Anthracyclines and taxanes induce the upregulation of thymidine phosphorylase in breast cancer cells

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We have investigated the immunohistochemical expression of thymidine phosphorylase before and after the administration of anthracycline, and/or anthracycline/taxane-based preoperative chemotherapy in a consecutive series of 55 patients with primary operable breast cancer. Pretreatment, large core breast biopsies and the corresponding surgical samples were retrospectively evaluated for thymidine phosphorylase immunoreactivity. Immunohistochemical expression was evaluated on tumor cells (nuclear and cytoplasmic staining) and on stromal cells (cytoplasmic staining). The cytoplasmic expression of thymidine phosphorylase was enhanced in the tumor cells after treatment ($P=0.04$). An increase in thymidine phosphorylase cytoplasmic tumor expression was observed in 33% (95% confidence interval: 19–50%) of patients after preoperative chemotherapy ($P=0.01$). No statistically significant nuclear staining changes were observed in response to treatment. Similarly, no significant changes of the enzyme expression were seen in stromal cells. This study provides further evidence that, at least in breast cancer, thymidine phosphorylase is upregulated after anthracycline and/or taxane-containing chemotherapy. Accordingly, it supports the hypothesis of a synergistic effect between thymidine phosphorylase-modulating and thymidine phosphorylase-targeting anticancer agents. Translational studies, specifically designed on the basis of this rationale, are eagerly waited. *Anti-Cancer Drugs* **18:883–888 © 2007 Lippincott Williams & Wilkins.**

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Introduction

Thymidine phosphorylase (TP), also known as platelet-derived endothelial cell growth factor, is a key enzyme of the three-step metabolic cascade that converts the oral prodrug capecitabine into the active form 5-fluorouracil (5-FU) [1]. Up to five times higher intratumoral concentrations of TP than in adjacent normal tissue determine a tumor-selective generation of 5-FU, making capecitabine a potential candidate for the club of so-called 'targeted therapy' [1–7]. Several studies showed that the target TP is expressed in a variety of human cancers [5,8] and its analysis has been proposed as a potential predictor of patient outcome and/or response to fluoropyrimidines [9–11]. In breast cancer, there is some evidence to suggest that TP expression in tumor cells correlates with microvessel density. The angiogenic activity of TP has been indicated as the main reason why TP-expressing tumors are less apoptotic [1]. Notably, TP expression has been observed not only in tumor cells but also in the stroma. In particular, several studies have reported that TP expression in stromal cells such as macrophages is associated with unfavorable prognosis [1].

Recently, it has been reported that some anticancer agents could upregulate TP [1,12,13]. The exact mechanisms of chemotherapy-induced TP upregulation are still unknown, although the induction of inflammatory cytokines seems to be crucial in the process. In addition, some chemotherapeutic drugs are directly involved in the transcriptional control of TP through activation of the transcription factor SP1 [1]. According to these observations, it is reasonable to hypothesize that TP-modulating chemotherapy would enhance the therapeutic activity of TP-targeting chemotherapy such as capecitabine [14–16] (Fig. 1). Specifically, among agents used in the treatment of breast cancer, paclitaxel and docetaxel have been reported to upregulate TP activity through elevation in tumor necrosis factor (TNF)-α levels, and to enhance antitumor effects of capecitabine in human cancer xenografts [12,17]. These preclinical findings have been supported by the 'in-vivo' demonstration that TP expression is significantly enhanced after certain neoadjuvant chemotherapies for breast cancer [13]. Furthermore, several clinical studies supported the hypothesis of a synergistic effect between TP-modulating (i.e. taxanes) and TP-targeting (i.e. capecitabine) anticancer agents

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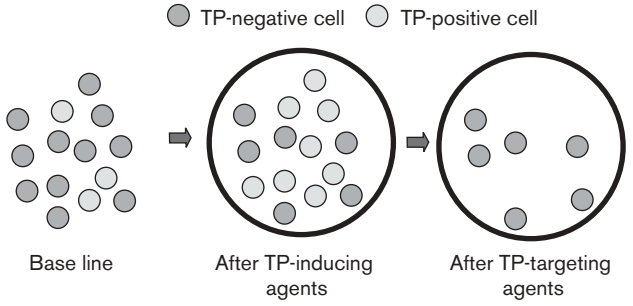
Fig. 1



TP modulation may enhance the therapeutic activity of TP-targeting chemotherapy. TP, thymidine phosphorylase.

