Evaluations of biomarkers associated with sensitivity to 5-fluorouracil and taxanes for recurrent/advanced breast cancer patients treated with capecitabine-based first-line chemotherapy

Hong-Yun Zhao, He Huang, Zhi-Huang Hu, Yan Huang, Su-Xia Lin, Ying Tian and Tong-Yun Lin

The aim of the present study was to investigate the gene expression of biomarkers associated with the sensitivity to fluoropyrimidine and taxanes in recurrent/advanced breast cancer patients treated with first-line capecitabine chemotherapy. We evaluated the clinicopathological/prognostic significance of thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD), thymidine phosphorylase (TP), class III β-tubulin (βIII-tubulin), and stathmin-1 or oncoprotein-18 (STMN1). Formalin-fixed, paraffin-embedded tumor specimens from 42 patients were used for analysis of TS, DPD, TP, βIII–tubulin, and STMN1 expression with a real-time reverse transcription-PCR technique. Patients were classified into the high-expression and low-expression groups according to the median value of the expression level of each biomarker. There was a significantly longer time to progression (TTP) in the high-TP group ($P=0.018$). The multivariate analysis revealed that the TP expression (hazard ratio for the low-TP group vs. the high-TP group, 2.873; 95% confidence interval, 1.143–7.223; $P=0.025$) is independent of prognostic factors for TTP. In the subgroup of patients treated with capecitabine plus taxanes as first-line chemotherapy, TTP was significantly longer in the low-βIII-tubulin group ($P=0.047$). The gene expression of TS, DPD, and STMN1 failed to have any significant impact on the outcome. These results provide further evidence that the TP expression may be a prognostic factor in breast cancer patients treated with capecitabine-based first-line chemotherapy, and βIII-tubulin can be used to predict the outcome of capecitabine in combination with taxanes as first-line chemotherapy. Therefore, these potential biomarkers should be further evaluated. *Anti-Cancer Drugs* 23:534–542 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: capecitabine, class III β-tubulin, dihydropyrimidine dehydrogenase, first-line chemotherapy, recurrent/advanced breast cancer, stathmin-1 or oncoprotein-18, taxanes, thymidine phosphorylase, thymidylate synthase

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Introduction

Breast cancer (BC) is the most common female cancer and the second most common cause of cancer-related death worldwide. Approximately 6–10% of patients have metastatic disease at the time of diagnosis and around 30% of patients initially diagnosed with early-stage BC will eventually suffer a recurrence [1]. The pyrimidine antimetabolic agent 5-fluorouracil (5-FU) has been used for nearly 50 years in both adjuvant and advanced disease settings for BC [2]. An oral fluoropyrimidine derivative, capecitabine, has been designed to generate 5-FU preferentially in tumor tissues and to mimic continuous infusion of 5-FU. Recent clinical studies have shown that capecitabine in combination with other cytotoxic agent(s) as first-line treatment was highly effective in treating locally advanced and/or metastatic BC [3–6]. Moreover, new cytotoxic agents, such as taxanes, have also improved the treatment of advanced BC. Taxanes-based therapy has become a standard of care for patients in whom anthracyclines have failed [7].

Despite the effective chemotherapy combinations in the first-line treatment with a response rate (RR) of approximately 50–70% and a median duration of 8–16 months [8,9], and the fact that long-term remissions are possible in individual cases today, the majority of patients displayed varying levels of resistance and could not maintain the response from the first-line treatment. Because an increasing number of patients are being treated with capecitabine and/or taxanes in the first-line metastatic setting, the development of resistance is a clinically important problem. There is a substantial need to understand the refractory mechanisms of these drugs and to implement novel strategies for overcoming resistance. Therefore, it is crucial to find reliable predictive markers of response to and prognosis for the effects of our backbone first-line regimen, so that we can improve the clinical outcome and avoid unnecessary toxicity in the future.

Inhibition of thymidylate synthase (TS) by the 5-FU metabolite fluorodeoxyuridine monophosphate has been

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identified as the major mechanism of 5-FU action [10]. Dehydropyrimidine dehydrogenase (DPD) is the first, rate-limiting, enzyme for the catabolism of 5-FU. The nucleoside cleavage enzyme, thymidine phosphorylase (TP), is involved in the conversion of capecitabine to 5-FU *in vivo*. Many previous reports have shown the relationships between 5-FU-related enzyme expression and the prognostic effects of fluorouracil derivatives on colorectal cancer, gastric cancer, and lung cancer [11–14]. The tubulin superfamily is a large group of molecules that play a complex role in the formation of cytoskeleton. One of these molecules, class III β-tubulin (βIII-tubulin), is a 50 kDa protein that participates in the formation of microtubules [15]. Overexpression of βIII-tubulin correlates with the resistance to microtubule-acting chemotherapeutics in some malignant tumors [16–18]. Stathmin-1 or oncoprotein-18 (STMN1) is a p53-regulated protein known to influence microtubule dynamics by participating in the control of microtubule polymerization, which can affect the binding of antimicrotubule drugs. STMN1 expression has been found in many human cancers, including BC, and has been associated with clinical outcome in breast and liver cancers [19–21]. *In vitro*, overexpression of STMN1 can decrease the sensitivity of BC cells to paclitaxel and vinblastine [22].

