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Predictive Role of Thymidine Phosphorylase Expression in Patients with Colorectal Cancer and its Association with Angiogenesis-related Proteins and Extracellular Matrix Components

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Abstract. *Background: Thymidine phosphorylase (TYMP) is an angiogenic factor that has potent chemotactic activity for endothelial cells and is involved in 5-fluorouracil (5-FU) metabolism. In colorectal cancer (CRC), previous studies evaluating the relationship between TYMP expression and clinicopathological features have yielded inconsistent results. The aim of this study was to investigate the prognostic value of TYMP, its association with other angiogenic factors, proliferation markers and, to our knowledge, for the first time its relationship with extracellular matrix components. Materials and Methods: Formalin-fixed, paraffin-embedded specimens from 97 patients with CRC were immunostained for TYMP, vascular endothelial growth factor (VEGF), microvascular density (CD34), proliferation marker (Ki-67), proliferating cell nuclear antigen (PCNA), p53 oncoprotein and extracellular matrix components (collagen type IV, fibronectin, tenascin and laminin). Survival curves were calculated with the Kaplan-Meier method. Results: Immunoreactivity was observed in the cytoplasm (cyt) and nucleus (n) of the tumor cells, as well in the stroma (st), endothelium and tumor-associated macrophages. High TYMPcyt expression was observed in 7.2% of the cases, moderate in 22.7% and weak in 59.9%, while 10.3% were negative. High TYMPst expression was observed in 58.8% of the cases. TYMPcyt expression was correlated with the VEGF expression of tumor cells and VEGF expression of vessels (p=0.014 and p=0.022, respectively). TYMPst expression was correlated with VEGF expression and tenascin (p=0.014 and p=0.011, respectively). Patients with higher TYMPcyt expression had a more favorable overall survival (p=0.041) in univariate analysis compared to patients without TYMP expression. Conclusion: These findings suggest that TYMP plays an important role in angiogenesis, extracellular matrix remodeling and in the prognosis of patients with CRC, but further studies are needed to clearly define its role in CRC.*

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Colorectal carcinoma (CRC) is the third most commonly diagnosed cancer and one of the leading causes of cancer-related deaths (1). The prognosis of CRC is dependent upon the extent of disease and approximately 60% of patients develop metastases after surgical resection. With a 5-year survival rate of less than 10% in patients with distant metastastatic disease, targeting of the metastatic process and sites should provide an effective treatment (1, 2). At present, the only curative treatment is surgical resection; however, it is often impossible to remove all cancer cells, especially those that have invaded the surrounding tissues. The penetration of tumor cells into lymphoid vessels and blood vessels leads to tumor metastasis and ultimately the tumor becomes fatal (2). The current major method for assessing the risk of metastatic recurrence and the need for adjuvant chemotherapy is to examine tumor resection specimens for evidence of metastasis to local lymph nodes. However, this approach may be of limited prognostic value as a sizeable fraction of CRCs have innate resistance to chemotherapy and 25% to 30% of the patients presenting with lymph node-negative tumors also develop fatal disease (3). The progressive growth of colonic cancer and the subsequent metastatic process is dependent on an angiogenic network. Thus, antiangiogenic strategies have emerged as effective therapies in patients with CRC, especially in the metastatic setting of the disease (4).

Tumor invasion and metastasis are the result of highly coordinated processes that involve multiple intracellular and extracellular factors. A large body of experimental and clinical evidence shows that tumor growth and metastasis depend on angiogenesis (5). Angiogenesis is a complex multistep dymanic process involving extravasation of plasma proteins, degradation of extracellular matrix (ECM), endothelial cell migration and proliferation, and capillary tube formation (5-7). It is now well-established that angiogenesis is orchestrated by a variety of activators and inhibitors that sequentially coordinate the complex series of processes in new vessel growth (8). Recent advances in vascular biology have identified some of the key factors that control vascular growth, including the intracellular enzyme thymidine phosphorylase (TYMP) (9). TYMP catalyzes the reversible phosphorolysis of thymidine and its analogs to their respective bases and 2-deoxyribose-1-phosphatase (10). TYMP has been demonstrated to be identical to platelet-derived endothelial cell growth factor (PD-ECGF), an endothelial cell mitogen initially purified to homogeneity from human platelets (10, 11). The mechanism by which PD-ECGF/TYMP induce angiogenesis has not been fully elucidated. Compared with adjacent normal tissues, TYMP activity has been reported to be increased in a variety of malignant tumor types including CRC, where it is expressed by both epithelial and stromal cells (11). Studies demonstrated that high TYMP expression is an indicator of worse prognosis in colorectal, gastric and breast cancer (12-14). TYMP not only serves as an indicator of angiogenic potential and a prognostic factor, but may also play an important role in cancer chemotherapy as a target for antiangiogenic agents (15, 16).

The prognostic role of TYMP as a predictor of the natural history of CRC has been assessed in several studies (11, 12, 15-20), but there are no data about its correlation with the ECM components. The ECM provides structural support for cells within a tumor, providing anchorage for cells and separating tissues; however, it also acts homeostatically to mediate communication between cells, and contributes to survival and promotion of differentiation signals (21). The ECM contains a basement membrane (BM) that separates cells from the interstitial matrix. At this junction, molecular components of the ECM can be found, including proteoglycans, non-proteoglycan polysaccharides, and various fibrous proteins (6). The carbohydrate polymers and proteins are organized in such a way that an interlocking meshwork exists and is the basic framework for the ECM (6). Alteration of ECM composition in cancer may be responsible for tissue remodeling processes and linked to cancer progression (21).

