SPANDIDOS PUBLICATIONS

Evaluation of 5-fluorouracil related genes in breast cancer to predict the effect of adjuvant therapy with oral fluorouracil derivatives

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Abstract. New anticancer drugs have been developed, and prediction of their effect is needed to perform tailor made chemotherapy. We investigated a selection of the predictive markers for oral adjuvant chemotherapy among 5-fluorouracil (5-FU) related genes. 5-FU related genes were examined by using a laser captured microdissection and real-time RT-PCR in 220 patients with invasive breast cancer. Sixty-six patients were treated with postoperative oral fluorouracil derivatives for 12 months or more, and we examined the prognosis of these patients according to the expression of 5-FU related genes. The median of thymidylate synthase (TS), dehydropyrimidine dehydrogenase (DPD), thymidine phosphorylase (TP) and orotate phosphoribosyltransferase (OPRT) mRNA values in the 220 specimens were determined for cut-off levels separating low and high gene expression. In 66 patients, 5-year disease-free survival (DFS) in the TP-high group (n=28) was significantly better than that in the TP-low group (n=38) (P=0.016). Of 220 patients, the 69 patients in TP-high group comprised 28 patients treated with oral fluorouracil derivatives and 41 patients with hormone therapy alone. The proportion of patients with lymph node involvement in the fluorouracil group was significantly greater than that in the hormone therapy alone group (P=0.003). Five-year DFS was not significantly different between the groups (P=0.80). Our results suggest that adjuvant oral fluorouracil chemotherapy may improve the prognosis of the patients with TP high expression breast cancer, and TP mRNA level in breast cancer may predict the effect of new oral fluorouracil derivatives for postoperative adjuvant chemotherapy.

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Key words: breast cancer, 5-fluorouracil related genes, oral fluorouracil derivatives, prognosis

Introduction

Adjuvant chemotherapy has been shown to improve the survival of patients with breast cancer (1-3), and 5-fluorouracil (5-FU) is commonly used in combination with anthracyclines and cyclophosphamide. New fluorouracil derivatives (capecitabine and S-1) have been developed and used alone for metastatic breast cancer (4,5). In Japan, monotherapy of fluorouracil derivatives still remains for oral adjuvant chemotherapy after breast cancer surgery. The Adjuvant Chemo-Endocrine Therapy for Breast Cancer (ACETBC) trial in Japan demonstrated the efficacy of uracil and tegafur in the postoperative adjuvant treatment of node-negative breast cancer patients (6).

5-FU is metabolized intracellularly to FdUMP, which binds to thymidylate synthase (TS) in the presence of reduced folate cofactors. This binding inhibits the formation of thymidylate from uridylate, which is required for DNA synthesis. Dehydropyrimidine dehydrogenase (DPD) is the first, and rate-limiting, enzyme for the catabolism of 5-FU. Activation of 5-FU into its nucleotides requires phosphorylation by three pathways involving orotate phosphoribosyltransferase (OPRT), uridine phosphorylase (UP) or thymidine phospho-rylase (TP). There are many studies on the 5-FU related genes and the prognostic effect of fluorouracil derivatives in colorectal cancer, gastric cancer and lung cancer (7-9), however, there are only a few studies in breast cancer, and it is still controversial which molecular marker predicts the efficacy of fluorouracil derivatives for adjuvant chemotherapy.

The aim of this study was to select the predictive marker of oral adjuvant therapy among 5-FU related genes. A laser-capture microdissection (LCM) and real-time RT-PCR (RT-PCR combined with LCM) for formalin-fixed paraffin-embedded (FFPE) samples was adopted for analysis of gene expression in this study. The associations between 5-FU related genes (TS, DPD, TP and OPRT) and the clinicopathological factors in breast cancer were evaluated, and we also examined the prognosis of patients treated with oral fluorouracil derivatives after surgery according to the expression of TS, DPD, TP and OPRT.