On the basis of previous results, these proteins may be used as potential predictive markers of first-line treatment for recurrent/advanced BC. However, there are only a few studies in BC about these biomarkers associated with 5-FU and taxane sensitivity. Furthermore, it still remains controversial as to which marker predicts the efficacy and prognosis of fluorouracil derivatives and/or taxanes for first-line chemotherapy.

Therefore, we conducted a retrospective clinical study to identify molecular markers that are potentially useful for the outcome of capecitabine-based first-line chemotherapy in patients with recurrent/advanced BC. Using the real-time reverse transcription-PCR method, we evaluated the gene expressions of five biological markers: TS, DPD, TP, βIII-tubulin, and STMN1. We further investigated the relationship between the gene expression of these five biomarkers and the clinicopathological/prognostic significance in BC.

Materials and methods

Patients

This is a retrospective analysis of 42 cases of metastatic BC patients treated with capecitabine-based first-line chemotherapy between May 2002 and March 2006 at the Cancer Center, Sun Yat-Sen University. Tumor tissue samples and follow-up data were available for all included cases. Patients with a history of malignant diseases and who had been previously treated with first-line chemotherapy were excluded. The study was approved by the Medical Ethics Committee of Sun Yat-Sen University.

Table 1 Patients' characteristics

Patients' characteristics	*N* (%)
Age at diagnosis (years)	Median 46 (range 29–64)
Patients' disease status before first-line chemotherapy	
Recurrent	39 (92.9)
Advanced	3 (7.1%)
Ki67	
0–+	13 (31.0)
+ + –+ + +	14 (33.3)
Unknown	15 (35.7)
P53	
Negative	8 (19.1)
Positive	19 (45.2)
Unknown	15 (35.7)
Adjuvant chemotherapy ($n=39$)	
Yes	38 (97.4)
CT	16 (41.0)
HT + CT	22 (56.4)
No	1 (2.6)
Anthracycline in adjuvant setting ($n=38$)	
Yes	35 (92.1)
No	3 (7.9)
First-line chemotherapy	
Capecitabine	1 (2.4)
Capecitabine plus gemcitabine	12 (28.6)
Capecitabine plus vinorelbine	5 (11.9)
Capecitabine plus taxanes	24 (57.1)
Capecitabine plus docetaxel	22 (91.7)
Capecitabine plus paclitaxel	2 (8.3)
Capecitabine dose reduction	
Yes	10 (23.8)
No	32 (76.2)

CT, chemotherapy; HT, hormonal therapy.

These patients comprised 42 advanced or recurrent female BC patients with a median age of 46 years (29–64 years) (Table 1).

Microdissection in primary tumors

A formalin-fixed, paraffin-embedded (FFPE) tumor specimen from each lesion was selected by a pathologist (S.X.L.) after examination on hematoxylin and eosin staining.

Preparation of cyclic DNA and the detection of quantitative mRNA expression of thymidylate synthase/thymidine phosphorylase/dihydropyrimidine dehydrogenase

RNA was extracted using a QIAGEN FFPE Tissue Kit (Cat No. 74404; Qiagen, Shanghai, China) according to the user manual. cDNA was derived from each sample according to the method described previously [23]. The 20 μl PCR reaction system consisted of 0.2 μl reverse transcription primers (50 μm/μl), 0.5 μl deoxyribonucleotide triphosphate (25 mmol/l/μl), 0.5 μl Taq enzyme (10 U/μl), 8.8 μl $MgCl_2$ (25 mmol/l), and 10 μl buffer A (pH 8.0). The PCR protocol was as follows: 95°C for 5 min, and 42°C for 1 h, followed by incubation on ice. The quantitative measurement of TS, TP, DPD, and the internal reference β-action was carried out using real-time reverse transcription-PCR with Stratagene MX3000P (Santa Clara, California, USA). The 25 μl PCR reaction system consisted of 10 μmol/l of primers and probes each, 1 U Taq enzyme, 200 nmol/l

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deoxyribonucleotide triphosphate, 0.25 mmol/l $MgCl_2$, and 5 × buffer (pH 7.5). (All the detection kits were kindly provided by Amoy Diagnositcs Co. Ltd, Xiamen, China.) The PCR protocol was as follows: 94°C for 5 min; 94°C for 15 s; 60°C for 20 s; 72°C for 20 s, 10 cycles; 94°C for 15 s; 58°C for 35 s; and 72°C for 15 s. The sequences of primers and probes are shown in Table 2.