The objective of the present study was to demonstrate immunohistochemically the patterns of tumor cell TYMP expression, both cytoplasmic and nuclear (TYMPcyt and TYMPn, respectively) expression and stromal TYMP expression (TYMPst) in CRCs, to define its prognostic significance, and to establish any relationship with clinicopathological features such as: tumor type, histological grade, lymph node involvement, proliferative activity [(Ki-67) and proliferating cell nuclear antigen (PCNA)] and p53 expression. The identified alterations have been further correlated with tumor neovascularization assessed by CD34, and vascular endothelial growth factor (VEGF) expression, and finally with the expression of the ECM components tenascin (TN), fibronectin (FN), collagen type IV (Coll) and laminin (LN), in order to elucidate the interrelationships and the possible role of these proteins in colorectal carcinogenesis.

Materials and Methods

Patients. The study population consisted of 97 patients whose tumors were completely removed surgically at the University Hospital of Ioannina (Greece). None had received prior chemotherapy or irradiation. The patients included in this study had no other cancer. The clinicopathological characteristics of the 97 patients with colorectal adenocarcinoma, investigated in this study, are summarized in Table I. The average age at the time of diagnosis was 64.92 years (range=26-86 years). There were 53 (54.6%) males and 44 (45.4%) females. The majority of patients were ≥60 years old. Tumors were classified histopathologically according to the World Health Organization (WHO) criteria (22). When more than 50% of the tumor volume was mucin, the tumor was defined as mucinous carcinoma. Histopathological diagnosis was made routinely at the Department of Pathology of Ioannina University Hospital. All routine sections were carefully investigated to identify venous or lymphatic invasion. The largest diameter of the tumor was defined as the tumor size. The extent of tumor invasion/metastasis was based on the Astler and Coller modification of Dukes' classification system (22, 23). Dukes' A cases were those in which the growth was confined to the submucosa of the colorectal wall and these were not included in this study. In Dukes' B cases, the growth spread by direct continuity into the extracolorectal tissues, but the lymph nodes were free from metastases. Dukes' C cases were those in which lymph node metastases were found (22, 23). The mean observation time was 4.7 years (range=0.5-13.5 years), our of these patients, 33.7% had recurrence or distant metastases and 31.7% were dead from the disease or other causes after 5 years of follow-up.

Immunohistochemistry. Immunostaining was performed on formalin-fixed paraffin-embedded tissue sections (2-4-μm thick) by the labeled streptavidin-avidin-biotin (LSAB) method. In brief, tissue sections were de-paraffinized in xylene and de-hydrated. For the detection of TYMP, VEGF, CD34, p53 and Ki-67, slides were immersed in citrate buffer (0.1 M, pH 0.6) in plastic Coplin jars and subjected to microwave irradiation twice for 15 min. The heat-mediated antigen retrieval method was not used for PCNA staining. For the detection of TN, FN, Coll and LN, slides were pre-treated with 1 μl/ml pronase (Dako, Denmark) for 10 minutes at room temperature. Subsequently, all sections were treated for 30 min with 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity and then incubated with primary antibodies. Mouse primary monoclonal antibodies were incubated on tissue

Table I. *Patients' characteristics*

Variable	Patients	
	n	%
Gender		
Male	53	54.6
Female	44	45.4
Age		
<60 years	34	35.05
≥60 years	63	64.94
Differentiation grade		
Well	19	19.58
Moderate	67	69.07
Poor	11	11.34
Dukes' Stage		
B	56	57.73
C	41	42.26
Size		
≤5 cm	39	40.20
>5 cm	58	59.79
Tumor type		
Mucinus	21	21.64
Non-mucinous	76	78.35

Table II. *Antibodies used in this study*

Antibody	Supplier	Dilution	Incubation time
TYMP: (P-GF44C)*	Neomarker (USA)	1:800	Overnight
VEGF: (JH121)*	Neomarker (USA)	1:50	1 hour
CD34: (QBEnd/10)	Novocastra (UK)	1:50	1 hour
Tenascin (TN2)+	Dako (Denmark)	1: 50	1 hour
Fibronectin (clone, 568)+	Novocastra (UK)	1: 100	1 hour
Collagen IV (clone, CIV22)+	Dako (Denmark)	1: 50	1 hour
P53 (DO-7)*	Dako (Denmark)	1: 50	1 hour
Ki-67* (MD722)	Dako (Denmark)	1:10	1 hour
PCNA (MD879)	Dako (Denmark)	1:20	1 hour
Laminin (An No 078 P)*	Menarin (UK)	1:1000	1 hour

*With microwave oven antigen retrieva l+ Incubation with pronase.

sections overnight at 4˚C, then extensively washed in 0.05 M Tris-buffered saline (pH 7.6), before the addition of biotinylated secondary antibodies (goat anti-mouse). Sections were again washed and incubated with horseradish peroxidase-conjugated streptavidin (Dako; dilution 1:100) and the immunoreactivity was revealed by diaminobenzidine (DAB) substrate. The slides were counterstained in Harris' haematoxylin, dehydrated and mounted. To assess the specificity of the reaction, negative controls were included, where tumor sections were not incubated with the primary antibodies. The antibody sources and dilutions are shown in Table II.