[16,18], whereas others were specifically designed on the basis of this rationale [14,15].

To provide further evidence of the chemotherapy-induced TP modulation in human tumor tissues, this study investigated the immunohistochemical TP expression before and after the administration of neoadjuvant chemotherapy in a consecutive series of patients with primary operable breast cancer.

Patients and methods

From January 1999 to December 2004, 55 patients with operable breast cancer ($T \geq 2$ cm, N0–1, M0) were consecutively treated with neoadjuvant chemotherapy, at the Department of Medical Oncology, University Hospital of Udine, Italy. Treatment consisted of anthracycline-based (all patients) and anthracycline/taxane-based ($n = 40$ cases) chemotherapy (Table 1). After written informed consent was obtained and before starting chemotherapy, all patients underwent 14-gauge core breast biopsy. For each breast neoplastic lesion, at least five cores were obtained. Each specimen was fixed in buffered neutral formalin and embedded in paraffin wax. After the final diagnosis was formulated by the pathologist, a representative specimen was subjected to immunohistochemical analysis for estrogen (ER) and progesterone receptor (PGR) status, HER-2/*neu* status (rabbit polyclonal, Hercept Test, Dako kit, Denmark) and proliferating activity (MIB-1 expression; mouse monoclonal, Ki-67, Ventana; 1 : 100) according to previously reported method [19]. In addition, all core biopsy samples were retrospectively processed for TP expression (see below for description of the method) and formed the basis of this study.

Histological evaluation and immunohistochemical analysis for TP were also performed on the corresponding postchemotherapy surgical samples.

Table 1 Regimens of neoadjuvant chemotherapy

Therapeutic regimens	Number of patients
Doxorubicin 60 mg/m^2 and cyclophosphamide 600 mg/m^2, day 1, every 3 weeks × 2 cycles followed by docetaxel 100 mg/m^2, day 1, every 3 weeks × 2 cycles	35
Doxorubicin 60 mg/m^2 and cyclophosphamide 600 mg/m^2, day 1, every 3 weeks × 4 cycles	13
Epirubicin 75 mg/m^2 and docetaxel 75 mg/m^2, day 1, every 3 weeks × 6 cycles	5
Epirubicin 90 mg/m^2 and cyclophosphamide 600 mg/m^2, day 1, every 3 weeks × 4 cycles	1
5-Fluorouracil 600 mg/m^2, epirubicin 100 mg/m^2, cyclophosphamide 600 mg/m^2, day 1, every 3 weeks × 4 cycles	1

Immunohistochemical analysis of thymidine phosphorylase

Immunohistochemistry was performed at a single central laboratory using a primary mouse anti-TP monoclonal antibody (Roche Diagnostics, Mannheim, Germany).

TP expression was assessed on 5-μm sections of paraffin-embedded tissue samples. Endogenous peroxidase activity was inhibited by incubating the slides in 0.3% H_2O_2 in absolute methanol for 5 min. Antigen retrieval was carried out in a water bath at 98°C [40 min in 1 mmol/l ethylenediaminetetraacetic acid (EDTA) buffer, pH 8.0]. The tissues were blocked with 20% goat serum for 20 min and then incubated overnight at 4°C with mouse monoclonal anti-TP antibody diluted 1 : 100 (Roche Diagnostics). For staining detection, the EnVision + Detection System (D) was used according to the manufacturer's recommendation. In each experiment, a negative control was included in which primary antibody was replaced by mouse ascites. Liver Kupffer cells served as a positive control.

Evaluation of immunohistochemistry

TP expression was evaluated on tumor cells (nuclear and cytoplasmic staining) and on stromal cells (cytoplasmic staining). Nuclear expression of TP was expressed as percentage of positive cells. Staining intensity as well as percentage of stained cells was considered in assessment of cytoplasmic staining according to the method described by Tsuda *et al.* [20]. The intensity of cytoplasmic and stromal immunoreactivity was scored as 0, 1, 2 or 3 denoting negative, weak, moderate or strong staining, respectively. Then, the percentages of areas of each category (0–3) of staining were estimated and, regardless of positive or negative staining, the category of the largest area of cancer or stromal tissue was reported as the representative score for each case. Therefore, for statistical analysis, the TP expression level for each case was putatively defined as positive if the predominant intensity was 2 or 3. The staining of normal mammary ductal epithelial cells was used as reference for moderate intensity.