mRNA expression of class III β-tubulin and stathmin-1 or oncoprotein-18

Firstly, the FFPE tissue samples were homogenized in a mixture of homogenizing solution at 65°C for 2 h. The homogenate was centrifuged to remove residual paraffin and debris, and then the supernatant was transferred to a fresh microcentrifuge tube. Homogenate (40 µl) was added to each well of a 96-well plate that contained the following reagents: 18.5 µl RNase-free water, 33.3 µl lysis solution, 2 µl blocking reagent, 1 µl capture beads, and 5 µl target-gene-specific (βIII-tubulin and STMN1) probe set (SP/SE/AE probe, Table 3). The plate was sealed and incubated at 54°C for 18 h on a shaker with 750 rpm. The hybridization mixture was then transferred to a filtered 96-well plate. The unbound RNA and other debris in the wells were removed by washing three times with 250 µl wash buffer (0.1 × saline sodium citrate and 0.03% lithium lauryl sulfate) under a vacuum system. Signals for bound target mRNA were developed using the following steps: (a) incubate in 100 µl preamplifier solution at 50°C for 1 h; (b) wash twice with 200 µl wash buffer; (c) incubate in 100 µl amplifier solution at 50°C

Table 2 Sequences of primers and probes

Target gene	Forward primers	Reverse primers	Probes
TS	GCGCTACAGCCTGAGAGA	CTCTTTAGCATTTGTGGATCCCTT	FAM-CGCCCTCTGCTGACAACCAAACGTGTGAGGGCG-Dabcyl
TP	CATGTGGCTGCAAGGTGC	CAGCAGCACTTGCATCTGC	FAM-5′-TGCCCCGGACGTGGTCTGGGGCA-3′-Dabcyl
DPD	CCAAAACTTTCTCTCTTGATAAGGAC	AATGCTAGCAATCACAATGTTGTC	FAM-5′-CCCCCAGAATCATCCGGGGG-3′-Dabcyl
β-Actin	ATTGCCGACAGGATGCAGA	CAGGAGGAGCAATGATCTTGAT	FAM-5′-CTGCCCTGGCACCCAGCACAATGGGCAG-3′-Dabcyl

Reverse primers represent reverse transcription primers.
DPD, dihydropyrimidine dehydrogenase; TP, thymidine phosphorylase; TS, thymidylate synthase.

Table 3 Sequences of probes specific for class III β-tubulin and stathmin-1 or oncoprotein-18 genes

Target gene	SP sequences (5′→3′) NH_2 + poly(dT) + P1
Support probes (SP)	
βIII-tubulin	5′NH_2-TTTTTTTT – CTCAAATACTCAAATC
STMN1	5′NH_2-TTTTTTTT – TTCTATATCAACATCT
	SE sequences (5′→3′) P2 + poly(dT) + P3
Support extend probes (SE)	
βIII-tubulin	CCGAGTCGCCCACGTAGTTG TTTTT GATTTGAGTATTTGAG
	TTCCGGGTTCCAGGTCCA TTTTT GATTTGAGTATTTGAG
	AGTTGTTGCCGGCCCCA TTTTT GATTTGAGTATTTGAG
	GCAGTTTTCACACTCCTTCCGC TTTTT GATTTGAGTATTTGAG
	AACGTGCCCATGCCGGAG TTTTT GATTTGAGTATTTGAG
STMN1	CACGCTTCTCCAGTTCTTTCACC TTTTT AGATGTTGATATAGAA
	GGGAAAGGGGGAATTCTGGA TTTTT AGATGTTGATATAGAA
	ACTTCTTTCTCGTGCTCTCGTTTC TTTTT AGATGTTGATATAGAA
	CCTCTCGGTTCTCTTTATTAGCTTCC TTTTT AGATGTTGATATAGAA
	CTTTGTTCTTCCGCACTTCTTCA TTTTT AGATGTTGATATAGAA
	AE sequences (5′→3′) P4 + poly(dT) + P5
Amplification extend probes (AE)	
βIII-tubulin	CCAGAACTTGGCCCCGATC TTTTT CCTATGCCTCCCGTGTCTA
	GGTCGATGCCATGCTCATCAC TTTTT CCTATGCCTCCCGTGTCTA
	TAGACGCTGATCCGCTCCAG TTTTT CCTATGCCTCCCGTGTCTA
	GGCACGTACTTGTGAGAAGAGGC TTTTT CCTATGCCTCCCGTGTCTA
	GCCCCTGAGCGGACACTG TTTTT CCTATGCCTCCCGTGTCTA
	CTGACCAAAGATGAAATTGTCAGG TTTTT CCTATGCCTCCCGTGTCTA
	CCTCCGTGTAGTGACCCTTGG TTTTT CCTATGCCTCCCGTGTCTA
	CACATCCAGGACCGAATCCAC TTTTT CCTATGCCTCCCGTGTCTA
	AGCGAGTGGGTCAGCTGGAAG TTTTT CCTATGCCTCCCGTGTCTA
	GGGATACTCCTCACGCACCTTG TTTTT CCTATGCCTCCCGTGTCTA
STMN1	ATCAGCTCAAAAGCCTGGCCT TTTTT CCTATGCCTCCCGTGTCTA
	GATTCTTTTGACCGAGGGCTG TTTTT CCTATGCCTCCCGTGTCTA
	TGCGTCTTTCTTCTGCAGCTTC TTTTT CCTATGCCTCCCGTGTCTA
	TCAAGACCTCAGCTTCATGGGA TTTTT CCTATGCCTCCCGTGTCTA
	TTGTTGTTCTCTTCTATTGCCTTCTG TTTTT CCTATGCCTCCCGTGTCTA
	GGGTCAGTTTCTCTTCTGCCATTTT TTTTT CCTATGCCTCCCGTGTCTA
	TCCAGTTTGGCAGCCATTTG TTTTT CCTATGCCTCCCGTGTCTA
	GCTTATCCTTCTCTCGCAAACG TTTTT CCTATGCCTCCCGTGTCTA
	TCTCGTCAGCAGGGTCTTTGG TTTTT CCTATGCCTCCCGTGTCTA
	AGGAAGGGGATGGGGAGAAAGT TTTTT CCTATGCCTCCCGTGTCTA

