The Prognostic Significance of Tryptophanyl-tRNA Synthetase in Colorectal Cancer

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Introduction

Colorectal cancer (CRC) is the third most common cancer in western countries. In Sweden, CRC is the second most frequent cancer in women, after breast cancer; corresponding to 8.0% of the cases in 2006 and the third most common type in men, after prostate and skin cancers, constituting 6.8% of the cases.7

CRC is most often staged according to the tumor-node-metastasis system (1), and stage is the most important prognostic indicator and the principal element used for selecting treatment and follow-up of patients (2). Histologic factors such as tumor differentiation and vascular invasion are also of prognostic value (3). Many tumor markers have prognostic significance in CRC; however, in clinical practice, carcinoembryonic antigen (CEA) is the only recommended tumor marker determining prognosis, surveillance following curative resection, and potentially monitoring therapy in advanced disease.

The tissue microarray (TMA) technique enables multiple immunohistochemical analyses of normal and cancer tissues in a high-throughput fashion. The most common assay in TMA is immunohistochemistry; however, the lack of validated antibodies toward the wide range of proteins encoded by the human genome is a drawback for global protein expression studies. One way to circumvent this problem is the advent of large-scale antibody-based proteomics initiatives, such as the Human Protein Atlas (HPA) program,8 which aims to generate a comprehensive atlas of protein expression patterns in normal and cancerous human tissues (4). The main objective of the HPA program is to produce specific antibodies to all nonredundant proteins with a high-throughput method involving the cloning and expression of protein epitope signature tags (5) and to use these antibodies to create a map of human protein expression. In addition to

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displaying a map of protein expression patterns, this atlas can be used as a tool for in silico biomarker discovery (6, 7). Tryptophanyl-tRNA synthetase (TrpRS) belongs to the aminoacyl-tRNA synthetase family; is involved in protein synthesis, regulation of RNA transcription and translation, and cytokine activities in inflammatory and angiogenic signaling pathways (8); and is induced by IFNγ (9, 10). The native enzyme lacks angiogenic activity. By proteolysis or alternative splicing of the native protein, T2-TrpRS or mini-TrpRS act as potent fragments and are active in the inhibition of new vessel development without affecting preestablished vasculature (11). These fragments work through the vascular endothelial-cadherin (VE-cadherin) receptor and Akt signaling pathways (12, 13).

The aim of this study was to analyze the correlation of TrpRS to the prognosis of patients diagnosed and treated for CRC within a defined population.

Patients and Methods

Patients. The identification of TrpRS as a prognostic marker in CRC was by HPA (4). In this program, the antibody was screened in TMAs, representing 48 types of normal tissues; 216 human tumors, representing the 20 most common forms of human cancer; and 47 cell lines. With HPA, TrpRS expression was generally weak to moderate in normal tissues and negative to weak in normal colonic and rectal mucosa. The staining intensity was generally stronger, and the fraction of positive cells higher in cancer tissues. In CRC, TrpRS was differentially expressed, varying from negative to strong staining, and further evaluation in a test cohort of 122 patients with CRC revealed that a low intensity was associated with worse overall survival (OS). This led to the analysis on the present population-based cohort with a long and complete follow-up.

Between August 2000 and December 2003, 320 patients undergoing operation for CRC at the central district hospital in Västerås, Sweden, were asked to participate in a prospective study of tumor markers. Information on tumor size, lymph node status, lymphatic or vascular vessel invasion, mucinous components, and grade of tumor differentiation was obtained from pathology reports. Information on clinical stage, cancer recurrence, death, and causes of death of the cohort was obtained by matching with the Regional Oncology Registry and from surgical and oncological hospital records.

TMA Construction. Before TMA construction, all cases were histopathologically reevaluated on H&E-stained sections by one pathologist (K.J.), and areas representative of invasive cancer, normal adjacent mucosa, and, when present, adenomatous lesions and lymph node metastases were marked on the slides. TMAs were constructed as described (14). In brief, for each case, two 1.0-mm cores from the invasive tumor component and one 1.0-mm core from normal mucosa, adenomatous mucosa, and lymph node metastases were taken with a manual arraying device (MTA-1, Beecher Instruments).

