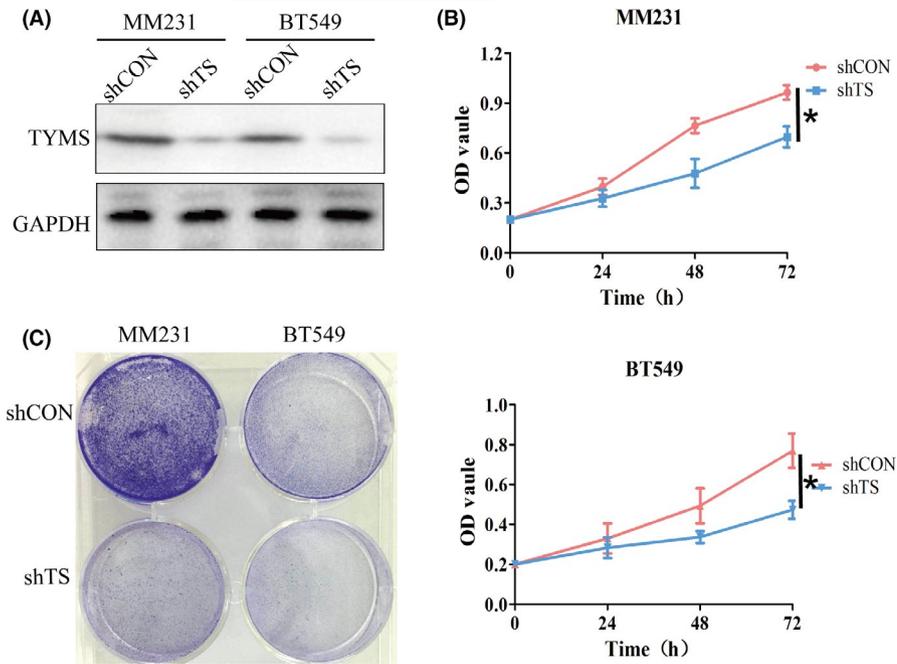


(B) non-TNBC breast cancer tissue



TNBC breast cancer tissue





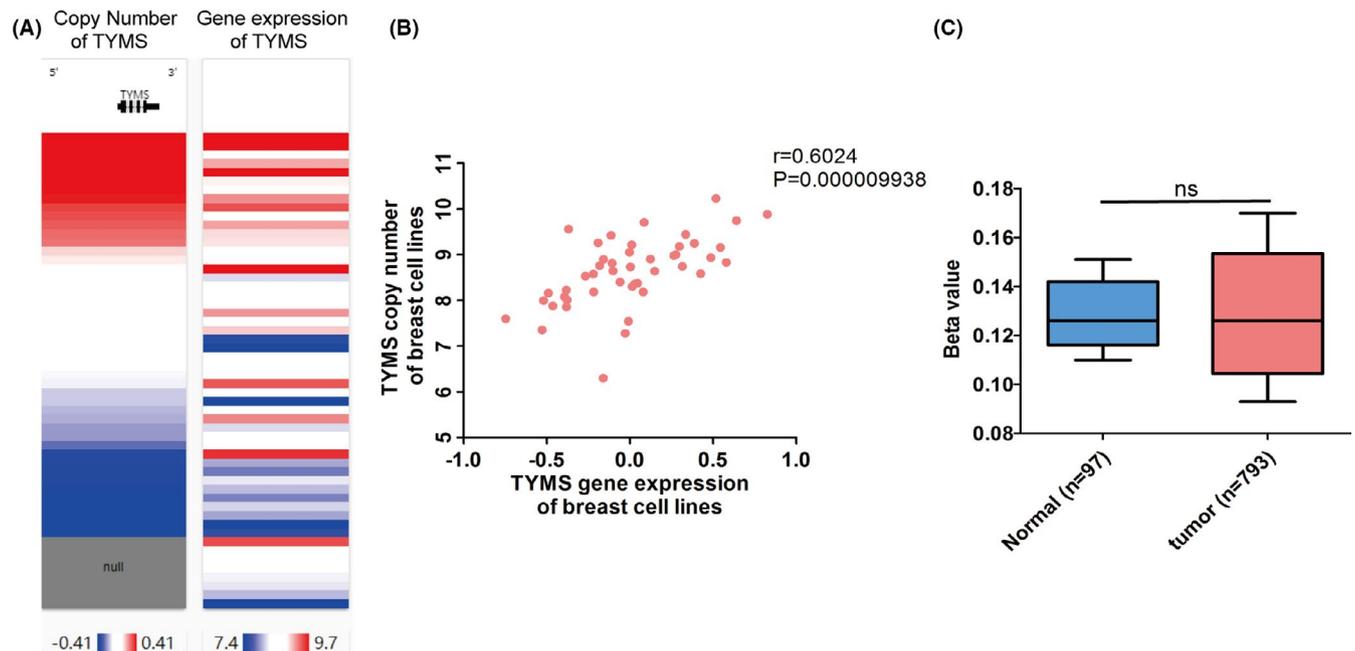
**FIGURE 5** Knockdown of thymidylate synthase (TYMS) inhibits the proliferation of breast cancer cells. A, TYMS silencing in MDA-MB-231 and BT-549 cells. B, TYMS silencing reduces the viability of breast cancer cells. C, TYMS silencing inhibits the clonogenicity of breast cancer cells

## 4 | MATERIALS AND METHODS

### 4.1 | BCIP integrative platform

The BCIP (<http://www.omicsnet.org/bcancer/>) is an integrative platform, which has multi-omics data and performs strict quality control using uniform normalization methods.<sup>23</sup> The BCIP has collected

data of 9005 breast tumours and 376 adjacent noncancerous breast tissues, which form the NCBI Gene Expression Omnibus (GEO).<sup>24</sup> The Cancer Genome Atlas (TCGA)<sup>25</sup> and the European Genome-phenome Archive (EGA) of the EMBL European Bioinformatics Institute (EMBL-EBI)<sup>26</sup> provide additional resources. This integrative platform provides flexible tools for analysis of gene expression, patient survival, pathologic stage, histological grade, metastasis status, ER/PR/HER2 status, tumour size, and prognosis.