βIII-tubulin, class III β-tubulin; STMN1, stathmin-1 or oncoprotein-18.

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Table 4 Comparison of clinicopathological factors for high and low levels of TS, DPD, and TP mRNA expression

Variables	Low TS (*n*)	High TS (*n*)	*P*-value	Low DPD (*n*)	High DPD (*n*)	*P*-value	Low TP (*n*)	High TP (*n*)	*P*-value
Menses status									
Pre/postmenopausal	12/9	13/8	0.753	11/10	14/7	0.346	10/11	15/6	0.116
Lymphoma status									
0–3/ ≥ 4	11/10	10/11	0.758	11/10	10/11	0.758	9/12	12/9	0.355
Tumor size at diagnosis									
<2 cm/ ≥ 2 cm/unknown	4/17/0	4/14/3	0.193	4/15/2	4/16/1	0.833	4/16/1	4/15/2	0.833
Estrogen receptor									
Negative/positive	12/9	11/10	0.537	10/11	12/9	0.537	12/9	10/11	0.537
Progesterone receptor									
Negative/positive	7/14	10/11	0.346	9/12	8/13	0.753	9/12	8/13	0.753
HER-2									
0–1/2̃3	4/17	17/4	<0.0001	7/14	14/7	0.031	8/13	13/8	0.123
Visceral metastasis at the beginning of capecitabine treatment									
Yes/no	11/10	10/11	0.758	8/13	13/8	0.123	10/11	11/10	0.758
Best response from first line									
CR + PR	12	11	0.828	8	15	0.095	9	14	0.298
SD	8	8	–	11	5	–	10	6	–
PD	1	2	–	2	1	–	2	1	–

CR, complete response; DPD, dihydropyrimidine dehydrogenase; HER-2, human epidermal growth factor receptor-2; PD, progressive disease; PR, partial response; TP, thymidine phosphorylase; TS, thymidylate synthase.

Table 5 Comparison of clinicopathological factors for high and low levels of class III β-tubulin and stathmin-1 or oncoprotein-18 mRNA expression

Variables	Low βIII-tubulin (*n*)	High βIII-tubulin (*n*)	*P*-value	Low STMN1 (*n*)	High STMN1 (*n*)	*P*-value
Menses status						
Pre/postmenopausal	13/8	12/9	0.753	12/9	13/8	0.753
Lymphoma status						
0–3/ ≥ 4	9/12	12/9	0.355	10/11	11/10	0.758
Tumor size at diagnosis						
<2 cm/ ≥ 2 cm/unknown	2/17/2	6/14/1	0.269	4/15/2	4/16/1	0.833
Estrogen receptor						
Negative/positive	13/8	9/12	0.217	12/9	10/11	0.537
Progesterone receptor						
Negative/positive	8/13	9/12	0.753	7/14	10/11	0.346
HER-2						
0–1/2–3	13/8	8/13	0.123	10/11	11/10	0.758
Visceral metastasis at the beginning of capecitabine treatment						
Yes/no	12/9	9/12	0.355	12/9	9/12	0.355
Best response from first line						
CR + PR	12	11	0.132	12	11	0.132
SD	6	10	–	6	10	–
PD	3	0	–	3	0	–

βIII-tubulin, class III β-tubulin; CR, complete response; HER-2, human epidermal growth factor receptor-2; PD, progressive disease; PR, partial response; STMN1, stathmin-1 or oncoprotein-18.

for 1 h; (d) wash twice with 200 µl wash buffer; (e) incubate in 100 µl labeled probe at 50°C for 1 h; and (f) wash twice with 200 µl wash buffer. The samples were then developed with 100 µl streptavidin–phycoerythrin solution at 50°C for 30 min. The fluorescence value of each sample was analyzed using the Luminex 200 system (Luminex, Austin, Texas, USA).