Immunohistochemistry and Annotation. From the TMA, immunohistochemistry was done on 4-μm sections with a polyclonal, monospecific IgG TrpRS antibody. Two forms of TrpRS exist: a cytoplasmic form (WARS) and a mitochondrial form (WARS2); the antibody used here is specific for WARS. The antibody had previously been validated by protein epitope signature tag array analysis with high specificity, and Western blot techniques revealed a single band corresponding to the predicted size (53 kDa; Fig. 1). The stained TMA sections were scanned in high-resolution scanners (ScanScope T2, Aperio Technologies) and separated into individual spot images representing the different cores in the TMAs. Histologic

Table 1. TrpRS score: a four-graded scale combining intensity of immune reactivity and fraction of positive cells

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Figure 1. Results from Western blot analysis of the TrpRS antibody show a single band of the predicted size (53 kDa). Strong band is seen in protein lysate from the cell line RT-4, whereas weaker, single bands of the same size are present in lysates from the U-251 cell line and from normal human tonsil tissue. The results from the Western blot are consistent with the immunohistochemistry results, where RT-4 shows a stronger staining intensity compared with U-251 (negative in immunohistochemistry) and tonsil (only weakly positivity). Liver tissue is negative in immunohistochemistry.
images were manually analyzed and all spot images were viewed and evaluated by a trained annotator (A.G.) with a web-based annotation system (i.e., imagescope viewer, a digital pathology system).9 The digital pathology system is used to present and visualize the high-resolution immunohistochemically stained images together with a form that can be manually filled in by the person responsible for annotation of immunohistochemical staining parameters. The annotation includes an estimation of intensity of immunohistochemical staining and an estimation of fraction (%) of positive tumor cells. Parameters annotated included intensity of immunoreactivity [negative (0), weak (1), moderate (2), or strong (3)], fraction of positive cells [<1% (0), 1-25% (1), 25-75% (2), or >75% (3)], and cellular localization of the staining. A combined TrpRS score for intensity and fraction was created with a four-graded scale (0-3; Table 1).

Figure 2. Examples of TrpRS expression on TMA from primary tumor tissue in patients with invasive colon cancer. The positive staining is mainly seen in the cytoplasm as a brownish color. A, a 1.0-mm core from the invasive tumor showing moderate staining primary tissue intensity for TrpRS. B to E, higher resolution showing negative (B), weak (C), moderate (D), and strong (E) staining intensity. A, the fraction of positive tumor cells is >75% in this example.

Statistics. For comparison of dichotomous variables, $\chi^2$ test was used. The Mann-Whitney $U$ test was used for comparison of nonparametric two group parameters, and the Kruskal-Wallis test for comparison of significance of more than two nonparametric groups. Relative risks (RR), with 95% confidence intervals (95% CI), were calculated by Cox proportional hazard model for univariate and multivariate analyses. The calculated hazard ratio was used as the RR estimate. In the multivariate analysis, the category I and IIA factors of histopathologic prognostic factors were included, as defined by the College of American Pathologists consensus statement 1999 (15).

Endpoint were defined according to the recommendations of Punt et al. (16). The OS was measured from the date of surgery to the date of death. Curatively treated patients were operated patients with stage I to III disease and microscopically free resection margins (R0). For curatively operated patients, disease-free survival (DFS) was calculated and measured from the date of surgery to the date of diagnosis of a local/distal recurrence or to the date of death due to CRC, and censored at the date of death due to reasons other than CRC. A second primary CRC/non-CRC was then ignored. All observations were censored at the end of the study period (November 15, 2008).

Survival was plotted graphically with the Kaplan-Meier method and tested for significance with the log-rank method. Statistically significant differences were assumed when $P < 0.05$.

Ethical approval (no. 00-001) was obtained from the Ethics committee at Uppsala University, Uppsala, Sweden.

Results

Staining and Annotation. Positive staining of TrpRS was mainly in the cytoplasm, not nuclei, of both cancer (Fig. 2A-E) and normal cells. In invasive tumor tissue and lymph node metastases, the fraction and intensity of the staining were generally higher than that in normal bowel mucosa (Table 2). Adenomatous tissue revealed more pronounced staining than in normal bowel mucosa,
similar to invasive tumor and lymph node metastases (Table 2).

