Prognostic Markers in Early-stage Colorectal Cancer: Significance of *TYMS* mRNA Expression

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Abstract. *Background: Several studies have recently indicated the prognostic or predictive role of several biomarkers in colorectal cancer. We sought to investigate the prognostic value of prostaglandin synthase 2 (PTGS2), cyclooxygenase 2 (COX2), thymidylate synthetase (TYMS), thymidine phosphorylase (TYMP), dihydropyrimidine dehydrogenase (DPYD) and topoisomerase I (TOPO1) in colorectal cancer patients treated with 5-FU-based regimens, such as De Gramont and FOLFOX in the adjuvant setting. Materials and Methods: In total, 96 formalin-fixed paraffin-embedded and 30 fresh-frozen tumor tissue samples were evaluated using immunohistochemistry, quantitative reverse transcription-polymerase chain reaction and microarray gene expression profiling, respectively. Results: The majority of tumors exhibited protein overexpression of COX2 (69%), TYMS (75%) and TOPO1 (75%). There was a significant association of TYMP protein expression with T classification, gender and stage (p=0.040, p=0.041 and p=0.011, respectively). TOPO1 protein expression was correlated with TOPO1 mRNA expression and was positively associated with stage (p=0.002) and lymph node infiltration (p=0.004). In univariate analysis, patients with high TYMS mRNA expression were shown to have a significantly lower risk for progression and death (Wald's p=0.030 and p=0.015, respectively). However, in multivariate analysis, only a trend for decreased risk for death was shown in patients with high TYMS mRNA expression (Wald's p=0.083), while patients with high PTGS2 mRNA expression had a trend for lower risk for progression (p=0.064). Using supervised hierarchical clustering, based on the expression in fresh-frozen tumor tissue of PTGS2, TYMS, TYMP and DPYD, our 30 patients were separated into two clusters. One of the clusters was enriched with patients with infiltrated lymph nodes (p<0.05), suggesting that these genes might have an impact on the tumor's ability to metastasize. Conclusion: These findings indicate a possible prognostic role of TYMS mRNA expression and highlight a cluster of genes associated with nodal metastases that warrant further investigation in a larger cohort of patients with colorectal cancer treated with 5-FU-based adjuvant chemotherapy.*

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Key Words: PTGS2, TYMP, DPYD, TOPO1, COX2, TYMS, 5-FU-based adjuvant chemotherapy, hierarchical clustering, stage II colorectal cancer, stage III colorectal cancer.

Table I. *Patient, treatment and survival data (formalin-fixed paraffin-embedded tumor tissue cohort).*

N=96		
Median age at surgery (range)	70.2 (32.5-90.0)	
	N	%
Gender		
Male	49	51.0
Female	47	49.0
Primary site		
Colon	55	57.3
Rectum	41	42.7
T Classification		
T1	4	4.2
T2	14	14.6
T3	72	75.0
T4	6	6.3
N Classification		
N0	57	59.4
N1	27	28.1
N2	12	12.5
Stage (7th AJCC Edition)		
I	16	16.7
II	41	42.7
III	39	40.6
Histological grade		
I	26	27.1
II	53	55.2
III	17	17.7
Obstruction	5	5.2
Perforation	7	7.3
Total number of patients that received adjuvant CT	59	61.5
5-FU/folinic acid	26	27.1
FOLFOX	18	18.8
FOLFOXIRI	1	1.0
FOLFIRI	13	13.5
Capecitabine monotherapy	1	1.0
Median number of cycles (range)	6 (1-12)	
Overall survival		
Deaths N (%)	30 (31.2)	
Median (95% CI)	102.7 (not reached yet)	
Percent 3-year rate (95% CI)	84.2 (76.9-91.4)	
Range	2.1-102.7	
Disease-free survival		
Events N (%)	38 (39.5)	
Progressions N (%)	21 (21.9)	
Median (95% CI)	Not estimated yet	
Percent 3-year rate (95% CI)	78.1 (69.7-86.3)	
Range	2.1-84.6	
Median follow-up in months (range)	82.7 (16.1-95.2)	

N, Number of patients; CI, confidence interval; CT, chemotherapy; AJCC, American joint committee on cancer, cancer staging.

Table II. *Patient, treatment and survival data (fresh-frozen tumor tissue cohort).*

N=30		
Median age at surgery (range)	71.4 (32.5-84.0)	
	N	%
Gender		
Male	18	60.0
Female	12	40.0
Primary site		
Colon	16	53.33
Rectum	14	46.67
T Classification		
T1	1	4.0
T2	4	16.0
T3	19	76.0
T4	1	4.0
N Classification		
N0	15	60.0
N1	3	12.0
N2	7	28.0
Stage (7th AJCC Edition)		
I	5	20.0
II	9	36.0
III	11	44.0
Histological grade		
I	9	30.0
II	17	56.67
III	4	13.33
Obstruction	0	0.0
Perforation	1	3.33
Total number of patients that received adjuvant CT	12	40.0
5-FU/folinic acid	3	10.0
FOLFOX	7	23.3
FOLFOXIRI	1	3.3
FOLFIRI	0	0
Capecitabine monotherapy	1	3.3
Overall survival		
Deaths N (%)	6 (20.0)	
Median (95% CI)	49.8 (33.8-not reached yet)	
Range	2.8-64.6	
Disease-free survival		
Events N (%)	12 (40.0)	
Progressions N (%)	10 (33.3)	
Median (95% CI)	47.5 (25.8-not reached yet)	
Range	0.1-64.6	
Median follow-up in months (range)	56.6 (0.1-84.6)	

N, Number of patients; CI, confidence interval; CT, chemotherapy; AJCC, American joint committee on cancer, cancer staging.