Clinicopathological variables

The clinicopathological variables in our study are some clinical and pathological characteristics of primary tumor and adjuvant therapy, including menses status at diagnosis, lymphoma status, tumor size at diagnosis, estrogen receptor, progesterone receptor, human epidermal growth factor receptor-2 (HER-2), ki67, p53, adjuvant therapy, visceral metastasis at the beginning of capecitabine treatment, and response to first-line chemotherapy. Some of these variables were stratified by TS, DPD, TP, βIII-tubulin, and STMN1 mRNA expression separately, as listed in Tables 4 and 5.

Treatment

All patients were treated with capecitabine (Xeloda; Hoffmann-La Roche, Nutley, New Jersey, USA) alone or in combination with other chemotherapeutic agents (Table 1). The drug administration protocols were as follows: capecitabine, 1500 mg/m^2/day, days 1–14, every 21 days; docetaxel, 75 mg/m^2/day, day 1, every 21 days; paclitaxel, 175 mg/m^2/day, day 1, every 21 days; gemcitabine, 1000 mg/m^2/day, day 1 and 8, every 21 days; vinorelbine, 25 mg/m^2/day, day 1 and 8, every 21 days. The usage of these drugs was consistent with NCCN breast cancer Clinical Practice Guidelines 2007. Dose reductions were applied if clinically indicated.

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Parameters of capecitabine-based first-line treatment efficacy

Treatment activity (RR) was evaluated according to Response Evaluation Criteria In Solid Tumors [24]. Tumor response was assessed at baseline and every two cycles of treatment with capecitabine-based first-line chemotherapy.

Time to progression (TTP) was defined as the period from the date of capecitabine-based first-line chemotherapy to the date of disease progression. If no disease progression was observed by the end of the follow-up, the time interval was defined as the period from the capecitabine-based chemotherapy to the last time of tumor evaluation. Overall survival (OS) was defined as the period from the date of capecitabine-based first-line chemotherapy to death or last follow-up. The end date of the follow-up study was 20 October 2010. The median follow-up was 20.7 months (range: 2.57–68.7 months).

Statistical analysis

SPSS 16.0 software (SPSS Inc., Chicago, Illinois, USA) was used for data analysis. The chi-square test was used to assess the potential association between mRNA levels of TS, TP, DPD, βIII-tubulin, and STMN1 and the categorical clinicopathological parameters. Univariate analyses using the Kaplan–Meier method and log-rank test were carried out by comparing the TTP/OS of some of the clinical variables and the five biomarkers. The Cox proportional hazards regression model was used to determine the independent prognostic value. A P value of less than 0.05 was considered statistically significant.

Results

Forty-two patients received at least one cycle of capecitabine-based chemotherapy as first-line treatment, and the median number of cycles received was four (range 1–6). Tumor and previous/current treatment characteristics are summarized in Table 1.

Distribution of biomarkers in breast cancer

According to the median values of mRNA levels of the five biomarkers, all patients were classified into the high-expression (≥ median value) and low-expression (< median value) groups. The median values of 2.74 (0–25.7) for TS, 0.028 (0–1.26) for DPD, 1.51 (0.0127–16.1) for TP, 0.142 (0.30–2.77) for βIII-tubulin, and 2.65 (0–9.69) for STMN1 in 42 cases were selected as the cut-off levels to separate the high and low mRNA expression levels.

Baseline clinicopathological parameters and mRNA levels of the five biomarkers

Within the entire population, the overall RR was 54.8% (95% confidence interval (CI), 40–70%). We found that the high-TS group and the high-DPD group were significantly associated with BC with HER-2 (0–1) ($P < 0.0001$, $= 0.031$, respectively) but not with other clinicopathological factors or best response from first-line chemotherapy, although the high-DPD group tended to include more patients with complete response and partial response response ($P = 0.095$) than the low-DPD group. In contrast, there was no significant difference in baseline clinicopathological parameters or best response between groups of different expression levels of TP, βIII-tubulin, and STMN1 (Tables 4 and 5).