Assessment of response

To evaluate response rate, a complete tumor assessment [comprising clinical evaluation of palpable tumor and

lymph nodes, mammography, breast ultrasound, and breast magnetic resonance imaging (optional)] was performed at baseline (before treatment) and before surgery.

The clinical response to chemotherapy was classified into the following categories, based on the 'response evaluation criteria in solid tumors' (RECIST) [21], using the measurements obtained by both palpation and by the most appropriate imaging method:

Responders

1. Complete response: complete disappearance of all tumor signs in the breast.
2. Partial response: reduction in size of the tumor $\geq 30\%$.

Nonresponders

1. Stable disease: reduction in size of the tumor $< 30\%$.
2. Progressive disease: increase in size of tumor or development of new, previously undetected lesions.

Axillary lymph node evaluation was not included in the assessment of clinical response.

Pathologic response was defined according to Chevallier categories [22]:

1. *Grade 1*: no microscopic evidence of residual tumor cells (invasive or noninvasive) in all resected breast specimens and axillary lymph nodes.
2. *Grade 2*: only residual noninvasive (in-situ) tumor.
3. *Grade 3*: residual invasive tumor with evidence of stromal alterations.
4. *Grade 4*: minimal tumor changes or tumor progression.

Statistical analysis

The χ^2 and Fisher's exact tests were used to compare the baseline characteristics (categorical variables) between the subgroups.

Spearman's rank test was used to assess the correlation between continuous variables.

Wilcoxon two-sample test was used to evaluate if the sums of the rankings for two groups were different from an expected number (Kruskal–Wallis test was used in case of more than two groups).

McNemar test was chosen to test the difference between paired proportions in the part of this study with 'before and after' design.

Wilcoxon signed-rank test was used to test the difference between pretreatment and posttreatment TP expression levels. $P < 0.05$ was considered statistically significant.

Participants with missing data were not included in the analyses, although this resulted in a substantial decrease in sample size. In fact, although alternative methods exist for treating missing values (e.g. imputation), subject deletion has the important advantage that, under the assumption that data are missing at random, it leads to unbiased parameter estimates. We preferred to reduce precision (i.e. the statistical tests were less likely to yield significant results) and not take the risk of compromising validity.

Results

Tumor characteristics are summarized in Table 2.

The predominant pattern of TP staining was cytoplasmic, although occasionally both cytoplasmic and nuclear staining was observed (Fig. 2).

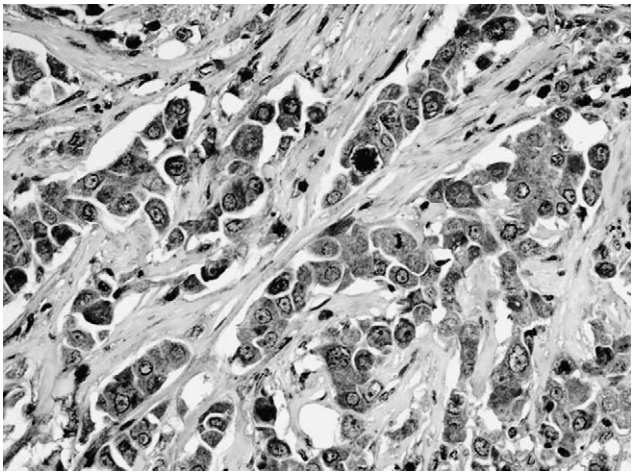
Descriptive statistics of baseline (pretreatment) and end-of-study (posttreatment) immunohistochemical expression of TP in tumor and stromal cells are described in Table 3.

