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The prognostic significance of thymidine phosphorylase, thymidylate synthase and dihydropyrimidine dehydrogenase mRNA expressions in breast carcinomas

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Summary. Thymidine phosphorylase (TP), thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) have been indicated as possible predictive markers for epithelial malignancies. All these three enzymes are actively involved in 5-FU metabolism. In this report, we investigated mRNA expression of these factors with real-time quantitative PCR in a series of 86 micro-selected breast carcinomas and 8 micro-selected tumour-adjacent normal breast epithelial specimens. Highly variable mRNA expressions of these factors were observed in both normal and cancerous samples. TP and TS mRNA expressions in breast carcinomas were elevated, but only TS mRNA expression showed a trend for statistical difference, compared with the expression in normal breast epithelial samples. Although the DPD mRNA expression range in tumours was also elevated, the average mean was reduced in tumours compared to that in normal samples.

No association between mRNA expressions of TP, TS and DPD and clinicopathological features such as histological grade, tumour size, node status, S-phase fraction, ploidy, and clinical stage was found. A negative association between DPD mRNA expression and age was, however, revealed. Ten-year follow-up analysis showed no association between TP and DPD mRNA expression and clinical outcome. An high level of TS mRNA expression, however, was associated with a shorter clinical survival, indicating its potential role as a clinical marker in breast carcinoma.

Key words: TP, TS, DPD, PCR, Breast carcinoma

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Introduction

Thymidine phosphorylase (TP), thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) interact with 5-FU pathways, indicating their predictor role for chemotherapeutic response (Lehman, 2002; Mizutaniet al., 2002; Nishimura et al., 2002; Rose et al., 2002; Anan et al., 2003). Furthermore, these enzymes have also been suggested to be potential prognostic markers.

The TP gene is located on human chromosome 22 (Hagiwara et al., 1991), and the product of this gene, previously known as platelet-derived endothelial cell growth factor (PD-ECGR), catalyses the reversible phosphorylysis of thymidine to deoxyribose-1-phosphate and thymine (Iltzsch et al., 1985; Krenitsky et al., 1981). TP plays an important role in the chemotherapeutic application of 5-FU because of its capability to catalyse the conversion of 5-FU to 5-fluoro-2-deoxyuridine (5-FdUR) and prodrug of 5-FU to 5FU (O'Brien et al., 1996). In various human solid tumours, different expression patterns of TP have been observed (Yang et al., 2002b) and linked to tumour progression, metastasis and prognosis (Salonga et al., 2000; Aishima Si et al., 2002).

TS is a DNA synthetic rate-limiting enzyme associated with cell division and proliferation. The TS gene is located on human chromosome 18p11.32 (Silverman et al., 1993). The TS enzyme catalyses the conversion of dUMP to dTMP, the critical source of thymidine nucleotides for DNA synthesis and repair .TS is also the target enzyme for 5-FU. Its activity is inhibited by the action of the 5FU metabolite and 5-fluoro-deoxyuridine-monophosphate (FdUMP). Competing with dUMP, FdUMP molecules occupy the same binding site on the TS protein through covalent force, preventing the binding of dUMP to TS. Accordingly, dUMP is deprived of the entry into DNA synthetic process and DNA synthesis is blocked. Recent

studies suggest that the variation of TS mRNA and protein expression in tumours has an important impact on both prognosis and drug-resistance for 5-FU-based chemotherapy (Cascinu et al., 1999; Edler et al., 2000; Salonga et al., 2000; van Triest et al., 2000).

The DPD gene is located on the centromeric region of human chromosome 1 between 1p22 and q21 (Takai et al., 1994). DPD is the initial and rate-limiting enzyme in the uracil and thymine catabolism, and also the principal enzyme responsible for degradation of 5-FU (Aishima Si et al., 2002; Diasio and Harris, 1989). High levels of DPD expression in tumour cells have been associated with low sensitivity to 5-FU chemotherapy (Diasio, 1999; Mizutani et al., 2001). In addition, DPD has also been indicated to be a potential prognostic marker in colorectal and breast cancers (Salonga et al., 2000; Horiguchi et al., 2002).