Immunohistochemical evaluation. The immunostaining was assessed by two pathologists (AM, EI), from numerically-coded slides, without any knowledge of survival or other clinical data. The evaluation of immunostaining for TYMP and VEGF was performed separately in both the parenchyma (cytoplasmic and nuclear staining of tumor cells), and stromal cells. To evaluate the expression of tumor cells, we used a combined score corresponding to the sum of both staining intensity (0: negative; 1: weak; 2: moderate; 3: strong) and the staining extent, i.g. the percentage of positive cells (0: 0%, 1: 1-25%; 2: 26-50%; 3: >50%). The sum of both qualitative and quantitative immunostaining reached a maximum score of 8. The combined scores were then divided into four main groups: 1: no immunostaining, score 0; 2: low immunostaining scores 1-2; 3: moderate immunostaining, scores 3-4; 4: strong immunostaining, scores 5-8.

Microvessel count. Microvessel density (MVD) was assessed using the CD34 antibody as previously described (25). Briefly, individual or clusters of cells with or without lumens, positively-stained by antibody to CD34 were considered microvessels. The lumen diameter had to be smaller than approximately eight red blood cells. Areas of fibrosis, necrosis, and inflammation, and vessels with muscle wall were excluded from counting. In each tumor, five areas with the highest vascularization "hot spot" were selected. Individual microvessel counts were then made on ×400 field (corresponding to an area of 0.63 mm^2) by two independent observers (AM, EI). The average count from the two observers was used as the final score.

ECM. Rgarding the evaluation of ECM components, the tumors were classified as positive when there was unequivocal immunostaining of the matrix components in at least one representative area of the tumor. The positive tumors were semiquantitatively scored as +, ++, +++ corresponding to weak, moderate and extensive immunoreactivity, respectively.

Statistics. Superior Performance Software System (SPSS Chicago IL, USA), was used to compare morphological features and protein expression data. Significant differences between the expressions of the target proteins with regard to clinicopathological parameters were computed by the *t*-test for paired or non-paired values, or ANOVA test if the data were normally distributed. Disease-free survival (DFS) was calculated from the date of surgery to the date of relapse, or death in the case of patients who died without relapse. Overall survival (OS) was defined as the interval between surgery and death or the date of the last follow-up evaluation. Both DFS and OS were calculated according to the Kaplan-Meier method. The contribution of prognostic variables to survival were analyzed using the log-rank test for univariate analysis and the Cox proportional hazards model for multivariate analysis. Six clinicopathological variables (sex, age, tumor size, grading, staging and lymph node involvement) and TYMP, VEGF, CD34, proliferative markers, p53, and ECM component expressions were examined for their association with the outcome. The log-rank test was used to measure the association between TYMP and survival. Statistical significance was assumed when *p*-values were equal to or less than 0.05.

Results

TYMP expression. Thymidine phosphorylase expression was detected in the nucleus (n) and/or in the cytoplasm of tumor cells (cyt) (Figure 1). Immunoreactivity was also often present in stromal (st), inflammatory, and endothelial cell

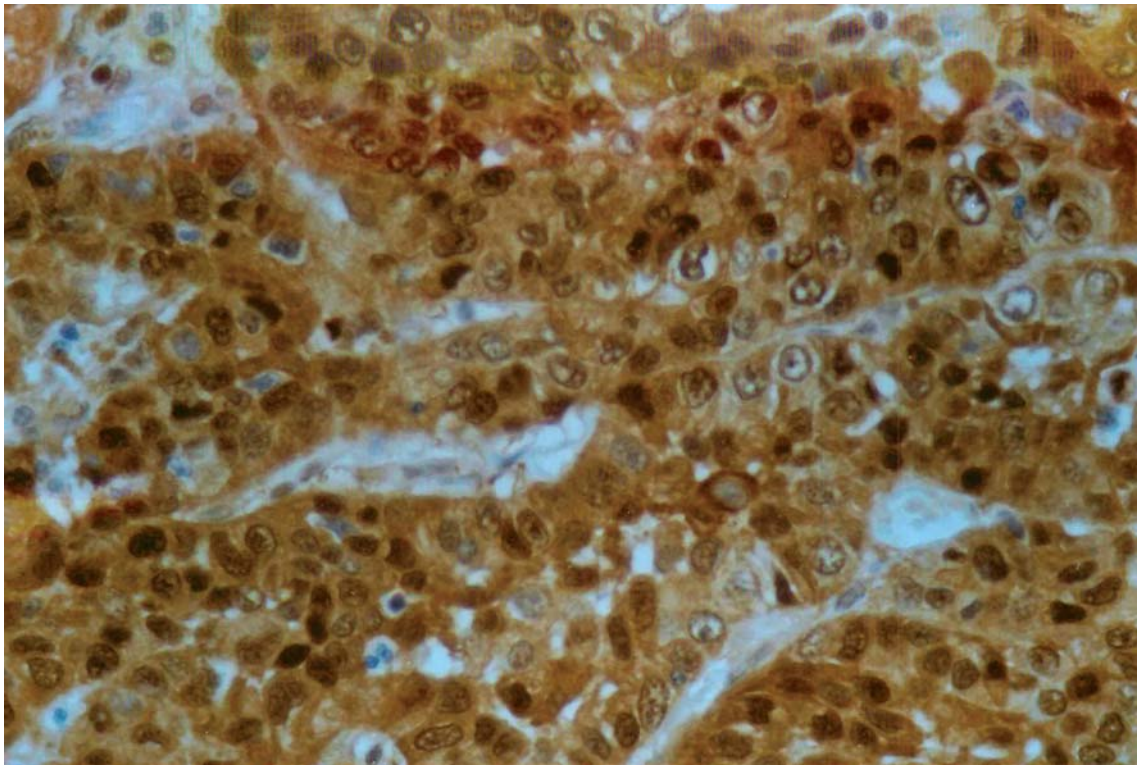

Figure 1. *Strong nuclear and cytoplasmic expression of thymidine phosphorylase in tumor epithelium of colorectal adenocarcinoma (×400).*