Pretreatment and posttreatment measurements of TP were available in 37 participants for nuclear and stromal expression and in 39 participants for cytoplasmic expression. Missing values were due to absence of residual tumor at surgery (five cases), absence of surgical samples (two patients underwent surgery at other hospitals) and

Table 2 Tumor characteristics

Characteristic	Number of patients	Percentage
Histotype		
Invasive ductal carcinoma	39	71
Invasive lobular carcinoma	7	13
Invasive ductal–lobular carcinoma	3	5
Other	6	11
Nodal status (clinical evaluation)		
Negative	22	40
Positive	33	60
Histological grade on core biopsy		
1	4	7
2	25	46
3	17	31
Not assessable	9	16
HER-2/*neu* status		
0	4	7
1+	27	49
2+	14	26
3+	10	18
Characteristic	Median value (25–75th percentiles)	
Tumor size (mm)	40 (30–50)	
Estrogen receptor (percentage of positive nuclei)	60 (0–90)	
Progesterone receptor (percentage of positive nuclei)	7 (0–66)	
MIB-1 (percentage of positive nuclei)	22 (9–35)	

Fig. 2



Representative immunohistochemical expression of thymidine phosphorylase with predominant cytoplasmic pattern.

Table 3 Baseline (pretreatment) and end-of-study (posttreatment) immunohistochemical expression of thymidine phosphorylase in breast cancer cells and stroma

TP expression	Pretreatment			Posttreatment		
	Mean value (%)	Median value (%)	25–75th percentile (%)	Mean value (%)	Median value (%)	25–75th percentile (%)
Nuclear TP	38	33	20–63	36	33	12–63
Cytoplasmic TP						
0	13	0	0–22	8	0	0–0
1	52	53	27–83	34	26	6–55
2	31	16	0–64	52	56	18–81
3	4	0	0–0	6	0	0–0
Stromal TP						
0	13	6	0–19	8	0	0–5
1	31	25	8–51	46	48	20–76
2	21	15	0–31	36	28	10–69
3	35	11	0–76	10	0	0–5

TP, thymidine phosphorylase.

technical problems (11 cases). Technical problems refer mainly to cases with scanty histological material.

The comparison between immunohistochemical expression of TP in the pretreatment large-core biopsy and the corresponding surgical specimens (Table 4) showed that the cytoplasmic expression of the enzyme was enhanced in the tumor cells after the neoadjuvant chemotherapy ($P = 0.04$, Wilcoxon signed-rank test). No statistically significant TP nuclear staining changes were observed in response to treatment ($P = 0.98$, Wilcoxon signed-rank test). In addition, stromal TP expression decrease was seen after chemotherapy, although it was not statistically significant ($P = 0.07$).

An increase in TP cytoplasmic tumor expression score was observed in 33% [95% confidence interval (CI): 19–50%] of patients after neoadjuvant chemotherapy (McNemar test, $P = 0.01$). Notably, increases in cytoplasmic TP score were more common in response to taxane-containing regimens (44%, 95% CI: 26–63%) than in regimens without taxanes (25%, 95% CI: 5–57%). The difference between the two treatment groups, however, was not statistically significant (Fisher's exact test, $P = 0.30$). Association between hormonal receptors, HER-2 status and TP stromal expression is described in Table 5. An association was observed between histological grade and TP stromal expression: grade 3 tumors resulted more frequently associated with higher TP immunoreactivity than grade 2 and 1 tumors (mean scores = 2.28, 1.43 and 2.00, respectively; $P = 0.06$, Kruskal–Wallis test).

Table 4 Thymidine phosphorylase changes in response to neoadjuvant chemotherapy

TP expression	Patients with both pretreatment and posttreatment measurements (*N*)	Difference (posttreatment – pretreatment)	
		Mean[a]	Wilcoxon signed-rank test *P* value
Nuclear (%)	37	2.02	0.98
Cytoplasmic (score)	39	0.28	0.04
Stromal (score)	37	– 0.30	0.07

TP, thymidine phosphorylase.
[a]Positive values indicate TP increase after chemotherapy.

Table 5 Association between hormonal receptors, HER2 status and TP stromal expression

Prognostic factor	TP expression			
	Mean score		Percentage of positive tumors	
ER-positive	1.55	$P=0.01$	45	$P=0.01$
ER-negative	2.41		82	
PGR-positive	1.43	$P=0.01$	39	$P=0.01$
PGR-negative	2.24		76	
HER-2-positive	2.25	$P=0.04$	75	$P=0.05$
HER-2-negative	1.57		46	

ER, estrogen receptor; PGR, progesterone receptor; TP, thymidine phosphorylase.

A statistically significant correlation was found between stromal TP expression and proliferative activity (MIB-1 immunostaining) of tumor cells ($\rho = 0.42$, $P \leq 0.01$, Spearman's rank test).