**Clinical and Histopathologic Variables.** The fraction of positive cells stained for TrpRS in invasive primary tumor tissue did not correlate to any clinical or histopathologic variables. A trend for increased age with higher staining intensity of TrpRS was seen ($P = 0.059$), and tumors located in the right colon more often had higher intensity than left-sided tumors ($P < 0.001$). Low intensity of TrpRS correlated to increased risk for lymph node metastasis ($P = 0.025$) and more advanced tumor (T) stage ($P = 0.001$). Higher TrpRS score of the invasive primary tumor tissue correlated to higher age ($P = 0.040$), right colon ($P = 0.001$), a trend for less vascular invasion ($P = 0.117$), and lower tumor stage ($P < 0.001$). For lymph node metastasis, high TrpRS fraction tended to correlate with distant metastasis ($P = 0.064$).

**Survival and Recurrence.** No statistically significant associations were observed between TrpRS (fraction, intensity, or score) and OS. Patients with high TrpRS score had a statistically nonsignificant trend toward better survival than those with low or medium TrpRS scores (Fig. 3A). There was an improved DFS and shorter time to recurrence for cases with a TrpRS score of 3 than for cases with TrpRS scores 1 and 2 (Fig. 3B and C).

A dichotomized variable was constructed, including TrpRS scores 1 and 2 combined, as these groups acted similarly in the survival analyses.

Cox multivariate analysis including TrpRS score, age at operation, stage of disease, histopathologic differentiation grade, lymphatic or vascular vessel invasion, and CEA revealed better DFS and a trend for decreased time to recurrence for curatively treated patients with TrpRS score 3 compared to patients with TrpRS scores 1 and 2 (Table 3).

Fewer recurrences were observed for cases with TrpRS score 3 than for cases with TrpRS score 1 and 2, for all CRC (Fig. 4A) and for colon cancer separately (Fig. 4B), with a reduced risk of recurrence in multivariate analysis (RR, 0.23; 95% CI, 0.07-0.80). The same trend, although not statistically significant, was identified for rectal cancer (RR, 0.27; 95% CI, 0.06-1.15; data not shown). Fewer recurrences in colon cancer patients with TrpRS score 3 were also determined when stage II (Fig. 4C) and stage III (Fig. 4D) were analyzed separately, although these were statistically nonsignificant for stage II. As there was only one recurrence in stage I patients, no separate calculations were possible for that group. Multivariate analyses for stages II and III revealed no differences of cancer recurrence between TrpRS scores 1 and 2 and TrpRS score 3.

Tissue cores from lymph node metastases were available from 45 of 102 stage III patients and from 22 of 45 stage IV patients. Only one patient had TrpRS score 3 in the lymph node metastasis in stage IV disease, and no differences were determined between TrpRS scores 1 to 3 with respect to OS. For stage III patients, OS and time to recurrence tended to be better for cases with TrpRS score 3 in the lymph node metastasis than for those with TrpRS score 1 and 2, but the differences were not statistically significant (data not shown).

Samples from normal mucosa adjacent to the tumor on the paraffin-imbedded specimen were available for 138 patients (Table 2). The fraction of positive cells was similar to invasive cancer, but the intensity of immunoreactivity was generally weaker, which also resulted in a lower TrpRS score (Table 2). No statistically significant differences were determined between OS and DFS or time to recurrence between groups of fraction, intensity, or TrpRS score in normal mucosa (data not shown).

**Discussion**

An increased expression of tumor-specific TrpRS in CRC was independently associated with improved prognosis. Furthermore, increased risk of lymph node metastases and a more advanced tumor stage for patients with
tumors exhibiting a low expression of TrpRS were identified. As clinical and histopathologic parameters do not provide sufficient prognostic or treatment-predictive information in CRC, a search for reliable molecular markers is warranted. The best evaluated tumor marker in CRC is CEA, which is still the only tumor marker recommended for use in clinical practice (2). The usefulness of CEA measurements in serum (S-CEA) is mainly for prognostic determination and postoperative surveillance (2, 17). Groups of patients will not respond with increased S-CEA, even when presenting with distant metastasis, which is especially true in the case of those with preoperatively normal S-CEA levels (18). Therefore, other modalities of prognostic evaluation and surveillance in CRC are necessary. In the present population, S-CEA levels of $\geq 6$ ng/mL were independently associated with increased risk for recurrence and a trend for worse DFS in curatively treated patients with CRC (data not presented). Microsatellite instability is associated with better prognosis; however, microsatellite instability is not routinely measured in Sweden except in cases with suspected hereditary CRC, and the present cohort has not been characterized according to microsatellite instability status.