Time to progression and overall survival

Among the entire population, the median TTP was 8.47 months (range 1.33–68.7) and the median OS was 20.7 months (range 2.57–68.7). The univariate analysis revealed that the high-TP group had a longer TTP (a median value of 25.8 months) than the low-TP group (a median value of 8.3 months, $P = 0.018$; Table 6, Fig. 1a). At the beginning of first-line chemotherapy, patients with nonvisceral metastasis presented a longer TTP than those with visceral metastasis ($P = 0.010$, Table 6). However, these two variables were not significantly associated with OS, although patients with visceral metastasis at the beginning of first-line chemotherapy had a decreased OS compared with patients with nonvisceral metastasis ($P = 0.070$, Table 6). Patients treated with capecitabine plus taxanes had a longer TTP and OS than patients treated with capecitabine plus nontaxanes ($P = 0.032$, $= 0.030$, respectively; Table 6). No association was revealed between TTP/OS and other clinical variables including ER, PgR, or HER-2 status (data not shown). In addition, there was no significant difference for TTP and OS among other groups of different expression levels of TS, DPD, βIII-tubulin, and

Table 6 Univariate analysis of time to progression and overall survival based on biomarkers

Variables	TTP (1 year)	Log-rank *P*-value	OS (2 years)	Log-rank *P*-value
Visceral metastasis				
No	78	–	68	–
Yes	36	0.010	49	0.071
First-line chemoregimen				
Capecitabine plus taxanes	70	–	67	–
Capecitabine plus nontaxanes	38	0.032	42	0.030
TS expression				
Low TS	55	–	56	–
High TS	59	0.722	58	0.305
DPD expression				
Low DPD	43	–	56	–
High DPD	68	0.079	59	0.315
TP expression				
Low TP	42	–	57	–
High TP	69	0.018	58	0.192
βIII-tubulin expression				
Low βIII-tubulin	65	–	64	–
High βIII-tubulin	46	0.098	53	0.478
STMN1 expression				
Low STMN1	62	–	57	–
High STMN1	50	0.892	59	0.983

βIII-tubulin, class III β-tubulin; DPD, dihydropyrimidine dehydrogenase; OS, overall survival; STMN1, stathmin-1 or oncoprotein-18; TP, thymidine phosphorylase; TS, thymidylate synthase; TTP, time to progression.

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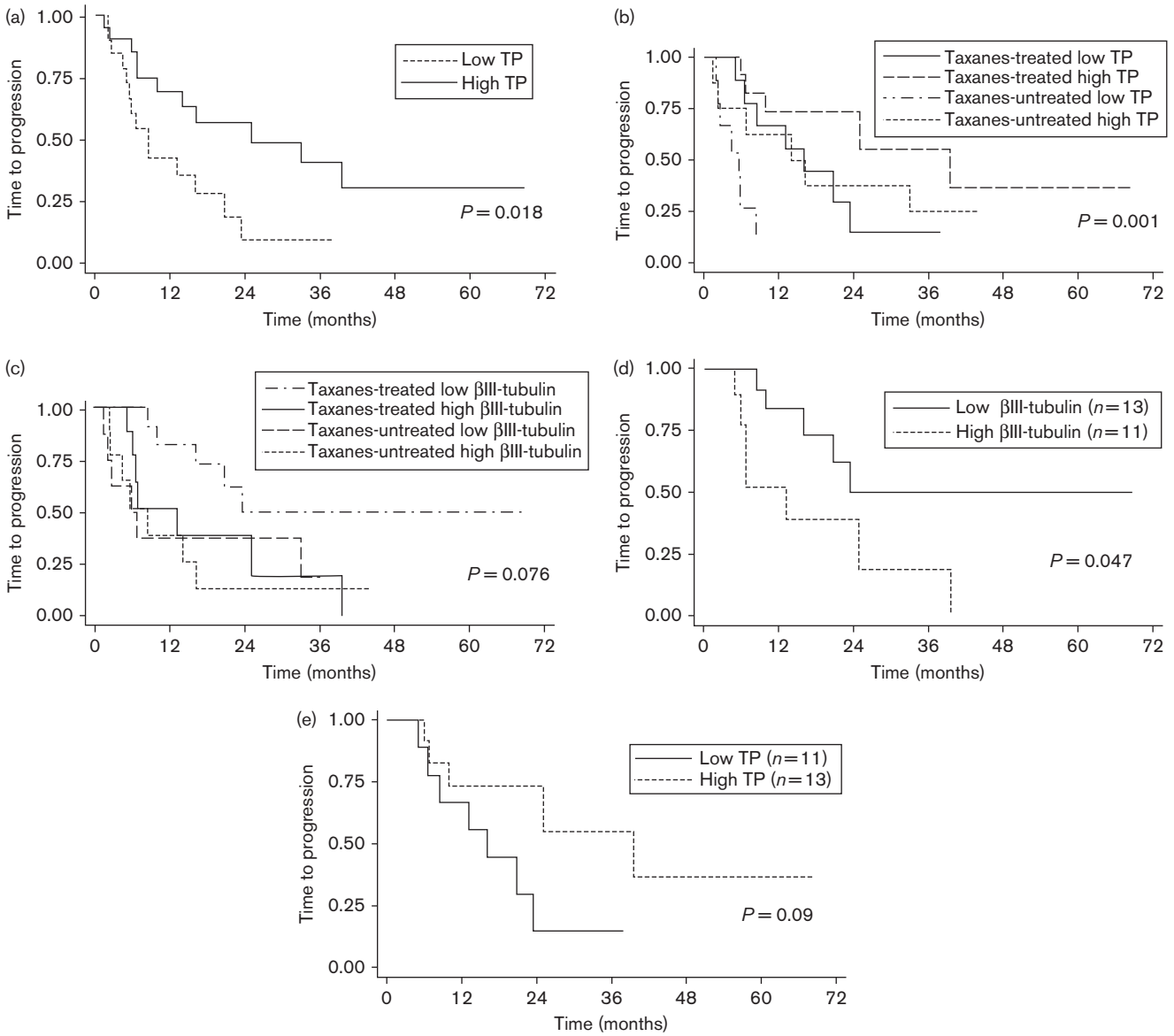
Fig. 1