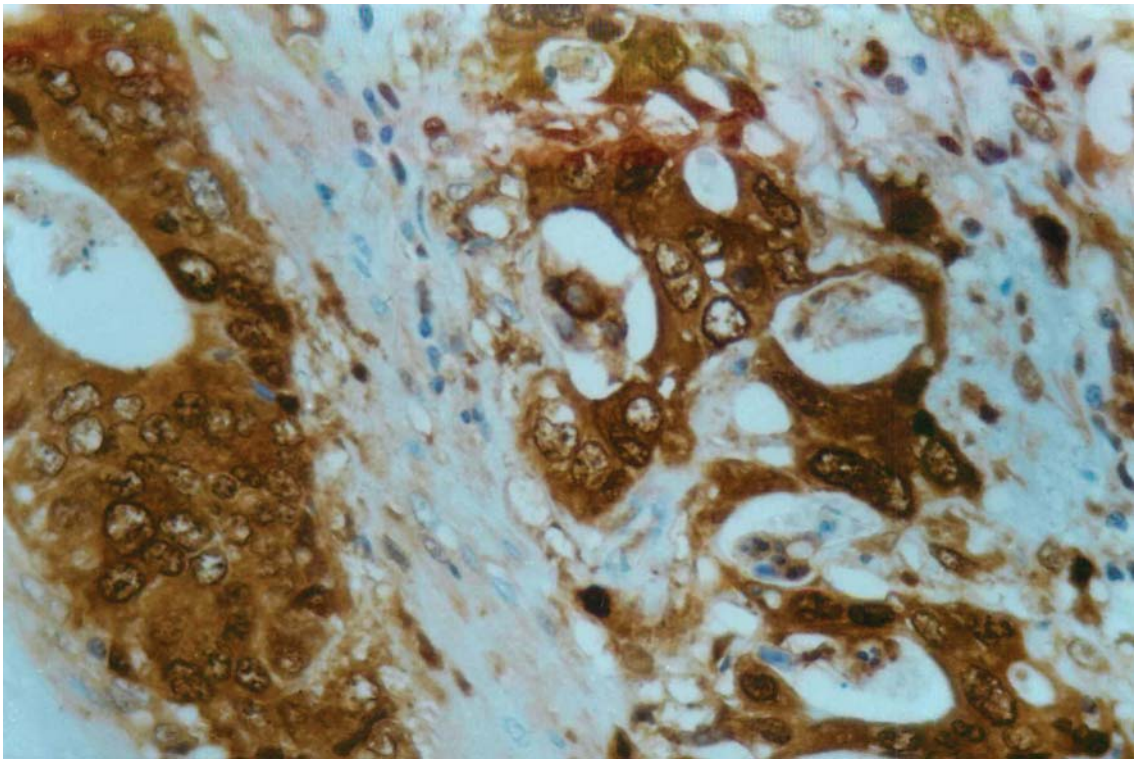

Figure 2. *Strong nuclear, cytoplasmic and some stromal cell expression of thymidine phosphorylase in moderately-differentiated colorectal adenocarcinoma (×400).*