According to the RECIST, five patients experienced a complete clinical response, 25 patients a partial response and 25 a stable disease. According to Chevallier classification, five patients obtained a complete pathologic response (grade 1), whereas 36 cases were classified as 'grade 3' and 12 cases as 'grade 4'. In two cases, patients underwent surgery in other hospitals and were lost at follow-up.

No significant association was observed between clinical or pathological response rate and TP expression in both

tumor and stromal cells. Similarly, no association was found between response rate and TP changes in both tumor and stromal cells.

Discussion

In the treatment of breast cancer, ER and PGR as well as HER-2 oncoprotein have been found to be important predictive factors of response to endocrine therapy and trastuzumab, respectively. Accordingly, these anticancer agents have been defined as 'targeted therapy' as their activity is dependent on a molecular target preferentially expressed by the tumor cells.

As recently pointed out by George Sledge [3], another important prerequisite to define a therapy as 'targeted' is the availability of routine methods that allow either quantitative or qualitative measurement of the target.

Several studies in breast and gastrointestinal carcinomas investigated the role of TP as a potential predictive factor of response to fluoropyrimidines and especially to capecitabine [10,11,23]. TP is an essential enzyme involved in the activation of the prodrug capecitabine to the active form 5-FU. Owing to the higher TP expression in tumor tissues compared with healthy counterpart, tumor-selective generation of 5-FU from capecitabine has been hypothesized.

Previous studies in mammary tumor animal models showed that several anticancer agents, such as taxanes, cyclophosphamide and mitomycin C can cause upregulation of TP [12,17]. More recently, similar observations were reported by two independent research groups that described TP changes in breast carcinoma cells in response to neoadjuvant chemotherapy. In particular, Kurosumi *et al.* [24] described enhancement of tumor immunoreactivity for TP in a small series of patients who received single-agent docetaxel for locally advanced breast cancer. Similarly, Toi *et al.* [13] demonstrated TP upregulation in epithelial and stromal tumor cells of 99 patients with operable breast cancer preoperatively treated with different anthracycline and taxane-based chemotherapeutic regimens.

Our study provided further evidence that, at least in breast cancer, TP is upregulated after anthracycline and/or taxane-containing chemotherapy. However, differently from Toi *et al.* [20], we, did not observe any significant increase of TP expression in stromal cells. Although no clear explanation of this difference is available, either different immunohistochemical score systems and techniques or inter-pathologist variability (i.e. reproducibility issues) in discriminating tumor stroma could be advocated as possible causes. On the other hand, these results could indicate a more selective TP-modulation with higher enhancement of the enzymatic expression in tumor than in stromal cells. Moreover, as previously reported [20,13], the absence of correlation in TP status between cancer cells and stromal tissue may suggest an intrinsic and biologically different role of the enzyme in these two components.

In fact, Nagaoka *et al.* [25] demonstrated that TP expression in breast cancer cells did not correlate with any prognostic factors, whereas TP-immunoreactive stromal cells were associated with angiogenesis.

Our observation of a significant association between TP expression in stromal cells and a more aggressive tumor phenotype (absence of ER and/or PGR immunoreactivity, HER-2 overexpression, high proliferative activity) further supports the existence of a different biological meaning of TP activity according to the tissue compartment. Notably, it is tempting to hypothesize a link between these findings and those of previous studies reporting that inflammatory cytokines (i.e. interferon-α, TNF, etc.) and other micro-enviromental factors may upregulate TP expression in various cell lines, especially in macrophages [26,27]. In turn, breast cancer infiltration by TP-positive macrophages was reported to highly correlate with increased vascular endothelial growth factor expression and microvessel density [28]. Indeed, Han *et al.* [16] recently reported the results of a translational study in patients with nonsmall cell lung carcinomas treated with capecitabine plus docetaxel. They found that the tumor response to chemotherapy inversely correlated to stromal TP expression (mainly resulting from TP-stained macrophages). In contrast, tumor cell TP expression was significantly associated with tumor response and better prognosis.

Recently, several clinical studies supported the hypothesis of a synergistic effect between TP-modulating (i.e. taxanes) and TP-targeting (i.e. capecitabine) anticancer agents [16,18], whereas others were specifically designed on the basis of this rationale [14,15]. Although further prospective confirmation of chemotherapy-induced TP modulation is warranted, this strategy appears particularly appealing in improving the therapeutic index of capecitabine-based regimens.

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