Time to progression of patients with recurrent/advanced breast cancers in relation to biomarkers. (a) Thymidine phosphorylase (TP) status in the whole group; (b) TP status and taxane-based chemotherapy in the whole group; (c) βIII-tubulin status and taxanes-based chemotherapy in the whole group; (d) βIII-tubulin status in the subgroup with taxanes plus capecitabine chemotherapy ($n=24$); (e) TP status in the subgroup with taxanes plus capecitabine chemotherapy ($n=24$).

STMN1 (Table 6). The parameters that showed significant differences in the univariate analysis were further examined. The univariate hazards ratio analysis revealed the following findings: the low-TP group had a higher risk of disease progression compared with the high-TP group (hazard ratio of low TP vs. high TP, 2.641; 95% CI, 1.145–6.093; $P = 0.023$). Patients with visceral metastasis at the beginning of first-line chemotherapy and patients treated with capecitabine plus nontaxanes as first-line treatment had higher risk of disease progression (hazard ratio of patients with visceral metastasis vs. nonvisceral metastasis, 2.910; 95% CI, 1.250–6.771; $P = 0.013$; hazard ratio of patients treated with capecitabine plus nontaxanes vs. capecitabine plus taxanes, 2.312; 95% CI, 1.050–5.093; $P = 0.037$).

Regarding the TP expression status and taxane-based first-line chemotherapy, the median TTP was 16.3 months in taxane-treated patients with low TP expression, 5.5 months in taxane-untreated patients with low TP expression, 39.6 months in taxane-treated patients with high TP expression, and 16.0 months in taxane-

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untreated patients with high TP expression ($P = 0.001$, Fig. 1b). Moreover, regarding the role of βIII-tubulin isoform in taxane treatment [16–18], another analysis of TTP-based βIII-tubulin expression and taxane-based first-line chemotherapy found that the median TTP was 60.0 months in taxane-treated patients with low βIII-tubulin expression, 6.5 months in taxane-untreated patients with low βIII-tubulin expression, 13.1 months in taxane-treated patients with high βIII-tubulin expression, and 8.1 months in taxane-untreated patients with high βIII-tubulin expression ($P = 0.076$, Fig. 1c).

Subgroup analysis

On the basis of our results mentioned above, we conducted a subgroup analysis for patients with taxane plus capecitabine as first-line chemotherapy. In this subgroup ($n = 24$), TTP was significantly longer in the low-βIII-tubulin group than in the high-βIII-tubulin group (median TTP, 42.6 vs. 17.2 months, $P = 0.047$; Fig. 1d). The high-TP group showed a slightly longer TTP, but the difference was not significant (median TTP, 39.1 vs. 17.6 months, $P = 0.09$; Fig. 1e), but neither OS nor the RR was associated with βIII-tubulin/TP expression (data not shown). Other biomarkers, including TS, DPD, STMN1, and HER-2, failed to significantly associate with TTP and OS (data not shown).

Multivariate Cox proportional hazards model analyses

Multiple Cox regression with forward elimination for the selection of prognostic factors for TTP, including the clinicopathological parameters mentioned above and mRNA levels of the five biomarkers discussed previously, revealed that high TP expression (hazard ratio for low-TP group vs. high-TP group, 2.873; 95% CI, 1.143–7.223; $P = 0.025$) and capecitabine plus taxanes as first-line chemotherapy (hazard ratio for capecitabine plus taxanes vs. capecitabine plus nontaxanes, 0.344; 95% CI, 0.147–0.803; $P = 0.014$) are independent prognostic factors for TTP.

Discussion

In the present study, we found that the TP gene expression could predict the outcome of capecitabine-based first-line chemotherapy, and the βIII-tubulin gene expression was also related to a better outcome in a subgroup of patients with capecitabine plus taxanes as first-line chemotherapy. However, we failed to either find or confirm previous indications of a clinical influence by the gene expression of TS, DPD, or STMN1 in first-line capecitabine-based regimens. Although previous studies have evaluated the expression of TP in patients with capecitabine alone (whatever line that patients received) or in combination with neoadjuvant or metastatic setting [9,25,26], our study, for the first time, evaluated the predictive value of TP and βIII-tubulin expression in BC patients treated with capecitabine-based first-line chemotherapy.