Patients and methods

Patients. A total of 220 women with invasive breast cancer who underwent surgery between 1995 and 1999 at our hospital were studied. This study was approved by the Ethics Committee of our university, and all patients gave written informed consent. The clinicopathological data of patients were collected from medical notes. The mean age at the time of surgery was 60.1 years, ranging from 30 to 90 years. Number of patients <50 years was 56, and ≥50 years was 164. Pathological examination revealed that axillary lymph node status was negative in 132 patients and positive in 81. The tumor size of the resected specimen was ≤2 cm in 108 patients and >2 cm in 112 patients. Estrogen receptor (ER) content of tumors was measured using an enzyme immunoassay at the time of surgery. Of 220 patients, 128 were ER-positive, 73 were ER-negative and 19 were ER unknown. Clinical examination of all patients did not reveal any metastasis from breast cancer at surgery. None had received chemotherapy or hormone therapy before surgery. All patients underwent modified radical mastectomy or breast-conserving surgery with axillary dissection. Patients with conservative surgery were treated with postoperative irradiation. 5-FU-based chemotherapy was administered to patients with lymph node involvement or ER-negative tumor. Sixty-six patients were treated with postoperative oral chemotherapy including fluorouracil derivatives for 12 months or more, and 9 patients for shorter than 12 months. Of 66 patients, 21 were given only fluorouracil derivatives, and 45 were given hormone therapy. The intravenous chemotherapy containing CMF or CAF was given to 53 patients. Seventy-three patients received hormone therapy for at least 2 years, 71 of whom were given tamoxifen and 2 an aromatase inhibitor. No adjuvant therapy was administrated to 19 patients (Fig. 1). The disease-free survival (DFS) was calculated as the period from surgery until the date of first recurrence. The median follow-up period was 90 months (range, 12-123 months).

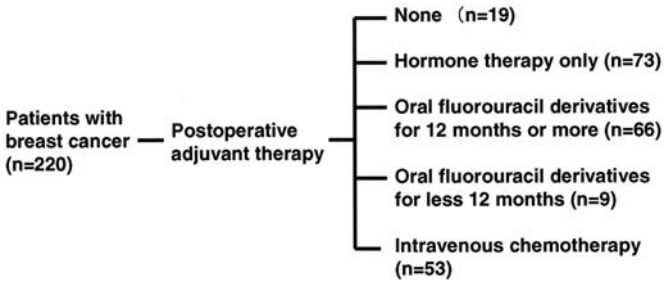

Figure 1. Schema of breast cancer patients with adjuvant therapy.

Microdissection and RT-PCR. A representative formalin-fixed, paraffin-embedded tumor specimen was selected by a pathologist (M.T.) after examination of the hematoxylin and eosin-stained slides. Sections, 10-μm thick were stained with nuclear fast red to enable visualization of histology for LCM (P.A.L.M. Microlaser Technologies AG, Munich, Germany). Cancer cells were dissected using the LCM technique. RNA was isolated from these cells using a novel, proprietary procedure (Response Genetics, Los Angeles, CA: United State Patent No. 6.248.535). After RNA isolation, cDNA was derived from each sample according to a previously described procedure (10). Target cDNA sequences were amplified by quantitative PCR using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System, TaqMan; Applied Biosystems, Foster City, CA) as previously described (11,12). The 25 μl PCR reaction mixture contained 600 nmol/l of each primer, 200 nmol/l each of dATP, dCTP and dGTP, 400 μmol/l dUTP, 5.5 mmol/l $MgCl_2$ and 1X TaqMan buffer. A reference dye was included (all reagents were supplied by Applied Biosystems). The primers sequences used were: TS primers: GCCTCG GTGTGCCTTTCA and CCCGTGATGTGCGCAAT, probe TS: 6FAM-TCGCCAGCTACGCCCTGCTCA; DPD primers: AGGACGCAAGGAGGGTTTG and GTCCGCCG AGTCCTTACTGA, probe DPD: 6FAM-CAGTGCCTACA GTCTCGAGTCTGCCAGTG; TP primers: CCTGCGGACG GAATCCT and GCTGTGATGAGTGGCAGGCT, probe TP: 6FAM-CAGCCAGAGATGTGACAGCCACCGT; OPRT primers: TCCTGGGCAGATCTAGTAAATGC and TGCTCCTCAGCCATTCTAACC, probe OPRT: 6FAM-CTCCTTATTGCGGAAATGAGCTCCACC; ß-actin primers: TGAGCGCGGCTACAGCTT and TCCTTAATGTCACGC ACGATTT, probe ß-actin: 6FAM- ACCACCACGGCCGAG CGG. The PCR conditions were 50˚C for 10 sec and 95˚C for 10 min, followed by 42 cycles at 95˚C for 15 sec and 60˚C for 1 min. TS, DPD, TP and OPRT gene expression in each of the tumors was quantified as ratios between two absolute measurements (gene of interest/ß-actin).

