# Thymidine Kinase and Thymidylate Synthase in Advanced Breast Cancer: Response to Tamoxifen and Chemotherapy[1]

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## ABSTRACT

Thymidylate synthase (TS) is a crucial target for 5-fluorouracil (5-FU) in the *de novo* pathway of pyrimidine synthesis, which is necessary for DNA synthesis. Thymidine kinase (TK) plays a key role in the complementary or alternative salvage pathway of pyrimidine synthesis in acute or pathological tissue stress. In the present study, the activity levels of TS and TK were determined in 257 primary breast tumors of patients who received tamoxifen as first-line systemic therapy after diagnosis of advanced disease. In 155 (60%) responding patients, the median response duration was 23 months for tumors with low TK activity, 15 months for tumors with intermediate TK activity, and 13 months for tumors with high TK activity ($P = 0.003$). In Cox multivariate analysis corrected for classical predictive factors including estrogen receptor and progesterone receptor, patients with intermediate and high levels of TK activity in their tumors showed a rapid disease progression ($P = 0.0002$) and an early death ($P = 0.002$) after start of tamoxifen treatment. Tumor TS activity levels were not significantly associated with the efficacy of tamoxifen treatment. In 121 patients who became resistant to tamoxifen or additional endocrine treatments and who received 5-FU-containing polychemotherapy, tumor TK activity was not significantly related to the efficacy of chemotherapy. Of the 13 patients with low tumor TS activity, only 1 (8%) responded favorably, whereas 46% (43 of 93) of those with intermediate and 73% (11 of 15) of those with high TS activity responded ($P = 0.001$). In Cox multivariate regression analysis in which TS was the only significant variable, intermediate and high TS activities were associated with a slow disease progression ($P = 0.005$) and prolonged survival ($P = 0.016$) on chemotherapy. In conclusion, for patients with recurrent breast cancer, high tumor TK activity is a significant marker of poor clinical outcome on tamoxifen therapy. Elevated tumor TS activity predicts a favorable outcome for 5-FU-containing polychemotherapy when applied after tumor progression on endocrine therapy.

## INTRODUCTION

The importance of tumor proliferation in determining response to chemotherapy and endocrine treatments has been clearly established in breast cancers. Although patients with a high growth fraction are likely to benefit from chemotherapy (1), patients with slowly proliferating tumors are likely to benefit from endocrine therapy (2). Pyrimidine incorporation is a limiting step for DNA replication. In cellular S phase, two pathways must simultaneously or independently be involved, the so-called *de novo* and salvage pathways of pyrimidine synthesis. The proliferation rates of breast cancers have been analyzed with various methods, such as the S-phase fraction or Ki-67 index, that do not discriminate between the *de novo* and salvage pathways of pyrimidine synthesis. Such discrimination would be important to predict more precisely individual resistance or sensitivity

of the patients. TS[3] and TK are key enzymes for pyrimidine synthesis. In the *de novo* pathway TS catalyzes the reductive methylation of dUMP to dTMP, using 5,10-methylene tetrahydrofolate as cofactor. In the salvage pathway, TK directly catalyzes the phosphorylation of deoxythymidine released from cells by DNA catabolism (3). Expression of both enzymes has been associated with proliferation of breast cancer cells *in vitro* (4, 5) and with proliferation indices *in vivo* (6, 7). Two different forms of TK can be distinguished, a fetal isoform, which is located in the cytoplasm and at the molecular level is cell cycle-regulated (8); and an adult isoform, which is located in the mitochondria. High TK levels have been observed in fast-growing tissues (9) and in target organs (uterus and prostate) after stimulation by their corresponding hormones (10, 11). TK expression might correlate indirectly with neoangiogenesis because the catabolic products of TP, which plays a key role in the supply of TK substrate, are angiogenic (12). In primary breast tumors, TK levels were related to the synthesis of the fetal cytosolic isoenzyme and were found to be elevated in patients who developed a recurrence (13). TK has been shown to be an independent factor of poor prognosis in node-negative breast cancer patients not treated with adjuvant therapy (14, 15). TS is the target for fluoropyrimidines, such as 5-FU. In tumor cells, 5-FU is converted to 5-fluoro-dUMP, which in the presence of 5,10-methylene tetrahydrofolate, inactivates TS by formation of a covalent complex with the active site of TS (16). In node-positive breast cancer patients receiving adjuvant chemotherapy that includes 5-FU, TS was suggested to be related to a favorable response, whereas high TK levels were correlated with increased risks of relapse and death (15, 17). The efficacy of endocrine therapy might also be related to the tumor levels of DNA synthesis enzymes. In nude mouse MCF-7 breast cancer xenografts, tumor TS levels were reduced by treatment with a pure antiestrogen (18), and in breast cancer cells *in vitro* the TK gene has been shown to be under transcriptional control of estrogens and antiestrogens (4). At present, no studies of patients with advanced breast cancer are available in which tumor TK and TS levels have been correlated with the efficacy of systemic endocrine and subsequent chemotherapy. TK can be released from the tumor into the serum, and it has been shown in advanced breast cancer patients treated with endocrine therapy that serum TK levels were significantly lowered in the responding patients, whereas in patients who progressed serum TK levels were increased (19).