In vivo and in vitro studies have identified the important role of TrpRS as an inhibitor of angiogenesis (11, 19, 20). Recent advances in the development of antiangiogenic agents make this enzyme an attractive choice for further studies in cancer therapy and in a variety of other diagnoses, such as psoriasis, ischemic heart disease, rheumatoid arthritis, diabetic retinopathy, and macular degeneration (11, 21-24).

Studies on TrpRS in CRC are rare, and to our knowledge, the immunohistochemistry expression level of TrpRS in CRC has not yet been described; therefore, comparison of the present results to other studies was not possible. The expression of TrpRS seemed to be lower in normal mucosa of the colon and rectum than for invasive primary tumor and lymph node metastases, as observed in both the HPA screening cohorts and the

Figure 4. A comparison of time to recurrence for different immunohistochemical expression of TrpRS in invasive primary tumor tissue from curatively treated patients with CRC. TrpRS scores 1 and 2, low to medium TrpRS expression; TrpRS score 3, high expression. Log-rank test is used for comparisons between two groups. The number at risk is the number of cases that entered the respective interval alive, minus half of the number of cases lost or censored in the respective interval. A, colorectal, stages I to III; B, colon, stages I to III; C, colon, stage II; D, colon, stage III.
present cohort. This could reflect an increased angiogenic activity in tumor tissue. In the primary tumors, low expression of TrpRS correlated with more advanced tumor and lymph node stages. The relationship to the tumor stage could be explained by invasion of tumor cells through the bowel wall being facilitated by increased neovascularization.

The relation to nodal stage is more difficult to explain, although it is possible that an increased angiogenic potential is related to an improved ability of the tumor cells to be implanted in the lymph nodes. The association of increased expression of TrpRS with fewer recurrences and improved DFS is more understandable, suggesting that TrpRS prevents tumor cells from successful implantation in distant organs by inhibiting the neoangiogenic potential of the tumor. Patients with stage III disease have increased expression of TrpRS in lymph node metastases and tend to have fewer recurrences than those with low expression of TrpRS. Tumor staging and survival in colon cancer is dependent on the quality of the pathology department in investigating and measuring the number of lymph nodes (25).

The TMA technique provides an efficient tool for analyses of multiple tumor tissue samples in a high-throughput fashion. The use of 1-mm core biopsies in duplicate is considered adequate for revealing the relationship between the biomarkers investigated and clinical outcome (26). Several molecular markers have been studied with TMA technique to determine their possible prognostic or predictive value in CRC (3). However, there are still some concerns with TMA studies, as the reproducibility of the results is often difficult, partly due to the variability in immunohistochemical staining and scoring methods for TMA (3). It is therefore important to validate findings in TMA-based studies using other independent patient cohorts or other methods.

Tumor cells are dependent on angiogenic and inflammatory factors for growth. The expression of proangiogenic factors is associated with advanced tumor stage and poor prognosis (27, 28). The field of antiangiogenic drugs expanded after Folkman’s discovery (29) of the relationship between tumor growth and angiogenesis through inhibition of tumor angiogenesis factors. Vascular endothelial growth factor (VEGF) is one of the major proteins involved in angiogenesis, and overexpression of VEGF is associated with advanced tumor stage and poor prognosis in CRC (31, 32). Bevacizumab, a monoclonal anti-VEGF antibody, in chemotherapy combination improves survival and prolongs the time to progression in patients with metastatic CRC (33). VE-cadherin is a specific endothelial cell adhesion molecule that has an essential role in vascular development and is located at the junctions between endothelial cells.

The potential role of T2-TrpRS in antiangiogenesis, both in vivo and in vitro, is through complex binding to VE-cadherin, which prevents activation of the VEGF receptor, leading to inactivation of the protein kinase B (Akt) signaling pathway, and thereby inhibits the formation of new blood vessels (12, 13, 20). In CRC cell lines, TrpRS mRNA is downregulated by p53, probably independent of its TrpRS translation (34). As p53 mutation is associated with increased VEGF expression and vessel counts, resulting in the promotion of metastatic disease (35), and could theoretically be explained by the suppression of the inhibitory function of TrpRS by p53.

In conclusion, low expression of TrpRS in tumor tissue correlates with increased risk for recurrence and worse survival in patients with CRC. This can be related to its antiangiogenic properties and could aid in the future selection of new drugs in the treatment of CRC. Further studies on the prognostic effect of TrpRS in CRC, as well as its potential as a target in antiangiogenic therapy, are warranted.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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