Preoperative Radiotherapy and Concomitant Capecitabine Treatment Induce Thymidylate Synthase and Thymidine Phosphorylase mRNAs in Rectal Carcinoma

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This work is intended to study the effect of preoperative capecitabine and radiotherapy treatment on the levels of thymidylate synthase (TS), thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD) mRNAs in rectal carcinoma.

55 patients with locally advanced rectal carcinoma (cT3-4, N0, M0 or cT2-4,N+, M0) were treated with capecitabine 825 mg/m2 twice a day and pelvic radiotherapy 1,8 Gy daily up to cumulative dose of 45 Gy, boosting up to 50,4 Gy. Patients underwent surgery 6th week after the completion of chemoradiotherapy. Biopsies of rectal carcinoma were taken before starting therapy and 14 days after its cesation. Biopsies were examined for TS, DPD and TP mRNA levels. CEA in serum was examined to monitor relapses.

Both TP and TS mRNA increase two weeks after starting therapy ($p<0,001$). TP mRNA median levels were elevated 2,3x after starting therapy. Moreover responders exhibit 1,5x higher induction than non-responders both before and after starting therapy, but difference is significant before therapy only ($p=0,017$). Non-responders have most frequent TS induction. Complete remission was observed in 17% and substantial responses with microscopic residuum only in additional 19% of cases were achieved. The pathologic downstaging rate was 76%.

Our data show that TS and TP mRNA are induced by preoperative chemoradiotherapy in both responders and nonresponders. TP induction is in accordance with the expected role of TP in the activation of capecitabine and the known promoting role of TP in tissue fibrosis frequently associated with tumor regression.

Key words: Thymidine Phosphorylase Induction, Thymidylate Synthase, Preoperative Chemoradiotherapy, Capecitabine, Rectal Cancer

Capecitabine is a 5-fluorouracil prodrug that is used in monotherapy of several types of cancer [1] and that possesses radiosensitizing properties [2, 3]. These attributes led to the capecitabine application in combined chemoradiotherapy in the adjuvant or neoadjuvant adenocarcinoma treatment by several research groups [4, 5, 6]. The introduction of chemoradiotherapy early in the disease course in neoadjuvant regimen may theoretically bring a benefit of potential prevention of micrometastatic disease spreading. Capecitabine is preferentially activated in tumor tissue with elevated thymidine phosphorylase activity, like in rectal adenocarcinoma [7, 8]. This could bring an advantage of the capecitabine therapy over 5-FU, potentially enhancing specificity of its effect on primary cancer and resulting in shrinkage of micrometastases.

Rectal carcinoma is accessible to preoperative microexcision biopsy, which provides valuable chance to follow changes in biomarker levels during therapy, at acceptable risk to the patient. The pyrimidine metabolism enzymes thymidine phosphorylase (TP), thymidine synthase (TS) and dihydropyrimidine dehydrogenase (DPD) are involved in fluoropyrimidine turnover. Both their protein levels [9, 10] and their mRNA levels [11, 12] were evaluated as potential prognostic factors or predictors of chemoresistance to 5-FU.

Only few studies [13,14] have evaluated predictive or prognostic role of pyrimidine metabolism markers in preoperative capecitabine treatment, probably because it has been ex-

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pected that capecitabine as a 5-fluorouracil prodrug would give results similar or identical to the 5-FU. According to the earlier papers, low TS, TP or DPD mRNA levels were associated with favorable responsiveness to 5-FU therapy [15, 16]. However capecitabine as a prodrug, requires activation by liver carboxylesterases, cytidine deaminase and consequently by tissue thymidine phosphorylase [7]. It is expected that increased levels of thymidine phosporylase in tumor will preferentially activate capecitabine in tumor tissue, and that high levels of TP might be beneficial to the patient [7, 8]. This is contradictory premise to the results achieved with 5-FU [15, 17]. The change of pyrimidine metabolism biomarker mRNA levels during the course of neoadjuvant single capecitabine and/or concomitant radiotherapy of rectal cancer was not studied so far, according to our knowledge.

The carcinoembryonic antigen serum level is an important dynamically changing marker of tumor behaviour [18, 19]. We used CEA serum levels as an auxiliary marker to monitor the therapy response of cancer patients.