Statistical analysis. TS, DPD, TP and OPRT gene mRNA values were expressed as mean ± SD. Comparisons between values and clinicopathological parameters were made by Student's t-test. The statistical difference in gene expression in relation to clinicopathological parameters was assessed by χ^2 or Fisher's exact test. The Kaplan-Meier method was used to estimate the probability of DFS, and differences in DFS of subgroup were compared using the log-rank test. All tests were two-tailed and a P-value of <0.05 was considered to indicate statistical significance.

Results

Gene mRNA values in breast cancer. The mean values and standard deviations of TS, DPD, TP and OPRT in cancer cells were 2.91±2.47, 1.10±0.84, 12.06±8.39 and 1.31±0.72, respectively. The medians of TS, DPD, TP and OPRT mRNA values were 2.27 (range, 0.27-22.08), 0.87 (0.09-4.98), 9.495 (0.54-45.42) and 1.19 (0.18-4.33), respectively. Table I shows a comparison of gene mRNA values among the clinicopathological features in the 220 patients. DPD mRNA value in tumors with lymph node involvement was significantly lower than without lymph node involvement (P=0.024). OPRT mRNA value in tumors without lymph node involvement was significantly higher than with lymph node involvement (P=0.012). There were no significantly difference between TS, DPD, TP and OPRT mRNA values and age, tumor size or ER status.

lnicopathological features and TS, DPD, TP and OPRT mRNA expression levels in breast cancer.

Variables	No. of cases	TS (mean ± SD)	P-value	DPD (mean ± SD)	P-value	TP (mean ± SD)	P-value	OPRT (mean ± SD)	P-value
Age									
<50	56	2.99±1.94	0.78	1.16±0.95	0.53	12.27±8.70	0.83	1.39±0.71	0.32
≥50	164	2.89±2.63		1.03±0.80		11.98±8.31		1.28±0.72	
Tumor size									
≥2 cm	108	2.79±2.65	0.47	1.17±0.84	0.24	12.80±9.42	0.19	1.29±0.79	0.69
<2 cm	112	3.03±2.30		1.04 ±0.84		11.33±7.24		1.33±0.64	
Lymph node involvement									
Absent	132	2.86±2.05	0.60	1.20±0.97	0.024	12.75±8.46	0.054	1.39±0.69	0.012
Present	81	2.70±2.17		0.93±0.57		10.53±7.52		1.15±0.67	
Estrogen receptor									
Absent	73	2.92±2.53	0.97	1.09±0.83	0.98	10.57±7.05	0.12	1.19±0.62	0.067
Present	128	2.94±2.59		1.09±0.81		12.47±8.81		1.39±0.79	
Total	220	2.91±2.47		1.10±0.84		12.06±8.39		1.31±0.72	
Median	220	2.27		0.87		9.495		1.19	
Min-Max	220	0.27-22.08		0.09-4.87		0.54-45.42		0.18-4.33	

Table II. Comparison of the clinicopathological features in relation to TS, DPD, TP and OPRT expression in the treatment with adjuvant oral fluorouracil derivatives (group, n=66).