**FIGURE 6** A, The relationship between thymidylate synthase (TYMS) expression and gene copy number is analyzed by heat map in 46 breast cancer cell lines. B, The correlation of TYMS expression with gene copy number is analyzed by Pearson's test. C, Methylation levels of TYMS genes in breast cancer and adjacent normal tissues

**TABLE 1** Clinicopathological characteristics of breast cancer patients

Parameter	No. of patients (%)
Age (years)	
<60	42 (60)
≥60	28 (40)
T-stage	
cT1	27 (39)
cT2	42 (60)
cT3	1 (1)
N-stage	
N0	36 (51)
N1	10 (14)
N2	19 (28)
N3	5 (7)
M-stage	
M0	70 (100)
M1	0 (0)
TNM phase	
I	16 (23)
II	31 (44)
III	23 (33)
ER status	
-	30 (51)
+	29 (49)
PR status	
-	30 (47)
+	34 (53)
HER2 status	
-	30 (60)
+	20 (40)
Ki67 status	
-	29 (59)
+	20 (41)

## 4.2 | UCSC Xena integrative platform

University of California Santa Cruz Xena (<https://xenabrowser.net/heatmap/>) is a functional genomics explorer and online data analysis and visualization platform that provides integrated analysis, visualization, and production of Galaxy data sets.<sup>27-29</sup> The existing 1098 common data sets of 91 queues, including TCGA, ICGC, TARGET, GTEx, and CCLE, are standardized. This integrative platform can display correlations of the expression of single and multiple genes, mutations, copy number variations, and sample attributes.

## 4.3 | UALCAN integrative platform

UALCAN (<http://ualcan.path.uab.edu/index.html>) is a simple, fast, and effective TCGA data mining and analysis integrative

platform<sup>30-32</sup> that is mainly based on TCGA data. It can help medical personnel to analyze biomarkers, gene expression profiles, and survival. It also provides links to related information in the database query.

## 4.4 | Tissue microarrays and patients

Tissue microarrays (HBreD077Su01 and HBreD140Su01) were obtained from the National Engineering Center for Biochip (Shanghai, China). A total of 70 breast cancer tissue samples and 45 adjacent noncancerous tissue samples were examined (Table 1). All patients underwent surgery between January 2005 and September 2012. The 70 breast cancer patients were followed up for 31 to 132 months (January 2005 to January 2016).

## 4.5 | IHC and scoring

Tissue sections were de-paraffinized, rehydrated, and subjected to antigen retrieval using 0.01 mol/L sodium citrate in a microwave. Sections were then blocked with 10% goat serum and incubated with a primary anti-TYMS antibody (1:200, 9045S; Cell Signaling Technology) at 4°C overnight. After washing, the bound antibodies were detected with a horseradish peroxidase (HRP)-conjugated secondary antibody, and stained with 3,3'-diaminobenzidine, followed by counterstaining with hematoxylin. All sections were visualized under a Leica microscope (SCN 400; Leica), and each IHC image was scored in three different fields. The TYMS expression score was assessed as the percentage of tumour cells with positive staining (0, <10%; 1, 10–40%; 2, 40–70%; or 3, >70%) and the intensity of staining was scored as 0 (negative), 1 (weakly positive), 2 (moderately positive), or 3 (strongly positive).

## 4.6 | Cells and transduction

Human breast cancer cells MDA-MB-231 and BT-549 were obtained from the American Type Culture Collection (ATCC). They were cultured in DMEM medium (Hyclone) supplemented with 10% FBS (Hyclone). All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. MDA-MB-231 and BT-549 cells were transduced with control lentivirus or lentivirus for expression of TYMS-shRNA (GeneChem).

## 4.7 | MTT and Colony formation assay

Cells ( $1 \times 10^4$  cells/well) were cultured in 96-well plates. During the last 4 hours culture, individual wells of cells were exposed to 20  $\mu$ L of MTT solution (20 mg/mL) and the generated formazan was dissolved in DMSO, followed by measuring absorbance at 490 nm in a microplate reader.

Cells ( $1 \times 10^4$  cells) were cultured in 6-well plates for 10 days. The cells were stained with 0.5% crystal violet solution. The numbers of colonies were counted in a blinded manner.

#### 4.8 | Statistical analysis

The differences among groups were determined using a two-tailed t-test and the differences between two groups were analyzed using the Wilcoxon signed-rank test. The survival of patients was analyzed using the Kaplan-Meier method, and differences were determined using the log-rank test. All statistical analyses were performed using GRAPHPAD PRISM (version 5). A *P*-value below .05 was considered statistically significant.

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#### PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1111/1440-1681.13415>.

#### DATA AVAILABILITY STATEMENT

The publicly available data sets used to support the findings of this study have been deposited in the BCIP, UCSC Xena, UALCAN integrative platform. The URL is <http://www.omicsnet.org/bcancer/>, <https://xenabrowser.net/heatmap/>, <http://ualcan.path.uab.edu/index.html>

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