High tumor TK activities may be indicative of a poor response to antimetabolic chemotherapy protocols (15, 17). TS has been suggested to be related to the natural history of the disease (17), and endocrine therapy merely targets the cell cycle but experimentally does not select for the *de novo* and salvage pyrimidine pathways of DNA synthesis. We therefore wished to study clinically whether the efficacy of tamoxifen treatment could be related with TK or TS activity, thus involving some specific pathways uncontrolled by steroid hormone receptors. The aims of the present study were also to

investigate in patients with advanced breast cancer the relation of tumor TS expression to the efficacy of polychemotherapy including 5-FU after the occurrence of endocrine resistance.

## MATERIALS AND METHODS

**Patients and Treatment.** The medical ethics committee of the University Hospital Rotterdam approved our study design. A series of 257 patients with primary operable breast cancer who underwent resection of their primary tumors between 1979 and 1991 and who showed a recurrence that was treated with tamoxifen was selected. The median age of the patients was 62 years (range, 29–92 years), and 197 patients (77%) were postmenopausal. The dominant site of relapse was soft tissue in 35 patients (14%), skeletal in 119 patients (46%), and visceral in 103 patients (40%); 178 patients (69%) had a disease-free interval of >12 months between primary tumor removal and first recurrence. None of the patients had received neoadjuvant therapy, had been exposed to hormonal adjuvant treatment, or had been treated with prior systemic treatment for advanced disease. Only 43 patients had been treated with adjuvant polychemotherapy (CMF in 23 patients; FAC or 5-FU plus cyclophosphamide plus epirubicin in 20 patients). Irrespective of ER status, all 257 patients received tamoxifen (40 mg daily) as first-line endocrine therapy after diagnosis of advanced disease. The median follow-up of patients still alive after start of tamoxifen treatment was 47 months (range, 4–100 months); fifty-six patients were still alive at the end of the present study, whereas 201 patients had died. On tamoxifen therapy, tumor progression occurred in 235 patients (91%) during follow-up. Of these patients, 175 were subsequently treated with one or more additional hormonal agents (mostly high-dose progestins), and to date, 121 patients have received systemic 5-FU-containing polychemotherapy (CMF in 78 patients, FAC in 43 patients) after the development of hormonal resistance.

The length of PFS was defined as the time from the start of treatment for advanced disease until the start of next treatment because of progressive disease or until the time of intercurrent death. All patients were assessed by standard Union International Contre Cancer criteria for complete and partial response (objective response). Patients with no change for >6 months (stable disease) have a OS similar to patients with partial response (20, 21). Therefore, for overall response, objective response and stable disease were combined.