Patients and methods

The patients aged 33-76 years, ECOG performance status of 0-2 [20], who had histologically confirmed rectal adenocarcinoma, were included. Stage II-III patients (cT3 – cT4, cN0 or T2 –T4, cN+) according to lUCC [21] were eligible for this study. The following were exclusion criteria: 1. prior malignant disease, or serious disorders such as uncontrolled hypertension, pregnancy and lactation. 2. the evidence of distant metastatic disease and any prior chemotherapy or radiotherapy. The Ethic Committee of the Masaryk Memorial Cancer Institute approved the treatment protocol. All patients gave written informed consent. Pretreatment disease evaluation included physical examination transrectal ultrasonography and computer tomography (CT) of the abdomen, pelvis and chest.

Capecitabine Treatment. Preoperative capecitabine was administered orally, at a dose of 825 mg/m^2 twice a day. The morning dose was taken approximately two hours prior to radiotherapy at the same time every day for approximately 5.5 weeks from the first to the last day of radiotherapy.

Radiotherapy. Three-dimensional conformal pelvic radiotherapy was delivered using linear accelerator with 18 MV photon beams and with an isocentric technique (source-axis distance of 100 cm). Radiation therapy was given in conventional fractionation in locally curative dosage. The daily fraction dose was 1.8 Gy, applied in five days per week up to cumulative dose of 45 Gy, boosting up to 50,4 Gy, during the period of 5.5 weeks. The dose was referred to the axis intersection (International Commission on Radiation Units and Measurements Report 50).

Bioptic Samples. Tumor microexcisions (1-3 mm^3) were taken before starting the therapy and again after fourteen days. Tumor samples were immersed immediately in RNA Later solution according to manufacturer recommendations (Quiagen GmbH, Germany).

Surgery. The standard total rectal resection or amputation [22], leaving tumor-free resection margins including total mesorectal excision (TME) was performed. Sphincter-preserving anterior resection or the low rectal tumour abdominoperineal resection (APR) with permanent colostomy was performed according location of tumor and feasibility of sfincter preservation. All resections were performed within 6th week after completion of radiotherapy.

Pathological Criteria. Pathological examination involved the former tumor-bearing area and its macroscopic description.

Proximal, distal and radial margins, tumor mass, fibrotic and irradiation changes were carefully examined and recorded. Microscopic examination was performed using Mandard's tumor regression (TRG) criteria [23] adapted to colon cancer [24]. "Responders" for statistical analysis in our study are defined as patients with complete remission (TRG1) or substantial response with TRG2 and residing microscopic signs of disease only. "Non-responders" are patients having tumor regression grade TRG 3-5.

Determination of CEA. Carcinoembryonic Antigen (CEA) serum levels were determined by Enzyme Immunoassay CEA Test using Elecsys 2010 (Roche GmbH, Mannheim, Germany) [25].

Determination of Pyrimidine Metabolism Marker mRNA Levels. TS, DPD and TP mRNA levels in tumour biopsies were determined in pre-treatment samples and two weeks after starting therapy. RNA was isolated by means of TriReagent under manufacturer recommendation (MRC Inc., Cincinnati, USA). RNA quality was checked using denaturation electrophoresis [26] and only non-degraded RNA without DNA contamination signs was processed. One μg of isolated RNA was reversely transcribed by SuperScript II RNase H- Reverse Transcriptase and oligo 17 dT, (Invitrogen, Carlsbad, CA, U.S.A.) using standard protocols [26]. The cDNA corresponding to 60 ng RNA and TaqMan R Universal Master Mix (Applied Biosystems, USA) was used for Real Time PCR in total reaction volume of 25 μl.

Relative mRNA levels were determined as a ratio of given marker mRNA to GAPDH mRNA level (an internal control standard). The primers and probes originate from two different exons and were designed using Primer Express 2 software (Applied Biosystems, USA) and checked for homologies in Blast program [27].

Real Time PCR primer details are as follows. GAPDH primers and probe were Pre-Developed TaqMan R Assay Reagents Human GAPDH, Part No. 4326317E (VIC-MGB), Applied Biosystems, USA. The other primers and TaqMan ™ MGB probes were:

TS primers:TS 511F 5'-ggcttccagtggaggcattt-3'
TS584R 5'-tggtcaactccctgtcctgaat-3'
TS probe: FAM-5'-cagaatacagagatatggaatca-MGB-3'
DPD primers: DPDkF: 5'-ccagaaagggaaaccagttcc-3'

DPDkR: 5'-gattttcttgcgctgttccag-3'

DPDProbe: FAM-5'-tgaactcatggacaagaaactgcca-MGB-3'

TP primers: TPkF: 5'-caggaggcaccttggataagc-3'

TPkR: 5'-tctgacccacgatacagcagc -3'

TPProbe: FAM-5'- agcccagagcagatgcaagtgctgc –MGB-3'

Statistical Analysis. Statistical analyses were performed by means of Statistica TM [28]. Normality tests (Shapiro-Wilk), paired (Wilcoxon) and non-paired (Mann-Whitney) tests were calculated for datasets, after excluding outliers (values >3SD).