	TS			DPD			TP			OPRT		
Variables	Low	High	P-value	Low	High	P-value	Low	High	P-value	Low	High	P-value
Age												
<50	10	8	>0.999	10	7	0.696	8	10	0.297	11	7	>0.999
≥50	28	20		32	15		30	18		31	17	
Tumor size												
≤2 cm	21	13	0.645	23	10	0.657	20	14	>0.999	25	9	0.143
>2 cm	17	15		19	12		18	14		17	15	
Lymph node involvement												
Absent	19	21	0.072	24	14	0.815	20	20	0.197	22	18	0.122
Present	19	7		18	8		18	8		20	6	
Estrogen receptor												
Absent	19	13	>0.999	19	13	0.606	22	10	0.101	21	11	0.921
Present	18	13		20	9		14	17		19	12	

DFS analysis in patients with adjuvant oral fluorouracil derivatives for 12 months or more. The median values of 2.27 for TS, 0.87 for DPD, 9.495 for TP and 1.19 for OPRT in 220 cancers were selected for cut-off levels separating low and high gene expression. Table II shows a comparison of TS, DPD, TP and OPRT expression among clinicopathological features in 66 patients treated with oral fluorouracil derivatives for 12 months or more. This group was composed of 21 patients treated with oral fluorouracil derivatives alone and 45 patients with oral fluorouracil derivatives and tamoxifen or an aromatase inhibitor. The overall 5-year DFS was 83.3% in this group. Five-year DFS of the patients treated with oral

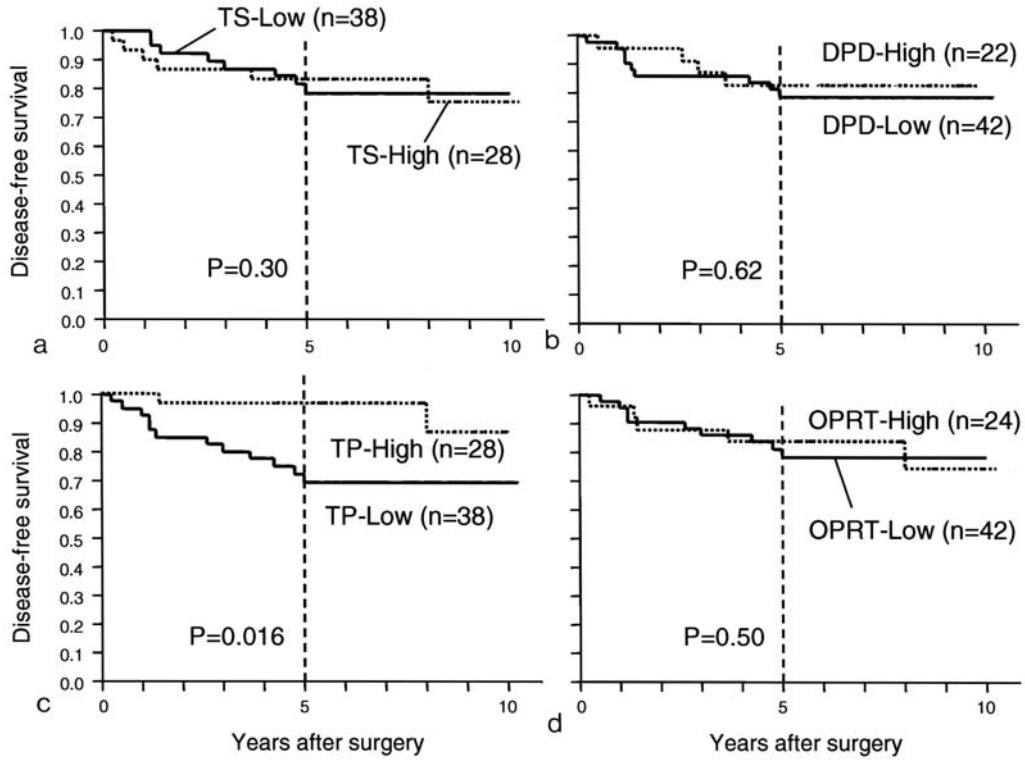

Figure 2. Disease-free survival in 66 patients with adjuvant oral fluorouracil derivatives for 12 months or more according to tumor (a) TS, (b) DPD, (c) TP and (d) OPRT expression.