**Tumors and Assays.** Tumors were collected and routinely assayed at the time of surgery for ER and PgR levels according to European Organization for Research and Treatment of Cancer guidelines, as described previously (22). When we used 10 fmol/mg of cytosolic protein as cutoff, 84% of the tumors were classified as ER positive and 74% as PgR positive. For TK and TS assays, new cytosols were prepared from stored tissues (liquid nitrogen) in European Organization for Research and Treatment of Cancer buffer containing 4 mM ATP and 8 mM MgCl$_2$ as has recently been recommended for stability (23). After it had been established that there was linearity between the activity levels in serial dilutions of a series of breast cancer cytosols, TK activity levels were measured with the adapted TK-REA assay (Ref. 23; kits were kindly provided by AB Sangtec Medical, Bromma, Sweden), and TS activity levels were measured with a tritium-release assay as described previously (17).

**Statistical Analysis.** The associations between ER, PgR, TK, and TS were studied with Spearman rank correlations ($r_s$). The nonparametric Kruskal-Wallis test was used to study the associations between TS and TK (used as continuous variable) with tumor size and nodal status at primary surgery and differentiation grade (used as grouping variables). The relation of response to therapy was examined with logistic regression analysis. Isotonic regression analysis (24) was applied to define cutpoints for TK and TS after it had been established in a test for trend using log-transformed TK and TS activity values that high TK activity was associated with poor PFS on tamoxifen therapy ($P = 0.002$) and that low TS activity was associated with a more rapid disease progression on chemotherapy after endocrine resistance ($P = 0.05$). Both uni- and multivariate analyses were performed using the Cox proportional hazards model. The assumption of proportional hazards was verified graphically. RHRs were calculated and presented with their 95% CIs. The likelihood ratio test in the Cox regression models was used to test for differences and for interactions. Survival curves were generated using the method of Kaplan and Meier, and the log-rank test for trend was used to examine survival data. All $P$ values are two-sided.

## RESULTS

**Levels and Associations.** The activity levels of TS and TK in the 257 analyzed tumors were 0–805 fmol [$^3$H]$_2$O/min/mg of protein (median, 24 fmol [$^3$H]$_2$O/min/mg of protein) for TS and 0–26.4 units/mg of protein (median, 0.41 units/mg of protein) for TK. TS and TK activity levels were not significantly correlated with the size or differentiation grade of the tumor or with nodal status at primary surgery. TS and TK activity levels were negatively correlated ($r_s = -0.26$; $P < 0.0001$), and those of ER and PgR were positively correlated ($r_s = 0.44$; $P < 0.0001$). There was no correlation between the activity levels of TS or TK with those of ER or PgR. Using isotonic regression analysis for both TS and TK we defined two cutpoints, 4 and 84 fmol [$^3$H]$_2$O/min/mg of protein for TS, and 0.16 and 0.93 units/mg of protein for TK.

**Response to Tamoxifen Treatment.** Of the 257 patients, 155 (60%) responded to tamoxifen. The median duration of response was 23 months in objective responders ($n = 47$; range, 3–84 months) and 15 months for stable disease ($n = 108$; range, 6–66 months). The ER status was known for 253 patients; the response rate in patients with ER-positive tumors was 65% (140 of 216), whereas 35% (13 of 37) of patients with ER-negative tumors responded favorably. The levels of TS activity were not significantly related to the rate of response (median, 24 fmol [$^3$H]$_2$O/min/mg of protein in both responders and nonresponders) or the length of PFS. A trend could be observed only between TS activity and the duration of response and the length of OS after start of tamoxifen treatment. In the 155 responding patients, the median duration of response was 12 months for tumors with low TS activity levels (21 patients), 16 months for patients with intermediate TS activity levels (113 patients), and 23 months for those with high TS activity (21 patients; $P = 0.08$). Compared with patients with low tumor TS activity, the RHRs were 0.71 (95% CI, 0.48–1.07; $P = 0.10$) for those with intermediate and 0.63 (95% CI, 0.37–1.07; $P = 0.09$) for those with high TS activity, respectively.