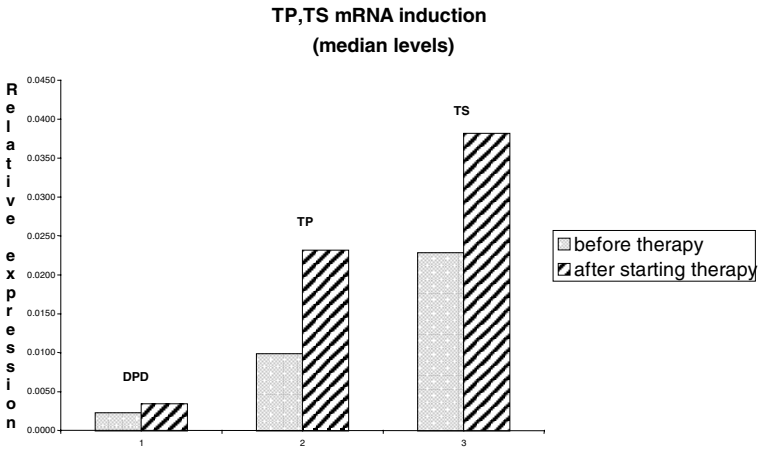
Results

Clinical Response, Surgery. Clinical response was based on the endorectal ultrasonography (EUS). EUS-based downstaging of T and N category combination was observed in 75% patients. Clinical response based on computer tomography was present in 37% patients; 3% patients showed disease progression according to CT.

Downstaging rate based on pathological findings was 76%. As many as 36% of patients achieved substantial pathological response with microscopic residual disease only and grade TRG2 or achieved complete pathological remission (TRG1). Sphincter saving surgery was performed in 67% cases including 46%patients with low rectum tumor. Table 1 summarizes frequency of different tumor regression grades TRG.

Carcinoembryonic Antigen Levels. There were 16% complete or partial responders (ypCR, ypR1) who had elevated pre-treatment CEA levels opposite to elevated CEA in 33% non-responders. The elevated CEA serum levels turned to normal in 90% of all cases after therapy completion. None responders but one had elevated CEA levels after finishing therapy.

Expression of Important Pyrimidine Metabolism Genes. DPD, TP and TS/GAPDH mRNA relative levels were determined in samples from 55 patients before starting therapy and in 47 paired samples taken before and two weeks after starting therapy. The arbitrary numbers representing relative ratios of gene expression were used in statistical testing. Normality tests Shapiro-Wilk (Royston), Kolmogorov-Smirnov (Lilliefors) [28, 29] showed non-parametric distribution at p<0,0001 and/or p<0,01 in all markers respectively.



Graph 1. Increases of median mRNA levels after starting therapy in paired samples (responders and nonresponders included).

TP and TS mRNAs were increased two weeks after starting therapy at the statistically significant level, both in responders and non-responders (Wilcoxon test). Median levels are presented in Graph 1. Statistical significance test shows Table 2. The most significant mRNA induction is seen in TP (2,3x), including both groups of responders and non-responders.

We investigated also the differences between responders and non-responders, which summarizes Table 3. The TP mRNA induction shows the statistically significant difference between responders and nonresponders as calculated by means of Mann-Whitney U-test.

All the important data about TP mRNA induction are included in Graph 2.