Table III. Correlation between the clinicopathological features and the difference of adjuvant therapy in TP expression group.

	TP-low group			TP-high group		
Variables	Fluorouracil derivatives	Hormone therapy	P-value	Fluorouracil derivetives	Hormone therapy	P-value
No. of cases	38	32		28	41	
Age						
<50	8	6	>0.999	10	8	0.219
≥50	30	26		18	33	
Tumor size						
≤2 cm	20	22	0.260	14	28	0.200
>2 cm	18	10		14	13	
Lymph node involvement						
Absent	20	25	0.0043	20	38	0.003
Present	18	4		8	1	
Estrogen receptor						
Absent	22	3	<0.0001	10	4	0.015
Present	14	29		17	34	

fluorouracil derivatives alone was 80%, and that of the patients with oral fluorouracil derivatives and tamoxifen or an aromatase inhibitor was 84.8%. There was no significant difference in 5-year DFS between the two groups (P=0.59). The expression of TS, DPD, TP and OPRT were low in 38, 42, 38 and 42, respectively. Those of TS, DPD, TP and OPRT were high in 28, 22, 28 and 24, respectively. There were not significant correlations in this group between the

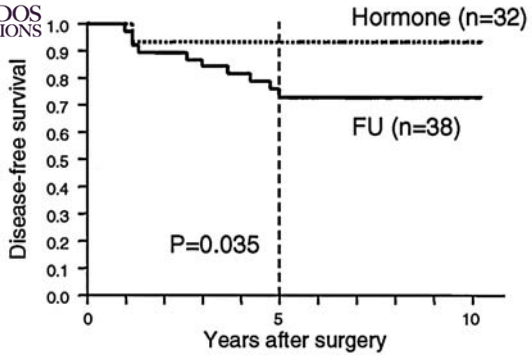

Figure 3. Disease-free survival in TP-low group (n=70) with respect to treatment with hormone therapy alone versus with oral fluorouracil derivatives (FU).

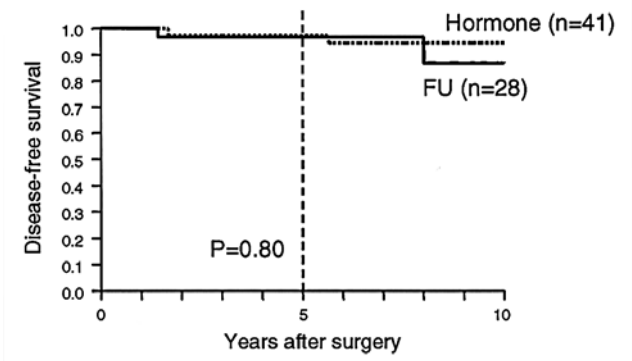

Figure 4. Disease-free survival in TP-high group (n=69) with respect to treatment with hormone therapy alone versus with oral fluorouracil derivatives (FU).

gene expression and clinicopathological factors (Table II). In TS-, DPD- and OPRT-high and low groups, 5-year DFS was 89.2 and 78.9%, 86.4 and 81%, 87.5 and 81%, respectively. There were no significant differences in 5-year DFS between the high and low expression in TS, DPD and OPRT gene groups. Five-year DFS of the patients in TP-high and low group was 96.4 and 73.7%, respectively. The patients in TP-high group had significantly longer disease-free intervals than those in the TP-low group (P=0.016) (Fig. 2).