TP is an enzyme responsible for nucleoside metabolism. In the three-step metabolic conversion of capecitabine to 5-fluoruracil, TP is the rate-limiting enzyme of 5′-deoxy-5-fluorouridine activation to convert 5′-deoxy-5-fluorouridine, which is an intermediate metabolite of capecitabine, to 5-FU. Hence, increasing TP gene/protein expression can enhance the sensitivity of tumor cells to prodrugs of 5-FU [27–29]. In addition, TP plays an important role in angiogenesis, cancer invasiveness, metastasis, and antiapoptosis [14]. TP is most frequently expressed in BC, followed by lung cancer, renal cancer, hepatocellular cancer, gallbladder adenocarcinoma, and the intestinal type of gastric adenocarcinoma [30]. One previous study found that higher levels of TP expression are associated with more extensive angiogenesis, unfavorable clinical and laboratory findings, and poor clinical outcome in colorectal cancer [31]. Later studies confirmed the prognostic role of TP in other types of cancer, such as transitional cell carcinoma of the bladder, cervical cancer, and gastric carcinoma [32–34]. However, the role of TP in BC is controversial. Some studies suggested that overexpression of TP was found to correlate significantly with poor prognosis [35,36]. Nevertheless, our results showed that patients with a high level of TP expression had a significantly longer TTP from first-line capecitabine-based chemotherapy, which is consistent with previous studies using oral fluorouracil derivatives in adjuvant or metastatic settings [9,25,37–39]. We also found that the low-TP group of patients had a higher risk of progression compared with the high-TP group, and that TP is an independent prognostic factor for TTP in the multiple Cox regression analysis. In contrast, our study did not show that TP expression correlates with therapeutic response. One explanation is that our sample size is small. Our observation is consistent with a recent study [26] on patients treated with capecitabine and docetaxel as neoadjuvant chemotherapy, but is in contrast with another study in which TP-positive patients with longer TTP showed a tumor response to capecitabine and docetaxel [25]; however, this study only involved qualitative and not quantitative analysis. Moreover, the survival benefit was not only associated with the RR, but was also associated with the disease control rate in the first-line setting for solid tumors [40]. Furthermore, our results did not show any statistically significant correlation between TP expression and clinicopathological parameters, which is consistent with previous studies on BC [41,42].

Together with other tubulin superfamily members, βIII-tubulin participates in the formation of microtubules. Overexpression of βIII-tubulin is associated with taxane resistance in BC cell lines, and some clinical studies has provided substantial evidence showing the relationship between poor response to taxanes and high βIII-tubulin expression level [43,44]. In the present study, we found that a low level of βIII-tubulin expression was associated

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with a longer TTP in the subgroup analysis of patients treated with capecitabine plus taxanes as first-line chemotherapy, but it was not associated with the whole group that included patients with nontaxane-based first-line chemotherapy. These observations suggest that analysis of the βIII-tubulin gene expression could assist in predicting clinical outcomes of taxane-based first-line chemotherapy in patients with recurrent/advanced BC.

First-line chemotherapy is very important for recurrent/advanced BC patients. However, the 'gold standard' regimen is yet to be determined [45]. One preclinical study suggested that taxanes upregulate TP in tumor tissues and showed synergy with capecitabine to inhibit tumor growth in colon and BC models [46], and this finding has been confirmed in women with primary BC who were treated with preoperative docetaxel [47]. Furthermore, capecitabine has been successfully combined with docetaxel in a recent phase III study [48]. These findings suggest that capecitabine/taxane combinations may be valuable. Our results also showed that capecitabine plus taxanes led to a longer TTP and OS compared with the capecitabine/nontaxanes combination. In particular, we found that high TP expression led to a slightly longer TTP in the capecitabine plus taxanes subgroup of patients, which might be partly because of the small number of patients studied ($n = 24$). However, even if the limitations of the small sample number and retrospective study apply, our study still provides solid evidence that capecitabine in combination with taxanes as first-line therapy is highly effective, especially in patients with high TP gene expression and low βIII-tubulin gene expression.

In conclusion, the present study did not reveal a significant clinical impact of the gene expression of TS, DPD, or STMN1 on recurrent/advanced BC patients treated with capecitabine-based first-line chemotherapy. However, we reported significantly different TTP according to the TP gene expression level in the whole group of patients compared with the βIII-tubulin gene expression level in the capecitabine plus taxanes subgroup of patients. Therefore, these two potential biomarkers should be further evaluated with regard to the biological and clinical aspects of BC.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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