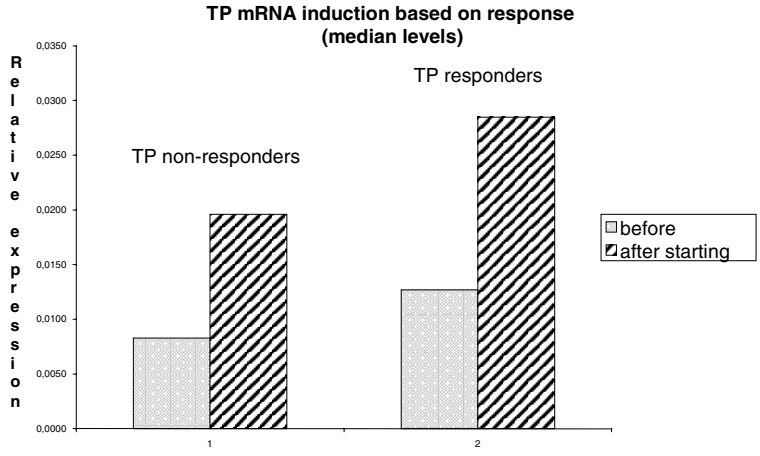
Because of expected role of TS in 5-FU treatment response [30, 31] and colorectal cancer prognosis [32], and possible association of TS induction with these events, we summarized data about TS induction in the graph 3. TS induction is marked TS>1 if there is an increase of TS mRNA level in paired sample two weeks after starting therapy. Adversely TS depression is marked TS<1 if there is an decrease of paired sample two weeks after starting therapy compared to status before. Both responders and nonresponders exert about 2-fold higher frequency of induction than depression in their groups two weeks after starting therapy. Irrespectively of response, there are 68% of TS>1 and 32% of TS<1 tumors in our study.

Table 1. Tumor regression grade (TRG) frequency

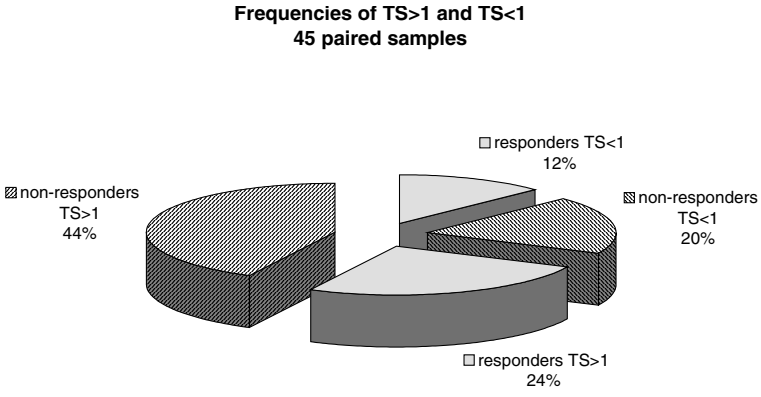
TRG	% of Patients
TRG 1	21
TRG 2	33
TRG 3	31
TRG 4	12
TRG 5	3

Table 2. Statistical significance of observed inductions according to Wilcoxon paired test without outliers, bold are significant

	Fold increase	N	Wilcoxon p-level
DPD/GAPDH_ before & DPD/GAPDH after	1.5	45	0.072
TP/GAPDH before & TP/GAPDH after	**2.3**	45	**0.001**
TS/GAPDH_ before & TS/GAPDH_ after	**1.7**	45	**0.001**



Graph 2. Median values of TP mRNA before and after starting therapy; differences between groups of responders and nonresponders.



Graph 3. Frequencies of TS>1 and TS<1 in responders vs. non-responders in paired samples

Discussion

Preoperative combined chemotherapy and radiotherapy have downstaging and downsizing effects in many patients and support curative surgery in locally advanced rectal cancer [4, 5]. According to our results, reduction of tumour mass is present in 76% of patients based on pathological examination. This is in good accordance with our EUS-based 75% downstaging of T and N category combination. These results are also in concordance with Kim and co-workers, who applied similar treatment schedule and found 76% downstaging under pathological criteria [6].

Low levels of DPD, TP and TS mRNA as determined by semi-quantitative methods, have been associated with tumor sensitivity to 5-fluorouracil [15, 16]. Some studies associated low mRNA or protein TS and TP levels with tumor sensitivity to fluoropyrimidines [16, 33]. On the other hand Kornmann et al. reported the link of high TS and low DPD mRNA levels to the patients prognosis [34]. The expression of TS and DPD proteins was not observed predictive for survival in patients with stage III colon cancer treated adjuvantly with 5-FU [35]. Our results do not show any significant relation between TS levels and immediate response to capecitabine (see table 3.). This is in agreement with the observation of Uchida [36] who did not observed any association of TS and DPD mRNA levels with conventional histopathological grade factors but revealed better prognosis in a group of 5-FU treated patients whose surgical specimens contained lower TS and DPD mRNA levels. Kornmann [31] observed higher median TS levels in 5-FU treated patients without recurrence. However in the group of recurrent patients those having high TS levels exhibited median recurrence – free survival by 7 months shorter.

