Comparative analysis of thymidylate synthase, thymidine phosphorylase and dihydropyrimidine dehydrogenase expression in colorectal cancer and surrounding normal tissue*

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Thymidylate synthase [TS], thymidine phosphorylase [TP] and dihydropyrimidine dehydrogenase [DPD] play the essential role in the activation and catabolism of the fluoropyrimidines used in cancer therapy. It's expression may influence the antitumor activity or toxicity of these drugs. We studied the expression levels of selected enzymes in colorectal tumors and adjacent normal mucosa.

The analysis of TS, TP and DPD gene expression was performed using quantitative Real time PCR technique (Roche) in 15 (TS), 64 (TP) and 12 (DPD) of 64 colorectal cancer patients.

The mean gene expression of TS, TP and DPD was found to be 3.29; 3.79 and 8.24 in tumors and 1.88; 3.80 and 19.69 in normal mucosa. The corresponding median gene expression was 1.87; 2.32 and 4.50 for tumors and 2.14; 2.63 and 11.64 for normal tissue.

We did not find any significant differences in TS, TP and DPD gene expression between colorectal tumor and surrounding mucosa.

Key words: thymidylate synthase, thymidine phosphorylase, dihydropyrimidine dehydrogenase, colon cancer

5-fluorouracil [5FU] and other fluorinated pyrimidines [e.g. capecitabine, UFT, floxuridine] are widely used for many decades in patients with various tumor types.

In colorectal cancer, 5FU is considered to be an active agent, but the metaanalysis showed response rates to 5FU alone about 11% with median survival of only 11 months [1]. A number of strategies have been attempted to increase the efficacy of 5FU-based regimens. The response rate was doubled, when continuous infusional schedules have been introduced into clinical practice [2]. These effective schedules are difficult to administer due to the need for indwelling devices and pumps. There is also patient preference for oral rather than intravenous treatment.

Recently, oral agents [3] capecitabine [Xeloda] or UFT [Orzel] have been developed to mimic continuous 5FU infusion. Capecitabine is fluoropyrimidine carbamate prodrug, that is converted into active metabolite 5FU by carboxylesterase, cytidin deaminase and thymidine phosphorylase. Capecitabine is deemed to be specifically activated in tumor tissue, because significantly higher TP activity was found in tumors than in normal surrounding tissue[4]. Unfortunatelly, there was no confirmatory study carried out to confirm these results. Thus, the aim of our study was to investigate the expression of thymidine phosphorylase in colorectal tumor tissue and in surrounding normal colonic mucosa. In selected samples, complementary analysis of TS and DPD expression have been performed.

Material and methods

The study population consisted of 64 patients with histologically confirmed colon or rectal cancer. The patients were diagnosed and treated between September 2002 and

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The samples have been taken immediately after surgical resection. The tumor sample and the control mucosa from each patient were divided into two equal portions of at least 100 mg each. Before subsequent handling, all necrotic tissue was removed. One portion of tumor and mucosa were immediately embedded in RNA later [Ambion, Inc., USA] and stored at 4 °C for one week. The samples were subsequently frozen at –20 °C until the RNA isolation. The second portion of tumor sample and control mucosa was embedded in paraffin for standard histological examination.

Total RNA was isolated using Perfect RNA Mini kit [Eppendorf Scientific, Inc., Germany], that utilizes a chaotropic guanidinium isothiocyanate solution for the cell lysis and rapid inactivation of cellular RNases. Samples were homogenized in sterile precautions before RNA isolation.

The quantification of target gene mRNA required two-step procedure and was performed with the use of commercially available kits for the quantitative RT-PCR: LightCycler TSmRNA Quantification Kit plus, LightCycler TPmRNA Quantification Kit plus and LightCycler DPDmRNA Quantification Kit plus (all kits Roche Diagnostics GmbH, Germany).

In the first step, cDNA is reversely transcribed from RNA using AMV reverse transcriptase and TP (TS, DPD) specific priming. Additionally, a second cDNA is reversely transcribed from RNA using AMV reverse transcriptase and glucose-6-phosphate dehydrogenase (G6PDH) specific priming. The G6PDH reaction product serves both, as a reference for relative quantification and as a control for RNA integrity.

In the second step a mRNA fragments are amplified from the cDNA by PCR in the LightCycler using specific primers.

The PCR started with an initial denaturation at 95 °C for 5 min. The cycling conditions were 10 s at 95 °C, 10 s at 62 °C, and 10 s at 72 °C for 45 cycles.

The amplicons were detected by fluorescence using specific pairs of hybridization probes. By using the Light Cycler relative Quantification Software, the amount of mRNA encoding for target genes is expressed as a relative ratio to the reference gene (G6PDH) in a sample relative to the TP:G6PDH ratio in a calibrator.

Univariate statistical analysis have been performed using SAS software.

Results

The isolation of RNA was performed successfully in all 128 samples of 64 patients. The TP analysis was carried out in all patients. TS and DPD analysis was determined in randomly selected 15, resp. 12 patients.