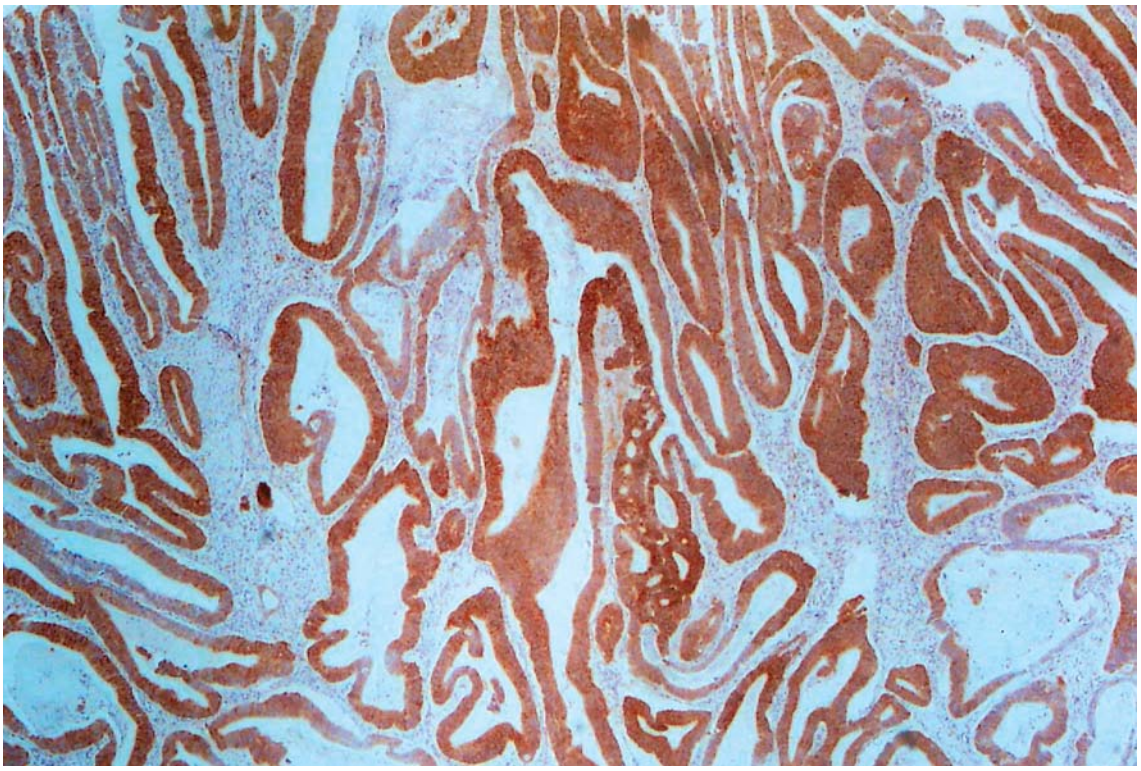

Figure 3. *Intense expression of vascular endothelial growth factor in tumor epithelium of colorectal adenocarcinoma (×100).*

elements (Figure 2). We assessed the TYMP expression separately in cancer cells and stromal cells. Cytoplasmic immunoreactions were more predominant than nuclear reactions. High TYMPcyt expression was observed in 7/97 (7.2%) of the cases, it was moderate in 22/97 (22.7%) of cases, weak in 58/97 (59.9%) cases, while 10/97 (10.3%) of the cases were negative. Moderate and high TYMPn expression was observed in 12/97 (12.4%) of the tumors and it was low in 30/97 (30.9%) cases, while 55/97 (56.7%) did not exhibit nuclear TYMP expression. High tumor TYMPst expression was observed in 57/97 (58.8%) of the cases, expression was moderate in 36/97 (37.1%) cases, and weak expression in 4/97 (4.1%) of the cases (Table III).

TYMP expression did not correlate with gender, age, tumor size, grade, Dukes' stage, lymph node involvement, proliferative markers, p53, and microvessel density (Table IV). TYMPst was positively correlated with the type of the tumor (p=0.037), TYMP being more frequently detected in non-mucinous carcinomas. TYMPcyt expression was positively correlated with TYMPst expression (p=0.011), while TYMPst expression was inversely correlated with TYMPn expression (p<0.0001).

VEGF immunohistochemical expression was detected in the cytoplasm and in some cases in the membrane of carcinoma cells, although with different percentages of stained cells. Specifically, 66 out of the 97 (68.1%) cases were strongly-positive (Figure 3), 17 (17.5%) were moderately-positive, 5 (5.2%) were weakly-positive, and 9 (9.3%) were negative. Strong immunoreactivity in the tumor vessels was observed in 43 (44.3%) cases, moderate in 17 (17.5%), weak in 23 (23.7%), and 14 (14.5%) cases were totally negative. CD34 immunoreactivity was detected in vascular endothelial cells. At the tumor site, the MVD ranged from 15 to 122 (mean±SD= 56.3±21.3). A positive association was observed between VEGF expression and MVD (p=0.0016).

TYMPst expression was positively-correlated with VEGF expression (p=0.014), as was TYMPcyt also positively correlated with VEGF expression of the tumor cells (p=0.014) and vessels (p=0.022). Patients with higher TYMP expression had a more favorable OS (p=0.041) (Figure 4) than patients without or with weak tumor cells and stromal TYMP expression in univariate analysis (p=0.076 and p=0.072, respectively).

TN expression. TN was found mainly in the stroma surrounding malignant cells, while tumor epithelial cells were negative. The staining was weak and occasional in 17 (17.6%) cases, moderate in 47 (48.45%) cases, and strong in

Table III. *Thymidine phosphorylase (TYMP) immunohistochemical expression in correlation with clinicopathological data in colorectal cancer.*

	TYMPcyt		TYMPn		TYMPst	
Type	+	++	+	++	+/++	+++
Mucinous*	11	5	2	2	25	47
Non Mucinous	47	24	3	5	11	7
Size						
≤5 cm	14	6	10	2	7	13
>5 cm	10	12	12	5	7	15
Grade						
Well	9	5	11	3	7	7
Moderate	40	15	42	2	22	33
Poor	2	5	21	1	3	4
Dukes' stage						
B	32	10	49	3	17	25
C	19	14	32	5	16	17
Lymph node status						
Negative	53	24	47	2	33	44
Positive	5	5	35	2	2	8
Ki-67						
<10%	39	19	42	3	23	34
≥10%	19	10	31	2	12	17
PCNA						
<50%	12	6	45	3	8	10
≥50%	46	22	35	2	27	41
P53						
<5%	25	8	45	3	12	21
≥5%	33	29	33	2	23	31

*Significantly different from mucinous tumors at $p=0.037$.

Table IV. *Correlation of thymidine phosphorylase expression with angiogenesis-related markers and extracellular matrix components in colorectal cancer tissue.*

	TYMPcyt		TYMPn		TYMPst		*p*-Value
	+	++	–/+	++	+/++	+++	
MVD							
Low	26	9	35	11	14	19	NS
High	38	16	47	9	23	33	NS
VEGF							
+	12	1	9	2	18	6	$p=0.022$
++	8	2	12	2	3	7	$p=0.014$
+++	45	27	49	3	23	45	$p=0.014$
Tenascin							
+	18	9	9	2	9	17	NS
++	25	9	12	2	17	18	NS
+++	19	9	49	3	13	19	$p=0.011$
Fibronectin							
–	3	3	8	2	4	5	NS
+	27	7	11	1	15	19	NS
+++	35	17	53	4	23	29	NS
Collagen IV							
+	24	14	8	2	11	27	NS
++	18	10	11	1	19	11	NS
+++	25	4	53	4	9	15	NS
Laminin							
+	43	8	7	2	38	28	NS
++	12	9	9	1	35	11	NS
+++	8	7	45	3	8	9	NS

23 (23.71%) specimens (Table IV). Tumor cell TYMP expression was inversely correlated with TN ($p=0.011$). There was no statistically significant association between TN immunoreactivity and any of the other clinicopathological variables.