However, to date, only few reports about the expressions of TP, TS and DPD genes in breast lesions are available in literature. In this study we have comparatively quantified the mRNA expressions of these genes in a series of 86 breast carcinomas and 8 tumour-adjacent normal breast tissues, and correlated their expressions in tumours with clinicopathological factors such as age, lymph node status, stage, tumour size, histological grade, S-phase fraction, ploidy and survival etc, in order to better understand their possible clinical impact.

Materials and methods

Patients and tissue samples

Tumours from 86 patients with breast carcinoma and 8 normal breast specimens were included in the present study. Surgically removed tumour tissues from these patients were partly frozen at -70 ºC, and partly fixed in buffered formalin and embedded in paraffin blocks. All the tumour samples were consecutively collected at the Department of Pathology, Örebro Medical Center Hospital, Sweden. All the follow-up data were retrieved from the medical journals of the hospital.

Ten of the 86 patients received standard adjuvant chemotherapy with CMF (cyclophosphamide, methotrexate and fluorouracil) for 6 courses. The eight normal breast tissues were dissected from the surgically removed breast cancer samples at The Norwegian Radium Hospital, Norway, and storaged in freezer at -70 ºC until use. All the samples were from female patients and the age of the patients with tumour ranged from 32 to 91 years old, with a mean age of 64.5 years. Patients and tumour parameters are listed in Table 1.

Light microscopy

Hematoxylin and eosin-stained paraffin and frozen sections were re-evaluated, and histological classification was made, in accordance with the WHO criteria and the tumours were graded following the recommendation of Elston and Ellis (1991).

S-phase fraction analysis

The published method (Vindelov et al., 1983) and a Facscan cytometer were applied in this study. S-fraction was calculated with the method as reported (Baisch et al., 1982).

Microselection of tumour cells from frozen materials for RT-PCR

Microselection of tumour cells from frozen materials was made according to the published method (Suo et al., 2001). Briefly, to select small tumour areas without normal epithelial cells, 4 mm frozen sections were cut and were stained with hematoxylin and eosin. Under light microscopy, the morphology of the sections was examined and areas with tumour were carefully selected. Both the frozen block and the corresponding 4 mm frozen section were orientated and marked. According to the selected tumour cell area on the section, the frozen block was trimmed to leave only the selected area. The trimmed frozen block was then re-embedded with OCT compound (Tissue-Tek, Torrance, California, USA) and

Table 1. Clinicopathological variables in 86 breast carcinoma patients.

VARIABLE	No. OF CASES
Age	
30-39	3
40-49	14
50-59	17
60-69	10
70-79	21
80-89	20
>90	1
Histological subtypes	
Invasive ductal carcinoma	72
Invasive lobular carcinoma	11
Medullary carcinoma	2
Adenoid cystic carcinoma	1
Histological grades	
Grade 1	7
Grade 2	45
Grade 3	34
Lymph node status	
With metastases	49
No metastasis	34
Unknown	3
Tumour size	
≤ 19 mm	35
≥ 20 mm	50
Unknown	1
Stage	
Stage 1	35
Stage 2	46
Stage 3	3
Stage 4	1
Unknown	1

then another 4 μm frozen section was made to ensure that no normal epithelial cells were inside the selected areas. Then, 20 μm frozen sections were made and collected in liquid nitrogen-chilled Eppendorf tubes. After a serial of 20 μm sections (up to 8 sections, depending on the area selected), one additional 4μm frozen-section was made to control morphology. If normal glandular structures appeared, the sections were not used, and other areas were reselected for microselection. Since all these genes are expressed in both epithelial (both normal and cancerous) and stromal cells of the breast, the micro-selection method was applied in this study, so that high density tumour cell (more than 90%) areas without normal epithelial cells or high density adjacent normal epithelial structures without prominent stromal cells (in that more than 60% of the cells were epithelial cells) were collected. In addition, areas with either visible micro-vessels or numbers of macrophages, which were known with TP and DPD expression, were avoided by this selection method.