In the 155 responding and the 107 nonresponding patients, the median tumor TK activity levels were 0.34 and 0.53 units/mg of protein, respectively ($P = 0.08$). Compared with the 64 patients with low tumor TK activity levels (70% response), the 79 patients with high activity levels showed a poor response to tamoxifen treatment, with 52% of the patients responding (odds ratio, 0.46; 95% CI, 0.23–0.91; $P = 0.03$). In the 114 patients expressing intermediate tumor TK activity, the response rate was 61% (odds ratio, 0.65; 95% CI, 0.34–1.25; $P = 0.19$; overall $P = 0.08$). The amplitude of the relation of TK activity levels with a poor response rate to tamoxifen treatment was mostly present in the ER-positive subgroup of patients. Of 57 ER-positive tumors with low TK activity, 42 (74%) responded, whereas 37 of 67 (55%) tumors with high TK activity responded. Of 92 ER-positive tumors with intermediate TK activity, 61 (66%) responded (overall $P = 0.09$). The response rates in ER-negative tumors with low ($n = 7$), intermediate ($n = 20$), and high ($n = 10$) levels of TK activity were 43, 35, and 30%, respectively (overall $P = 0.86$).

Additionally, the duration of response was inversely correlated with the level of tumor TK activity. In the 155 responding patients, the median duration of response was 23 months for tumors with low TK activity (45 patients), 15 months for tumors with intermediate TK activity (69 patients), and 13 months for tumors with high levels of TK activity (41 patients; $P = 0.003$). In Kaplan-Meier analysis of all 257 patients, when compared with tumors with low TK activity, those with intermediate and high TK activity showed a shorter PFS ($P = 0.0001$; Fig. 1A) and an earlier death after start of tamoxifen treatment ($P = 0.007$; Fig. 1B). The results of the Cox multivariate analysis (Table 1) show that, corrected for the classical predictive factors, TK was an independent variable to predict a poor PFS
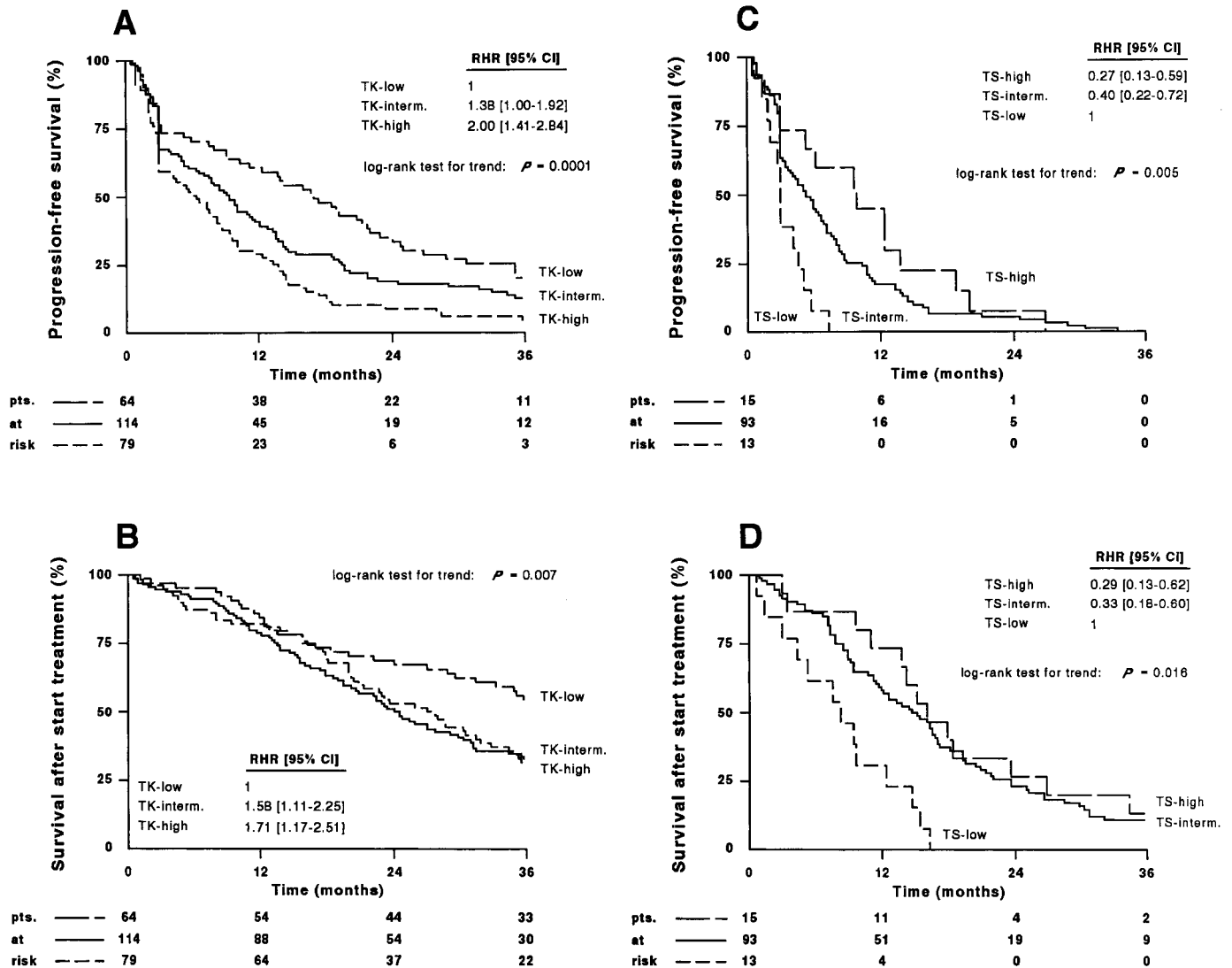
## Tamoxifen