The results of the quantitative expression of TS, TP and DPD measured in comparison with the reference gene G6PDH are shown in Table 1.

The difference between the TS expression in tumor and mucosa has the Gauss distribution and is not statistically significant (pair t-test, p=0.0952). In contrast, the non-Gauss distribution of difference was found between TP expression in tumor and mucosa (Wilxocon test, p=0.6940; median pair test, p=0.9007).

The difference in DPD expression between tumor and mucosa has also non-Gauss distribution and is not significant, when analysed with Wilxocon test [p=0.0962] or median pair test [p=0.3877].

Table 1. The results of TS, TP and DPD gene expression in colorectal tumors and surrounding normal mucosa

Gene	Sample	No	Mean[1]	SD[1]	Median[1]	Minimum[1]	Maximum[1]	p values
TS	tumor	15	3.29	2.99	1.87	0.46	10.41	pair t-test
	mucosa	15	1.88	3.54	2.14	0.27	4.41	p=0.0952
TP	tumor	64	3.79	4.91	2.32	0.11	31.43	Wilxocon test p=0.6940
	mucosa	64	3.8	4.69	2.63	0.04	29.94	median pair test p=0.9007
DPD	tumor	12	8.24	8.8	4.50	0.51	28.35	Wilxocon test p=0.0962
	mucosa	12	19.69	19.69	11.64	0.34	60.35	median pair test p=0.3877

SD – standard deviation; [1]The values represent the ratio of target gene expression to reference [G6PDH] gene expression.

Discussion

5-fluorouracil is the essential component of chemotherapeutic regimens in colorectal cancer. The novel antineoplastic pyrimidines, including capecitabine and UFT, have been shown to have similar activity as the continuous infusion regimens [3].

Capecitabine was the example of the rationally designed drug intended to increase the therapeutic ratio of fluoropyrimidines by the preferential activation of non-cytotoxic prodrug in tumor tissue. The purposed mechanism for this preferential generation of 5FU was the observation of higher enzymatic activity of TP in tumors than in normal tissue [4].

MIWA [4] and coworkers found significantly higher TP activity in various gastrointestinal, genitourinary and breast cancers than in corresponding normal tissue in Japanese patients.

In SCHÜLLER's [5] study, 3.66 times higher TP activity was found in eight primary colorectal cancer samples than in normal mucosa. The evaluation of the other eight patients did not show any significant differences in TP activity between normal liver samples and corresponding liver metastases (ratio tumor tissue/healthy tissue: 0.99). In the same study, 5FU concentrations have been determined in target tissues after capecitabine administration for 5–7 days. 3.2 times higher concentra-

tions of 5FU have been observed in primary colorectal tumors than in adjacent healthy tissue, but no differences have been reported for the 5FU concentrations in liver metastases and healthy liver tissue. At this moment, we do not have the same data on tumor/tissue concentrations of 5FU after its systemic administration.

Previously mentioned studies analyzed directly the enzymatic activity of TP in study samples. On the other hand, we decided to use quantitative Real-time PCR technique**.** On the homogenous sample of 64 pairs of tumor and control tissue, we were not able to confirm the preferential TP expression in primary colorectal cancers.

There are many reasons, that could lead to these conflicting results. Real-time PCR detects differences on the level of mRNA but it is unable to asses the potential postranslational modifications. These modifications may lead to higher enzymatic activity which is not detectable on the mRNA level. To exclude this possibility it is necessary to study the TP enzymatic activity and mRNA expression in parallel. From statistical point of view, the SCHÜLLER's study is highly underpowered, analysing only eight samples in each cohort. One can also speculate, that some racial differences in TP expression could exist and this issue should be further explored.

There are two more studies, that directly compared the TP expression in normal and tumor samples using immunohistochemical approach [6, 7]. Both found the highest TP staining in stromal lymphocytes and macrophages, and not in tumor cells. So, these data as well as the results of our study do not support the hypothesis on preferential capecitabine activation in tumor tissue.

On the other hand, both animal models and clinical studies showed, that TP expression is induced by ionizing radiation in tumor cells, but not in adjacent normal tissue [8, 9]. This observation can explain the synergy between capecitabine and radiotherapy.

TP expression is intesively studied also as a predictive factor of the clinical response to 5FU and other fluoropyrimidines. NISHINA [10] reported better overall survival in patients with gastric cancer with high TP/DPD expression ratio.

Similary, VAN TRIEST [11] examined TS, TP, p53, Ki67 and VEGF expression in 32 colorectal cancer patients and found TS and TP to be of prognostic significance.

In our study, the analysis of TS and DPD expression was performed on 12 and 15 patients, and no differences have been observed between tumors and normal mucosa. This result, probably due to low number of studied samples, is in contrast with the study of ODIN [12] and coworkers, who described higher TS expression in tumor biopsies compared with normal mucosa on 102 consecutive colorectal cancer patients.

In conclusion, further studies are warranted to explore the role of TP, TS and DPD as predictive or prognostic factors in colorectal cancer.

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