RNA isolation

Total RNA was isolated from the sections made from the microselected materials by using an RNeasy total RNA isolation kit (Qiagen, West Sussex, UK), following the supplier's protocol.

Real-Time Quantitative RT-PCR

LightCycler mRNA Quantification Kits for TP, Ts and DPD from Roche Diagnostics (Roche Diagnostics Gmbh, Mannheim, Germany) were applied in this study. A two-step RT-PCR procedure was conducted. First, complement DNA (cDNA) was reversely transcribed with 20 units of AMV reverse transcriptase, 0.08 units of random hexamer primers, and 0.4 mM dNTPs in a 20μl reaction volume from total RNAs for TP, TS, and DPD, respectively, with a conventional PCR machine with the following cycling conditions: annealing for 10 min at 25 ºC, reverse transcription for 60 min at 42 ºC and inactivation of reverse transcription for 5 min at 94 ºC. Thereafter, real-time quantitative PCR amplifications were performed in a 20μl reaction volume in glass capillaries by the use of target-specific, double-labelled fluorogenic probes and gene-specific primers in the faststart mixture provided by Roche Diagnostics (Roche Diagnostics Gmbh, Mannheim, Germany). The cycling profiles for DNA amplifications were as follows: pre-incubation at 95 ºC for 5 min; 40 cycles with denaturation at 95 ºC; annealing at 62 ºC; and extension at 72 ºC for 10 seconds, respectively, and then the reactions were cooled down for 30 seconds. Separate running for glucose-6-phosphate dehydrogenase (G6PDH) gene amplification was performed with the same cDNA as a control for RNA integrity and a reference for relative quantification in each reaction. The relative amount of TP, TS and DPD mRNA was calculated with the LightCycler relative quantification software version 1.0 (Roche Diagnostics Gmbh, Mannheim, Germany).

Statistical analysis

All the statistical analyses were performed with the software package SPSS for windows version 10.1 (SPSS Inc., Chicago, USA). The signifcance of differences among the means was determined by the independent Mann-Whitney test (two categories) and the one-way Kruskal-Wallis test of variance (three or more categories). Disease-free survival was determined as the interval between surgical resection and the first recurrence or metastasis or death. Cancer-specific survival was calculated as the time elapsed between surgical resection and the most recent clinical observation or death. The survival curves were constructed by the Kaplan-Meier method with the logrank test. The accepted level of significance was $p<0.05$. All p-values presented are two-sided.

Results

mRNA expression of TP, TS and DPD in tumours and neighbouring normal tissues

mRNA expression of TP, TS and DPD gene was highly variable among the specimens examined (Table 2). A broad range of TP mRNA expression in the normal breast tissues was seen, from the lowest, with 0.23, to the highest, with 51.44, relative copy numbers, showing a 233.7 -fold difference. Although elevated TP mRNA expression was present in the tumours, the TP expression pattern in tumours was similar to that in

Table 2. Comparative mRNA expression of TP, TS and DPD in tumour-adjacent normal and tumour tissues of breast.

	No. OF SAMPLE	MINUMUM VALUE	MAXIMUM VALUE	MEAN	STD. DEVIATION
TP (Normal)	8	0.23	51.44	15.9388	16.2642
TP (Cancer)	74	1.83	133.87	23.9932	24.3744
TS (Normal)	8	0.01	0.86	0.2663	0.2794
TS (Cancer)	85	0.03	4.36	0.5914	0.6912
DPD (Normal)	8	0.09	11.12	6.7900	4.3431
DPD (Cancer)	85	0.43	45.49	5.0734	6.7616

normal tissues, with a 73.15-fold difference. Comparatively, the lowest and the highest TP mRNA expressions in the tumours were 8- and 2.6-fold higher than those in normal tissues, respectively. However, no significant statistical difference of TP gene expression in normal and tumour tissues was observed (p=0.27).