Comparison of DFS in the TP-low and in the TP-high group. TP-low group comprised 38 patients treated with oral fluorouracil derivatives and 32 patients with hormone therapy alone. There were significantly fewer patients with lymph node involvement in the hormone therapy alone group than in the fluorouracil derivatives group (P=0.0043), and more tumors with ER-positive status in the hormone therapy alone group than in the fluorouracil derivatives group (P<0.001). The differences in age and tumor size between the groups were not found (Table III). The overall 5-year DFS was 82.9% in the TP-low group. Five-year DFS in the fluorouracil derivatives group (73.7%) was significantly poorer than that in the hormone therapy alone group (93.7%) (P=0.035) (Fig. 3). TP-high group comprised 28 patients treated with oral fluorouracil derivatives and 41 patients with hormone therapy alone. There were significantly fewer patients with lymph node involvement in the hormone therapy alone group than in the fluorouracil derivatives group (P=0.003), and more tumors with ER-positive status in the hormone therapy alone group than in the fluorouracil derivatives group (P=0.015) (Table III). The overall 5-year DFS was 97.1% in the TP-high group. Five-year DFS was 97.6% in the hormone therapy alone group, and 96.5% in the fluorouracil derivatives group. There was no significant difference between the groups (P=0.80). In the TP-high group, adjuvant oral fluorouracil chemotherapy significantly improved the 5-year DFS (Fig. 4).

Discussion

RT-PCR technology combined with improved methods for extraction of RNA from paraffin-embedded specimens has allowed the measurement of gene expression accurately (10,13). LCM provides selective isolation of defined cell populations from heterogeneous tissue sections (14). Ichikawa *et al* (15) reported a close correlation of TS and DPD gene expression between fresh-frozen tissues and paraffin-embedded tissues in gastric cancer. Gene expression using the RT-PCR method, which had the same primers as used in our study, combined with LCM from paraffin-embedded specimens of 4871 solid cancers including breast cancer in Japan were reported by Fukui *et al* (the 43rd Annual Meeting of Japan Society of Clinical Oncology 12: 2005). The medians of TS, DPD and OPRT mRNA values in all cancers were 2.05, 0.75, and 1.14, respectively. The medians of the mRNA values in 370 breast cancer were 2.15, 0.84 and 0.99, respectively, and those values were similar to the values in our 220 consecutive breast cancers. In this study, DPD mRNA value in tumors with lymph node involvement was significantly lower than without lymph node involvement. The expression of DPD in breast cancer using the immunohistochemical method was described by Horiguchi *et al* (16), in which there was no significant difference between DPD staining and lymph node status. The difference of results may originate from the different methodology, but DPD mRNA value of tumor with lymph node involvement (median, 0.75) in this study was not low, as it was similar to DPD mRNA value of colorectal cancer (median, 0.31) in the report by Fukui *et al* (the 43rd Annual Meeting of Japan Society of Clinical Oncology 12: 2005). Kakimoto *et al* (17) reported the relation between DPD mRNA value and fluorouracil sensitivity in breast cancer by using the RT-PCR method combined with LCM and the histocultural drug sensitivity test. It was shown that low DPD gene expression in cancer cells was sensitive to fluorouracil, although the difference of DPD mRNA values between the sensitive group (median, 1.1) and the resistant group (median, 1.8) was not significant. Concerning OPRT mRNA value, Ichikawa *et al* (18) described the gene expression influence on outcome following fluoropyrimidine-based chemotherapy for metastatic colorectal cancer, and median values of OPRT mRNA expression in primary colorectal cancers were 1.39 and 0.85 for responding tumors and non-responding tumors, respectively. In this study, OPRT mRNA value in tumors without lymph node involvement was signi-ficantly higher than with lymph node involvement. It may suggest that the breast cancer without lymph node involvement could be sensitive to fluorouracil derivatives.