## Chemotherapy



Fig. 1. PFS (*A*) and OS (*B*) after start of tamoxifen therapy as a function of the level of TK, and PFS (*C*) and OS (*D*) on chemotherapy after hormone resistance as a function of the level of TS. The numbers of patients at risk are indicated. *Interm.*, intermediate; *pts.*, patients.

($P = 0.0002$) and OS ($P = 0.002$). In a separate multivariate analysis with ER and PgR included as log-transformed continuous variables instead of dichotomized variables, the estimators of the effect of intermediate and high TK activity did not change. Furthermore, when ER, PgR, and TK were all analyzed as log-transformed continuous variables, TK remained an independent variable to predict early disease progression ($P = 0.0005$) and death ($P = 0.002$). The level of TS activity did not contribute to the multivariate models (data not shown). No statistically significant interactions of the activity levels of TK with those of TS, ER, or PgR in multivariate analyses for PFS and OS were observed.

**Response to Chemotherapy.** Of the 121 patients who received chemotherapy after failure to endocrine treatment(s), 55 (45%) responded. The median duration of response was 9 months for objective responders ($n = 22$; range, 5–34 months) and 11 months for stable disease ($n = 33$; range, 6–30 months). Irrespective of the type of treatment (CMF or FAC), there were no significant relationships between the levels of TK activity and the efficacy or duration of response to chemotherapy or length of PFS or OS.

Of the 13 patients with low TS activity levels, only 1 (8%) responded, whereas 43 of 93 (46%) of those with intermediate levels and 11 of 15 (73%) of the patients with high tumor TS activity levels responded ($P = 0.001$). In Cox univariate analyses of all 121 patients, when compared with patients with low tumor TS activity levels, those with intermediate and high levels were associated with a prolonged PFS ($P = 0.005$; Fig. 1*C*) and an improved OS ($P = 0.016$; Fig. 1*D*). In Cox multivariate analysis for PFS and OS, TS was the only significant variable.

## DISCUSSION

The benefits of endocrine treatment of breast cancer have been widely accepted. Nevertheless, ~50% of the tumors do not respond to (anti)hormonal treatment. Similarly, ~50% do not respond to chemotherapy. In the systemic treatment of patients with advanced breast cancer, the occurrence of acquired therapy resistance in responding patients is a major problem. Most patients will initially be treated with endocrine treatment, mainly tamoxifen, and the question remains