TS mRNA expression in the normal and tumour samples showed an 86- and a 145-fold difference, respectively. Similarly, both the lowest and the highest TS mRNA expressions in the tumours were higher than the corresponding TS mRNA expressions in the adjacent normal tissues. The mean TS mRNA expression was increased from 0.27 in the normal to 0.59 in the tumour tissues. A statistical difference trend was seen for TS expressions in normal and tumour samples (p=0.067).

DPD mRNA expression in the normal and tumour tissues showed a similar difference: a 123.6-fold difference in normal and a 125.8-fold difference in tumour tissues, respectively. Although both the lowest and highest expressions in the tumours were elevated in comparison with those in normal tissues, most tumours demonstrated similar or lower DPD expression, giving a

Table 3. Correlation between TP, DPD, TS and TP/DPD mRNA expressions and clinicopathological features.

VARIABLE	CATEGORIES	TP		DPD		TS		TP/DPD	
		N	P-value	N	P-value	N	P-value	N	P-value
Age	≤ 69	43		44		43		43	
	≥ 70	41	0.67	41	**0.016**	42	0.61	40	0.90
Tumour size	≤ 19mm	35		35		35		35	
	≥ 20mm	47	0.11	48	0.30	48	0.42	46	0.44
S-phase fraction	≤ 6.3	44		44		44		44	
	> 6.3	32	0.58	34	0.80	33	0.24	32	0.29
Ploidy	Diplid	34		35		34		34	
	Aneuploid	46	0.68	46	0.28	47	0.81	45	0.45
Nodal status	Negative	49		48		49		48	
	Positive	32	0.36	34	0.85	33	0.15	32	0.23
Histological grade	Grade I	7		7		7		7	
	Grade II	45		45		45		45	
	Grade III	34	0.90	34	0.52	34	0.26	34	0.77
Stage	Stage I	35		35		35		35	
	Stage II	46		46		46		46	
	Stage III and IV	4	0.16	4	0.97	4	0.51	4	0.67

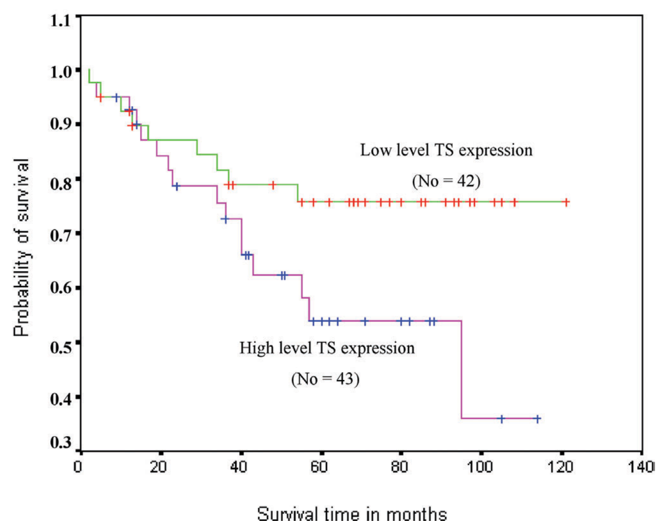

Fig. 1. Kaplan Meier plot of the effect of TS mRNA expression on disease-free survival for patients with breast carcinoma (p=0.069). Patients still alive or dead of other reasons than breast cancer are marked with a cross on the curves.

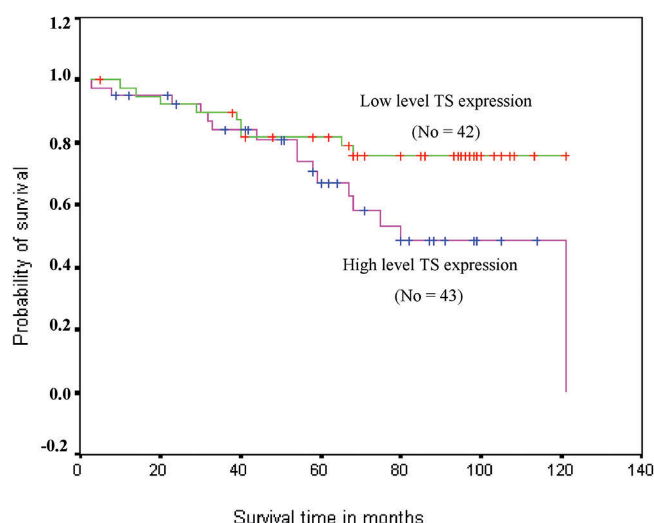

Fig. 2. Kaplan Meier plot of the effect of TS mRNA expression on overall survival for patients with breast carcinoma (p=0.049). Patients still alive or dead of other reasons than breast cancer are marked with a cross on the curves.