Coll IV expression. Significant loss of type IV collagen expression, represented as discontinuous or thin staining patterns, as well as limited or lack of Coll IV staining was noted in invasive CRCs. Limited or lack of Coll IV staining was seen in 72 (74.22%) of the cases, while reactivity for Coll IV was observed in 25 (25.78%) of the cases. No statistically significant association was found between Coll IV expression and Dukes' classification, grade of tumor differentiation, number of positive lymph nodes, or vasoinvasion. Moreover, the MVD and VEGF expression did not have a significant association with Coll IV expression. No statistically significant relation between the expression of stromal, cytoplasmic or nuclear TYMP and Coll IV was found in the present study.

FN expression. In CRCs, FN immunostaining showed positive reaction in the tumor stroma with a varying intensity, with orientation along connective tissue fibers as diffuse staining. In the cases of well-differentiated tumors, FN had patchy distribution along BM remnants around tumor glands and nests as a band-like pattern. The band-like pattern of FN immunoreactivity was preserved in the tumor center but was partly lost in the tumor periphery. The staining was absent or weak in 8 (8.2%) of the cases, mostly poorly differentiated adenocarcinomas, and moderate to strong in 89 (91.8%) specimens of well- and moderately-differentiated tumors. No statistically significant relation between the expression of stromal, cytoplasmic, or nuclear TYMP and FN was noted in the present study.

LN expression. Moderate and strong LN expression was observed in 71 (73.2%) cases of well- and moderately-differentiated colorectal carcinomas, with continuous membrane LN staining. Weak or absent LN expression was noted in 26 (26.8%) cases. Patches of LN immune-reaction was scattered throughout the tumor, mainly in poorly-differentiated adenocarcinomas. When the staining patterns of poorly-differentiated tumors were compared with those of well- and moderately differentiated tumors, a statistical significant difference was found ($p<0.01$).

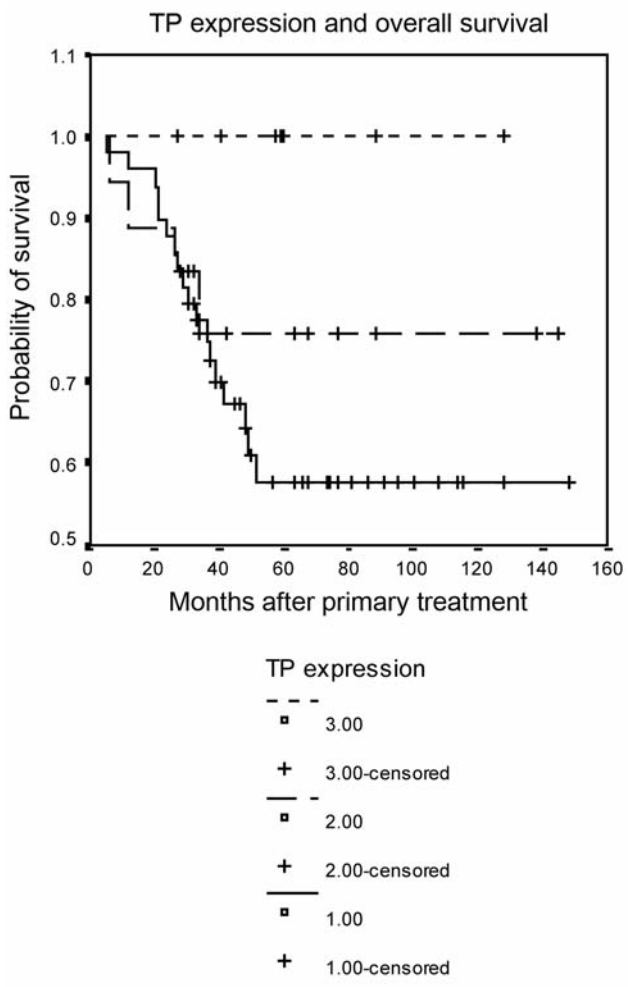

Figure 4. *Kaplan-Meier curves according to thymidine phosphorylase staining. Patients with higher tumor cells of thymidine phosphorylase expression show a favorable overall survival than patients without or with low thymidine phosphorylase expression (p=0.041).*

Discussion

TYMP has been demonstrated to be identical to PD-ECGF, an endothelial mitogen initially purified to homogeneity from human platelets (9). PD-ECGF has chemotactic activity for endothelial cells *in vitro* and is angiogenic *in vivo*, taking part in the induction of intratumoral microvessels. It has been suggested to play a crucial role in the remodeling of existing vasculature in the early phase of tumor development (10). TYMP not only serves as an indicator of angiogenic potential, but may also play an important role in cancer chemotherapy as a target for antiangiogenic agents and as an activating enzyme for pro-drugs of 5-fluorouracil (5-FU) (18). TYMP is an enzyme involved in pyrimidine nucleoside metabolism. It can catalyze the reversible phosphorolysis of thymidine, deoxyuridine and their analogs to their bases and 2-deoxyribose-1-phosphate. 5'-Deoxy-5-fluorouridine (5'DFUR), a pro-drug of 5-FU, must be activated by TYMP in cancer tissues and converted into 5-FU, resulting in the induction of anticancer activity, with few side-effects in normal tissues (15, 16).