Table 1 *Multivariate analysis in tamoxifen-treated patients*

| | PFS | | OS | |
|---|---|---|---|---|
| | *P* | RHR[a] | *P* | RHR[a] |
| Age and menopausal status | 0.005[b] | | 0.014[b] | |
| Age premenopausal[c] | | 0.52 (0.32–0.87) | | 0.40 (0.23–0.70) |
| Age postmenopausal[c] | | 0.84 (0.72–0.97) | | 0.94 (0.81–1.10) |
| Post *vs.* premenopausal | | 1.91 (1.02–3.60) | | 2.23 (1.11–4.49) |
| Visceral metastasis (*vs.* skeletal and soft tissue) | 0.099 | 1.26 (0.96–1.65) | <0.0001 | 1.88 (1.40–2.53) |
| Disease-free interval (>12 *vs.* ≤12 months) | 0.002 | 0.56 (0.41–0.75) | <0.0001 | 0.45 (0.33–0.62) |
| ER/PgR (+/+ *vs.* others)[d] | 0.008 | 0.66 (0.49–0.89) | <0.0001 | 0.43 (0.31–0.58) |
| TK | 0.0002[e] | | 0.002[e] | |
| TK-intermediate *vs.* TK-low[f] | | 1.47 (1.05–2.07) | | 1.69 (1.17–2.44) |
| TK-high *vs.* TK-low[f] | | 2.15 (1.50–3.09) | | 1.91 (1.29–2.83) |

[a] 95% CI in parentheses. The final multivariate models included 250 patients.
[b] Age and menopausal status combined.
[c] Tested in decades, separately for pre- and postmenopausal patients.
[d] +/+, ER and PgR ≥10 fmol/mg of protein (*n* = 180) *vs.* combined one (*n* = 24) or both <10 fmol/mg of protein (*n* = 46).
[e] TK-intermediate and TK-high combined.
[f] TK-low, ≤0.16 units/mg of protein; TK-intermediate, >0.16–0.93 units/mg of protein; TK-high, >0.93 units/mg of protein.

which treatment could be most effective after the occurrence of endocrine resistance. The main aim of the present study was to investigate whether in the future the knowledge of tumor TK and/or TS activity status could be helpful in designing individualized treatment strategies. In this report, we focused on the enzymes TS and TK, which play key roles in the *de novo* pyrimidine synthesis and the salvage DNA synthesis pathways, respectively, because specific chemotherapeutic agents interfere with these pathways.

Evaluation of the clinical importance of DNA-synthesizing enzymes in breast cancer showed that TK activity is significantly associated with the natural history of the disease. A high prognostic value was found with respect to disease-free survival and OS (7, 14, 15), especially in node-negative patients receiving no systemic adjuvant therapy (14). In an analysis of whether TK and TS are predictive factors for drug sensitivity in patients receiving 5-FU-containing adjuvant chemotherapy, our group showed an increase in the average time to relapse with decreasing levels of TK (15, 17). The subsequent question regarding the potential association of TK and TS activity with the efficacy to tamoxifen and chemotherapy in patients with advanced cancers was investigated in the present study.

In our breast cancer patients, TS and TK activity levels were log-normally distributed, in agreement with our previous study (17). In the present study, no positive correlation was found between the activity levels of TS and TK, suggesting that both pathway of DNA synthesis may take over each other's function. In some tumors, however, synthesis was not clearly predominant in one pathway. Data obtained in experimental models may support these observations. Indeed, a coordinated regulation of TK and TS, *i.e.,* a prolonged decrease in TK activity that coincides with TS inhibition, has been described in one subtype of murine colon cancer. (25). In contrast, an uncoordinated variation, *i.e.,* a rapid return of TK activity to the control level, was observed in another subtype (25). Thus, for tumors that escape TS blocking by agents such as 5-FU through a fast TK increase, blocking of both the TS and TK pathways should be targeted.

In the present study, we found no significant correlation between TK activity and the levels of ER. Only weak negative relationships (13) or an absence of association (14, 26) have been described previously, and also by us in specific subgroups of patients (7, 17). Absence of a correlation with ER was also observed in the present study for TS, in agreement with previous studies (6, 17, 27). In MCF-7 breast cancer cells *in vitro*, it has been shown that the expression of TK is transcriptionally regulated by estrogen and antiestrogens (4). The lack of an association between TK activity and ER levels in breast tumors may reflect the heterogeneous nature of breast