Associations with the gene expression and the effect of 5-FU-based adjuvant chemotherapy were studied by the method of immunohistochemistry or enzyme linked immunosorbent assay (ELISA) or RT-PCR assay. There are many reports on colorectal, gastric, and lung cancer, but only a few for breast cancer. In regard to breast cancer, Horiguchi *et al* (16) reported using immunohistochemistry that the patients with DPD-positive tumor had a significantly poorer DFS than those with DPD-negative tumor under the treatment of 5-FU or 5-FU derivatives, while Hakamada *et al* (19) described using ELISA that DPD expression was not associated with the survival of cancer patients treated with 5-FU-based chemotherapy. In this study, gene expression were examined by using RT-PCR method combined with LCM. DFS in patients treated with postoperative adjuvant oral fluorouracil derivatives for 12 months or more was analyzed according to gene expression. The patients in the TP-high group had significantly better DFS than those in the TP-low group, and there were no significant differences in DFS between the high and low group in TS, DPD and OPRT. The discrepancy might be due to the different antibody used, the different cut-off value adopted, or the contamination of intratumoral stromal cells. RT-PCR method combined with LCM was useful to examine the gene expression of cancer cells separated from cancer stroma and normal glands in cancer. The purpose of post-operative chemotherapy is to prevent metastasis, according to the anticancer effect against the circulating cancer cells. Our results may suggest that TP could play a role in the prevention for micrometastasis.

TP is considered not to be important regarding the pathways of 5-FU activation, because of the lack of its cofactor dRib-1-P at physiological concentrations (20). Apart from the function of TP in 5-FU phosphorylation, TP, which is identical to platelet derived endothelial cell growth factor, has been shown to play an important role in angiogenesis, cancer invasiveness, metastasis and antiapoptotic effect (21). TP is an angiogenic factor commonly expressed in breast cancer, and it is also known to show great heterogeneity in the expression of angiogenic growth factors in breast cancer (22). Tumor-associated macrophages (TAMs) produce angiogenic factors and in breast cancer are associated with poor survival. Leek *et al* (23) showed two alternative TAM-mediated angiogenic pathways, one was via induction of TAM hypoxia-induced factor-2α, and the other was via oxidative stress. TP may induce angiogenesis via oxidative stress (24), however, an inverse relationship was found between tumor TP expression and TAM hypoxia-induced factor-2α (23). These pathways seemed to be reciprocal in that one or another dominates, perhaps reflecting tumor hypoxia or oxygenation selecting the relevant pathway for angiogenesis (23). ACETBC trail revealed the effect of adjuvant therapy with UFT after surgery of breast cancer. The patients in the UFT group had significantly better 5-year survival than those in the non-UFT group (6). We analyzed 5-year DFS according to the difference of adjuvant therapy under the expression of TP in this study. In TP-high group, 5-year DFS in the oral fluorouracil derivatives group was similar to that in the hormone therapy alone group, although the patients in the oral fluorouracil derivative group had significantly more lymph node involvement and more ER-negative tumors than in the hormone therapy alone group. However, the patients in the oral fluorouracil derivative group had poorer 5-year DFS than in the hormone therapy alone group within TP-low group. The subgroup analysis in TP-high group showed a significant improvement of prognosis in the oral fluorouracil derivatives group. It may suggest that the oral fluorouracil derivatives could be useful to prevent micrometastasis from the circulating cancer cells with TP high expression.

Recently, low-dose and frequency chemotherapy has been recognized to be effective after surgery (6,9). The targets of low-dose and frequency chemotherapy seem to be cancer cells, endothelial cells, and endothelial progenitor cells (25). Oral fluorouracil derivative adjuvant therapy is an adequate administration for low-dose and frequency chemotherapy. This study probably revealed an important implication for TP expression and oral fluorouracil adjuvant therapy, however, randomized prospective translational treatment trial is needed to confirm the relationship of TP expression and the efficacy of oral fluorouracil derivatives in this limited retrospective study. It suggests that oral fluorouracil adjuvant therapy could be available as tailor made chemotherapy in the near future.

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