mean of 5.07 relative copy numbers in the tumours, compared with a mean of 6.79 relative copy numbers in the normal tissues. Such a difference was not statistically significant (p=0.158).

Relationships between TP, TS, DPD expression and clinicopathological features

Correlations between the classical clinicopathological factors (age, histological grade, node status, tumour size, stage, S-phase fraction and ploidy) and mRNA expressions of TP, TS and DPD and TP/DPD ratio in the 86 breast carcinomas are summarized in Table 3. Of all the factors studied, only age showed a statistically significant negative association with DPD expression (p=0.016). No correlation between TP, TS and DPD mRNA expression was observed.

Prognostic significance

Disease-free survial in the groups of patients with high and low TS mRNA expression in their tumours demonstrated a trend toward significance (p = 0.069) (Fig. 1). In addition, overall survival between high and low TS expression patients showed a statistically significant difference (p=0.0496), and patients with tumours with lower TS mRNA expression levels had a better clinical outcome (Fig. 2). There was no statistically significant difference in disease-free survival and overall survival between patients with tumours showing high and low TP and DPD mRNA expressions or high and low TP/DPD mRNA expression ratios. No survival (overall or disease-free) difference was found between patients received fluorouracil therapy and patients without fluorouracil therapy. However, the number of patients with chemotherapy was limited in the present series.

Discussion

In this study we have quantitatively characterized TP, TS and DPD mRNA expressions in a series of 86 breast tumours and 8 tumour-adjacent “normal” breast tissues.

Our results show a highly variable expression of these genes in both tumour-adjacent normal and tumour tissues. With a similar technique, Kanzaki et al. (2002) reported a very large variation in both UP and TP mRNA expressions in breast carcinomas, and reported a 1000-fold difference in UP expression. Unfortunately, they did not show the exact TP expression difference in the tumours, but Figure 3 in their report could indicate a 100-fold TP expression difference in breast carcinomas, which is in line with our present result. It should be borne in mind that highly variable TP gene expression also exists in the normal breast tissues, as shown here in our report. The highly variable TP expression in adjacent normal tissues may be caused by the lover purity in micro-selected normal samples. More elevated activity and gene expression of TP in many solid tumours (Takebayashi et al., 1996; 1999), including breast carcinomas (Fox et al., 1996; Toi et al., 1995; Yang et al., 2000, 2002a), than those in adjacent non-tumour tissues have been reported and have been confirmed in our present study in breast tissues.

Variable TS mRNA expression in different tumours has been observed (Horikoshi et al., 1992), with up to a 100-fold difference. We found a similar difference in TS mRNA expression in both normal and cancerous breast tissues. Although expression patterns in normal and cancerous tissues were also similar, higher levels of TS mRNA expression in the breast carcinomas (p=0.067) were observed, which is supported in the report by Nishimura et al. (1999). It should be noted that the adjacent normal tissues in our present study were not paired with tumours and were collected from different institution. This may influence the drawing of a conclusion . Furthermore, sample quality of adjacent normal tissues can be variable. The tissues are more heavily influenced by bio-factors secreted by the tumour cells which are located closer to carcinoma cells. Therefore, our present results should be explained with care when comparason of TS mRNA expression in normal and cancerous breast tissues is considered.