The induction of TS and TP after 5-FU treatment is already known phenomenon first discoverend in cell lines and xenografts [37, 38] but was not proved in clinical samples from preoperative chemoradiotherapy with capecitabine yet.

Discrepancies concerning predictive or prognostic role of TP, TS and DPD could have several different reasons. Older semi-quantitative methods must naturally generate much more variability than present standard quantitative Real Time PCR.

It is well documented that TP levels in tumor-associated (TA) macrophages are even higher in comparison to those in

Table 3. Median values of different markers before and after starting therapy splited into separate groups of responders and nonresponders. Mann-Whitney test of significance of differences between responders and non-responders. Unpaired samples, bold is significant.

	N Resp	Median Value	N Nonresp	Median Value	Mann-Whitney U-Test p-level (2*1sided)
DPD/GPD_ before	21	0,0029	32	0,0021	0,478
TP/GAPDH_ before	21	**0,01270**	32	**0,00828**	**0,017**
TS/GAPDH_ before	21	0,02755	32	0,02108	0,689
DPD/GPD_ after	15	0,00343	30	0,00352	0,762
TP/GAPDH_ after	15	0,02850	30	0,01958	0,438
TS/GAPDH_ after	15	0,05266	30	0,03624	0,347

tumor cells [39]. The inter- and intra-tumoral variability in the proportion of different tumor-associated cells like macrophages and stroma is also well known [39, 40]. The collecting method is also very important. Because of cell type-associated gene expression differences, the use of the laser captured microdissection method to obtain pure tumor cells could give results quite different from those using other collection methods where mixed cell populations are harvested. TS is strongly expressed in the sites of the tumor proliferation [41]. Therefore sample collection site could be crucial. According to our experience, the inter-sample difference between TS mRNA levels could be more than 50% (not published). Most studies were carried out on 5-FU treated cancers but apparently existing specific differences in predictive or prognostic value of concerned markers might associate with different 5-FU treatment regimens. It is obvious that profound differences could exist between 5-FU and capecitabine since the later acts as a prodrug.

Most tumors of different origin exhibit higher TP protein levels compared to surrounding non-tumorous tissue [8]. Thus presence of tumor cells and macrophages might account for preferential capecitabine activation. From this point of view, there could be adverse effect of TP on the response to 5-FU compared to capecitabine response. While low TP mRNA levels could be beneficial for 5-FU sensitivity in some patients [15], high TP levels could be beneficial for tumor sensitivity to capecitabine [42]. High TP levels could be also beneficial for low TS patients treated with folate-based TS inhibitors, because of thymidine depletion caused by TP phosphorylase activity [1, 42]. On the other hand, TP is also an angiogenic factor promoting tumor growth [42, 43, 44]. From this point of view, high TP levels could be unfavorable. Concerning the extent of capecitabine treatment response, in case of all tumor cells eradication, high TP levels would be beneficial to the patient. However, if some tumor cells sustain therapy (partial responders), the other aspect of high TP level could gain ground, promoting tumor growth after finishing therapy, owing to TP-associated angiogenic activity [45]. Based on this presumption, high TP level could be beneficial for complete capecitabine responders, however it could represent contemporarily bad prognostic factor in cases whom non-complete remission was achieved and/or in cases receiving drugs that are not activated by TP in tumor tissue.

As expected from the role of TP in capecitabine activation, we showed that complete or partial responders had statistically significant higher median TP mRNA levels as compared to non-responders before starting therapy. Median levels are 1,5 x higher in responders. Moreover, both responders and most non-responders exert at least partial response (85% response frequency if we include TRG1-3) as could be seen from table1. summarizing the frequency of different tumor regression grades (TRG). Contemporarily to at least minimal response in both groups, responders and non-responders together elicit 2,3x induction of TP after starting therapy (calculated from table 3. medians) compared to pretreatment values. It is in support of expected TP role in immediate response to capecitabine. The TP difference between responders and non-responders might be more significant if measured in samples taken from tumor-invasion wall, where highest TP levels are expressed [46], but this part is not accessible by simple surface micro excision. Taking micro-excision biopsy samples from tumor-invasion wall would be a complicated technical problem in rectum or colon in clinical situation and is not acceptable because of high health risk.

DPD, TP and TS are not the only factors involved in cellular response to fluoropyrimidines. Our results could only confirm the already well-known complex nature of response to fluoropyrimidines [47, 48] and highlight the need of multiple factors involvement in tumor response prediction [49, 50, 51].

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