TYMP is found in many normal tissues and cells, showing high levels in macrophages, stromal cells, reticulocytes, some epithelia, tissues of the digestive tract (esophagus and rectum), salivary gland, brain, bladder, spleen, lymph nodes and the lungs (24). Within the cell, TYMP is present in both the cytoplasm and the nucleus. Thus, in the nucleus it may modulate the pool for DNA synthesis, whilst in the cytoplasm it controls other effects through different enzyme systems (26). Blood platelets are one of the richest sources of TYMP, which suggest a role for the enzyme in normal healing. TYMP activity is also detected in plasma and serum, probably due to blood platelet damage or cell turnover (27). It is well-known that TYMP expression is found to be selectively increased in various malignant tissues compared with normal tissues in the same organs, when quantified using enzyme-linked immunosorbent assay (27).

The expression levels of TYMP in CRC are higher than those in colorectal adenoma and normal mucosa (28-30). However, the distribution of TYMP has been subject to controversy. There are reports that TYMP was expressed in the cytoplasm of many carcinoma cells and also in macrophages and fibroblasts of tumor interstitium (12, 17, 18, 31). On the other hand, many studies showed that TYMP was expressed mainly in the stromal cells of the tumor interstitium and a few tumor cells were immunoreactive to TYMP (19, 31-33). In the present study, we found high stromal TYMP expression in 59/97 (58.8%) of the cases, in tumor epithelium in 87/97 (89.7%) of the cases with strong expression in 7.2% of the tumors; in the nucleus strong TYMP immunostaining was noted in 12/97 (12.4%) of the cases. We were, thus, able to support previous data on higher TYMP expression in tumor tissues (12, 17, 34-38) (Figure 1). In CRC, there are reports that correlate TYMP expression with some clinicopathological features, while others did not find such a correlation. In particular, it has been shown that TYMP positivity was correlated with tumor size (12), stage of differentiation (12, 31, 37), lymph node metastasis (12, 37), lymphatic invasion (12, 37), hematogenous invasion (12, 31, 40, 41), MVD (12, 18, 39), depth of invasion (12), Ki-67 proliferation marker (40), and p53 protein expression (40, 41). However, other investigators did not find any correlation of TYMP expression with tumor size (19, 34-36), grade of differentiation (19, 34, 35), depth of invasion (19, 42), lymphatic invasion (19, 34, 35), venous invasion (19, 34, 35), p53 expression (39-41), Dukes' stage (29, 34), Ki-67 expression (41), or lymph node metastasis (34, 37). In the present study we failed to corroborate any relationship of tumoral or stromal expression of TYMP with gender, age of

patients, tumor size, grade, Dukes' stage, lymph node involvement, proliferative markers, p53 and CD34. A statistically significant association was observed between TYMPstr expression and the type of tumor (p=0.037). The percentage of TYMPstr immunostaining was higher in non-mucinous tumors than in mucinous adenocarcinomas in accordance with the study of van Halteren *et al*. (20).

A variety of factors have been identified as potential regulators of angiogenesis, most being proteins and cytokines. One is VEGF, identified as a mitogen that acts directly on endothelial cells and induces angiogenesis (5). The influence of neo-angiogenesis, as well as VEGF expression, has been related to relapse and poor prognosis in CRC, thus adding important information for the prognostic evaluation of this type of cancer (43). Knowledge of the angiogenic pattern, which may affect behaviour in CRC, would be valuable from a clinical standpoint, since it could be very useful in selecting different prognostic patterns and postsurgical treatments and could lead to the development of new specific anti-angiogenic drugs for the treatment of colon cancer. Moreover, patients with low VEGF expression have a significantly better survival rate than those with high VEGF expression (43). Preoperative serum VEGF concentrations in patients are significantly higher than those of healthy controls, reflecting clinical stage progression, depth of invasion, liver metastasis and lymphatic invasion (17). Detection of VEGF has been used as a potential marker for CRC progression and metastasis, independently of other markers (36). Our data clearly demonstrated that high TYMP expression both in carcinoma and stromal cells, correlated with tumoral VEGF expression (p=0.014), as did TYMPcyt, as well as with vessel expression of VEGF (p=0.022). These findings suggest that TYMP and VEGF may play important roles in the development of angiogenesis, with a significant relation to tumor growth, invasion, metastasis and prognosis. Although no factors have been reported to stimulate both VEGF and TYMP expression simultaneously, unknown mechanisms should be present to explain a high frequency of their co-expression in human CRCs (17, 36, 40). The localization of VEGF in cancer cells is different from that of TYMP, and this difference may suggest their different roles in angiogenesis in cancer invasion and growth. A recent study demonstrated that TYMP may induce VEGF secretion *in vitro* through inducing hypoxia. Transcription of VEGF is known to be driven by hypoxia-inducible factor-1α (HIF-1α). Under hypoxic conditions, the trancription factor HIF-1α is up-regulated and increases the expression of several target genes by forming a dimer with HIF-1β, which recognizes the hypoxia-responsive elements in the promoter region. In human bladder RT-112 cancer cells TYMP activity augmented the level of HIF-1α during *in vitro* hypoxia and TYMP and HIF-1α acted together to induce VEGF secretion (24).