tumors or the absence of the transcriptionally regulatory mechanism *in vivo*. It may also suggest that in breast cancers additional mechanisms are involved in TK regulation. In this respect, the E2F family of proteins plays a key role in progression of cells from the late $G_1$ into S phase. These E2F family members (E2F-1 through E2F-5) regulate transcription of genes that encode protein products that are required for DNA synthesis, such as TK, TS, dihydrofolate reductase, and ribonucleotide reductase (28). On the other hand, c-Myc has continued to emerge as centerpiece of cancer biology because of its ability to enhance the activities of specific enzymes involved in DNA metabolism, such as TK, and other metabolic pathways (29). Furthermore, *in vitro* studies have shown that activation of c-Myc specifically induced TK mRNA expression and enzyme activity throughout the cell cycle (30).

We showed that high activity levels of TK, independent of ER and PgR status, were related with a poor clinical outcome on first-line tamoxifen therapy. These results are consistent with the reported benefit of endocrine treatment in slowly proliferating breast cancers (2). Thus, the inhibitory effect of tamoxifen on tumor growth as a result of a blockade of ER function has likely been abrogated by the presence of high TK levels. There is only one very small study in the literature (12 patients) concerning TK and tamoxifen therapy (26). No conclusions can be drawn from that study because the patients also received different kinds of endocrine treatment (26). In our present study, the patients who failed directly on first-line tamoxifen treatment and those who showed a short duration of response when initially responding had the highest tumor TK activity levels. In hormone-refractory patients with relatively high tumor levels of TK, subsequent 5-FU-containing polychemotherapy failed in the small group of patients with high tumor TS activity levels. Although this first report should be interpreted with caution until it has been confirmed, this suggests that 5-FU will be effective only when its target protein is present in sufficient amounts, irrespective of the levels of TK. No comparable studies involving hormone-resistant advanced breast cancer patients are available in the literature. In breast cancers it was shown in a small study that treatment with 5-FU caused an increase in tumor TS levels and that increased free TS levels may cause 5-FU resistance (31). In the study of Pestalozzi *et al.* (27), in which TS expression was assessed by immunohistochemistry, node-positive patients with high TS expression demonstrated the most significant benefit of adjuvant CMF therapy. Furthermore, in rectal cancers, adjuvant 5-FU-based chemotherapy significantly improved disease-free survival and OS for patients with high TS levels, whereas it did not improve prognosis in patients with low levels (32). In other types of tumors in which TK measurements were not performed

simultaneously, such as colorectal cancer (33, 34), gastric cancer (34), and head and neck cancer (35), higher tumor TS levels were almost uniformly found in nonresponding patients or were associated with poor clinical outcome.

Concerning TK and chemotherapy, in the small study of Zhang *et al.* (26), higher levels were found in the tumors of 17 responding patients compared with those of 28 patients who did not respond. However, these data are difficult to interpret because the investigators included predominantly ER-negative patients who received a very heterogeneous mixture of chemotherapy regimens and who were not yet hormone refractory. In the present study, in hormone-refractory patients who already had the highest levels of tumor TK activity, the level was not further related to the efficacy of subsequent chemotherapy. Previously, we showed that in patients with primary breast cancer who were treated with adjuvant CMF or FAC, high TK activity levels were associated with a poor prognosis, which suggested that high levels of TK were associated with failure of antimetabolic chemotherapy protocols (15, 17). It thus may be a selective factor for innovative treatment protocols including, *e.g.*, taxanes. In our present study, we showed that for patients with recurrent breast cancer, high tumor TK activity levels are associated with a poor efficacy of tamoxifen therapy. We also showed for the first time a small subgroup of patients with high tumor TS activity who responded favorably to polychemotherapy that included 5-FU after they had become hormone refractory.

Our results suggest that in the future, treating patients individually based on the tumor levels of TK and TS may be considered. In addition, for hormone-refractory patients, administration of 5-FU or new TS inhibitors, which presently are under clinical evaluation, could be considered for tumors with high TS activity levels, whereas treatment with other drugs, such as taxanes, could be more effective in patients with high tumor TK activity levels but a less active *de novo* pyrimidine synthesis pathway.

## ACKNOWLEDGMENTS

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