DPD activity in breast carcinomas, detected catalytically, has been reported to be increased, compared with that in adjacent normal breast tissues (Hamaji et al., 2000; Anan et al., 2003). However, systemic mRNA level studies in breast tissues are lacking. Here we have reported a variable DPD gene expression in both normal and cancerous tissues. Although the DPD expression range was elevated in the tumours, the mean of DPD expression in the tumours decreased compared with that in the normal tissues. This may be due to the limited number of normal tissue specimens examined or the presence of highly varible DPD mRNA expression. In addition, mRNA expression does not necessarily correlate with protein expression, as reported by Kawakami et al. (2001). Finally, samples for catalytical DPD activity examination reported by Anan et al. (2003) and Hamaji et al. (2000) could contain large portions of stromal cells, which might give a different DPD expression pattern, since DPD expresses in monnucler, stromal and plasma cells (Takenoue et al., 2000).

No clinicopathological association was found between mRNA expression of these enzymes and tumour size, tumour histological grade, lymph node metastasis, S-phase fraction, ploidy or clinical stage. No association was observed between TP/DPD ratio and the above factors either. Our results are supported by the findings of TP in breast carcinomas (Toi et al., 1995; Kanzaki et al., 2002). However, a negative association between age and DPD mRNA expression in breast carcinomas was seen (Table 3), and this feature may be of significance when DPD expression in relation to 5-FU-based chemotherapy is considered. This feature was not observed in the normal tissues, but the correlation

between age and DPD mRNA expression in normal breast tissues is still a matter of study because of the limited non-paired normal breast tissues used in our present study.

There was no difference in survival (overall or disease-free) for patients with fluorouracil therapy and patients without fluorouracil therapy in our present series. However, the limited number of patients with fluorouracil therapy did not permit us to draw a conclusion about the importance to predict fluorouracil therapy response and influence on survival in this study. It merits additional studies with a larger number of breast cancer patients treated with 5-FU-based chemotherapy.

We did not find any survival association between patients with high and low TP and DPD mRNA expressions. It has been reported that TP-positive tumors have a significant increase in both disease-free survival and overall survival when such patients treated with CMF adjuvant therapy and no significant difference in either disease-free survival or overall survival is observed in patients without CMF treatment (Yang et al., 2002a). Similarly, no significant difference between DPD immunostaining and tumour size, lymph node status, clinical stage, oestrogen receptor status, histologic grade, or 5-fluorouracil administration is observed in breast carcinomas reported by Horiguchi et al. (2002). But when evaluated in patients treated with 5-fluorouracil or 5-fluorouracil derivatives, patients with DPD-positive tumours show a significantly poorer disease-free survival compared to those with DPD-negative tumour (Horiguchi et al., 2002). Such observations from the above-mentioned reports support our negative association between TP and DPD mRNA expressions and survival in breast carcinoma patients since the majority of the patients in our present study did not receive chemotherapy.

We did, however, observe an association between TS mRNA expression and clinical outcome for breast cancer patients. A negative association trend with disease-free survival ($p = 0.067$) and a significant negative association with overall survival ($p = 0.049$) were revealed for TS mRNA expression. TS is the rate-limiting enzyme in the synthesis of pyrimidine nucleotides necessary for DNA synthesis. Elevated TS expression is reported to be a powerfully unfavourable prognostic marker for several epithial malignancies such as colorectal cancer (Johnston et al., 1994; Bathe et al., 1999; Corsi et al., 2002; Johnston et al., 1994), bladder carcinoma (Mizutani et al., 2001) and node-positive breast carcinoma (Pestalozzi et al., 1997). All these findings highlight a predictive value of TS mRNA and most probably TS protein as well, for clinical outcome in breast carcinoma patients.

In summary, highly variable mRNA expressions of TP, TS and DPD were detected in both normal and cancerous tissues in our present study. Comparatively, TP and TS mRNA expressions were elevated in breast carcinomas. There was no association between mRNA expressions of TP, TS and DPD and clinicopathological features such as histological grade, tumour size, node status, S-phase fraction, ploidy, and clinical stage but a negative association between DPD mRNA expression and age was observed. Ten-year follow-up analysis showed no association between TP and DPD mRNA expression and clinical outcome. An higher level of TS mRNA expression, however, was associated with a shorter clinical survival, indicating its potential role as a clinical marker in breast carcinoma.

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