Mutant p53 has been reported to regulate VEGF-activating pathways, whereas after loss of wild-type p53, a decrease in angiogenesis inhibitors, such as thrombospondin-1 was observed (43). We did not observe any relationship with p53, which may be related to the discrepancy between p53 immunostaining and mutation analysis. Positive immunostaining for p53 may not reflect a mutated status of the protein.

In CRC, the relationship of TYMP expression with vascularity is controversial. Some authors reported that no relationship was found between MVD and TYMP expression carcinoma and stromal cells (19, 20), whereas others reported the opposite (18, 31, 37). In the current study, we failed to observe any correlation between TYMP expression and MVD, as estimated by CD34 immunostaining.

The ECM components are critical for normal vessel growth and maintenance, acting as both scaffold support, through which endothelial cells may migrate, and as a reservoir and modulator for growth factors. Cell culture experiments suggest that TN promotes cell growth by augmenting the mitogenic effect of fibroblast growth factor which is a prerequisite for epidermal growth factor-induced proliferation (44). The role of TN in angiogenesis has been also investigated. It has been found that TN promotes vascular migration and phosphorylation of focal adhesion kinases. Tenascin may also regulate angiogenesis in tumor through the regulation of VEGF expression (45). For the first time to our knowledge, in the current study, we demonstrated that the TYMP expression was correlated with the ECM component TN (p=0.011), contributing to stromal remodeling through the interaction with TN. In CRC, the distribution pattern of stromal tenascin has been previously reported to be a prognostic factor by itself. A diffuse stromal fibrillar staining has been correlated with poor survival, while a subglandular pattern has been associated with a more favorable prognosis (44). We did not observe any correlation between TN expression and OS of the patients. Our results indicate that immunohistochemical expression of TN is not of prognostic significance in CRC, despite its relationship with TYMP expression.

BMs are dynamic sheet-like structures of ECM that provide a supporting structure on which epithelial and endothelial cells reside (46, 47). Coll IV, along with LN, plays an important role in cell adhesion, migration, differentiation and growth. We observed loss of BM Coll IV in colorectal adenocarcinomas, but no correlation was found with TYMP expression. In the present study, we demonstrated that LN in CRC exhibited a progressive loss of intact BM. Well- and moderately-differentiated tumors exhibited a thin BM with intermittent disruptions, while poorly-differentiated tumors exhibit no areas of intact BM. No statistically significant association was noted with TYMP expression.

FN is a major mesenchymal ECM glycoprotein involved

in-cell-to matrix and cell-to-cell adhesion, and cell migration (48). In the current study, we also studied the distribution of FN, and found a very heterogeneous and patchy distribution in the tumor stroma. We suppose that the observed differences in distribution might be due to a down-regulation of FN secretion in some tumor regions, or due to FN degradation process that might have taken place in these areas. In the present report, no statistical correlation was observed between FN expression and TYMP immunoreaction, in accordance with our previous report on breast carcinoma (49).

The prognostic value of TYMP in patients with CRC remains a subject of contradiction and disagreement. Studies in which TYMP expression has been evaluated in tumor cells by immunohistochemical staining suggest that high expression of TYMP is related to poor prognosis (12, 20, 36, 37, 40). On the other hand, research in which TYMP expression is assessed within tumor stromal cells, with high TYMP levels in macrophages and fibroblasts suggest that high expression results in a significantly better prognosis and a lower rate of incidence of lymphatic and hematogenous metastasis (19, 42). Studies using Cox's regression analysis to determine independent prognostic strength found that high TYMP expression status in tumor cells was a significant and independent prognostic factor for DFS (31, 35). In the present study, in univariate analysis, we found that patients with high tumor cell TYMP expression had a more favorable OS than patients without or with weak tumor cell TYMP expression (p=0.041) (Figure 4). The discrepancies might be caused by differences in the histological type of tumor, stage of tumor (early versus advanced stage of disease), number of patients examined, assays used for TYMP, and different methodology for the evaluation of the immunohistochemical results. The existence of such a high variability supports the demand for a uniform evaluation of immunohistochemical expression. The use of a standardized scoring system which considers both staining intensity and pattern could be useful to more accurately evaluate the expression of the patterns studied that will allow a comprehensive collection of comparable data from multicenter studies.

As mentioned earlier, it has been found that TYMP interacts with pathways of 5-FU metabolism, indicating their predictive role for chemotherapeutic responses (16). In vitro studies have demonstrated that transfection of TYMP into cancer cells increases their sensitivity both to 5-FU-and-prodrugs requiring TYMP cleavage for activation to fluoropyrimidines, and reports have shown that low TYMP levels are associated with 5-FU sensitivity (15, 16). The correlation between high TYMP expression and non-response to 5-FU could be a consequence of the role of TYMP as an angiogenic agent. It has been shown that hypoxia which is known to stimulate the development of angiogenesis, can select for cells that are apoptosis-resistant. These observations suggest that high TYMP expression in tumors is a marker of other genetic and biochemical changes associated with a more aggressive and malignant tumor phenotype that has an increased resistance to cytotoxic agents due to the loss of apoptotic potential (16).

In this study, we showed that a) TYMP and VEGF expression were correlated, b) TYMP expression and the ECM component, TN were correlated, and c) TYMP expression in tumor cells has a prognostic significance in CRC, and can be considered as a marker of better prognosis. These findings suggest that TYMP plays an important role in angiogenesis, ECM remodeling, and in the prognosis of patients with CRC, but further studies are needed to clearly define its role.

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