Colorectal cancer (CRC) is the third most common cancer worldwide accounting for 10% of all malignant neoplasms and the fourth cause of death in the adult population (1, 2). Surgical resection followed by combination chemotherapy, including 5-fluorouracil (5-FU) and leucovorin or capecitabine and oxaliplatin, has improved the outcome of patients with stage III CRC by 25% (3, 4). With respect to stage II CRC, only a small percentage of patients with features reflecting a high risk of relapse, such as obstruction, perforation, inadequate lymph node sampling or T4 disease, may benefit from adjuvant 5-FU-based chemotherapy (5).

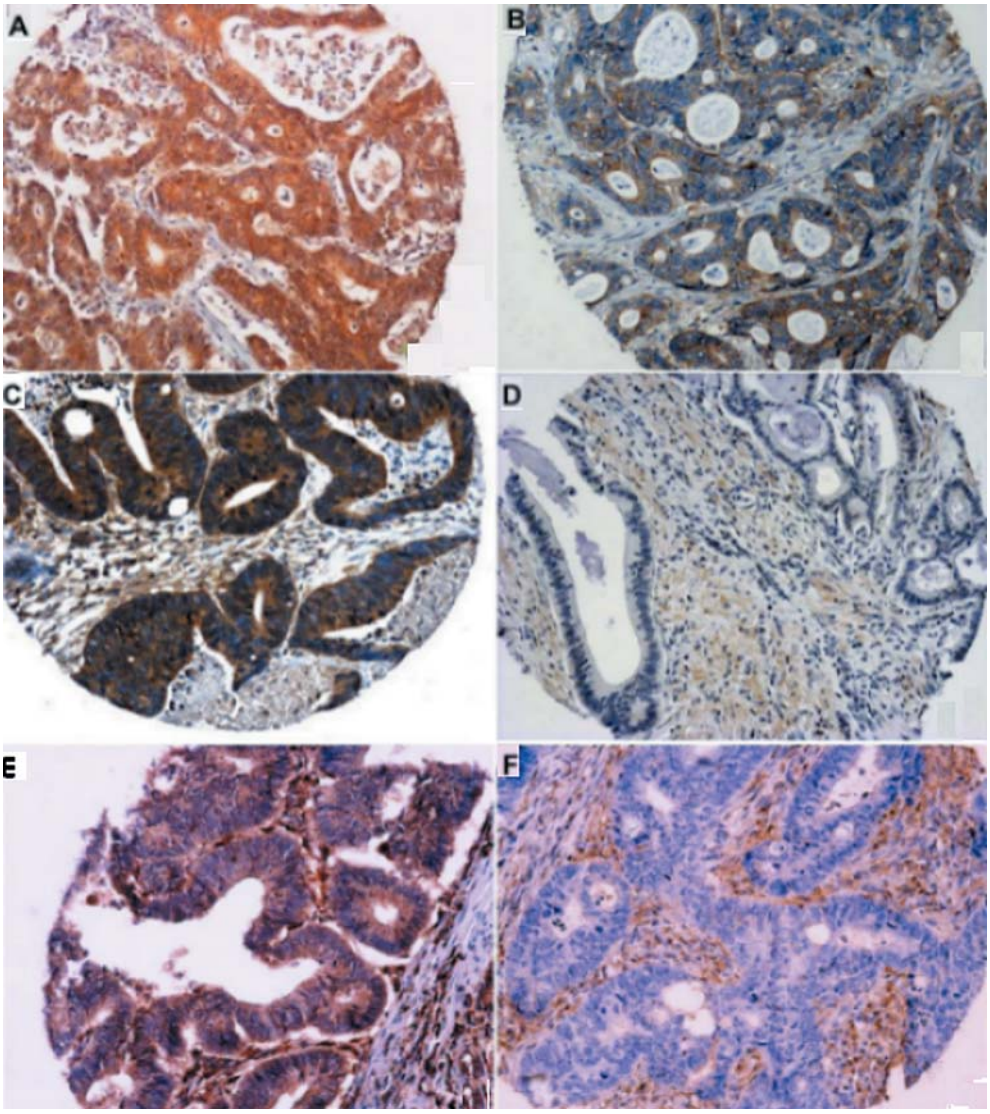

Figure 1. *Immunohistochemistry performed on tissue microarrays of invasive colorectal carcinomas obtained from the first cohort of patients (formalin-fixed paraffin-embedded tumor tissue cohort). A: Intense cyclooxygenase 2 (COX2) protein expression in tumor cells; B: moderate COX2 protein expression; C: strong cytoplasmic thymidylate synthetase (TYMS) protein expression in tumor cells; D: lack of TYMS staining; E: intense nuclear and cytoplasmic thymidine phosphorylase (TYMP) protein expression; F: lack of TYMP staining. Original magnification ×200.*

Several single molecular markers have been investigated for their prognostic role in CRC. Among them is the prostaglandin synthase 2 gene (*PTGS2*) encoding cyclooxygenase 2 (COX2), a key enzyme in prostaglandin biosynthesis, acting both as a dioxygenase and a peroxidase. COX2-inducible isozyme is regulated by specific stimulatory events, suggesting that it is responsible for the prostanoid biosynthesis involved in inflammation and tumorigenesis (6). COX2 converts free arachidonic acid to prostaglandin E2, which by activation of its receptor leads to signaling for enhancement of cellular proliferation, promotion of angiogenesis, inhibition of apoptosis and stimulation of invasion/motility in CRC (7). Numerous studies have shown that celecoxib, a selective COX2 inhibitor with Food and Drug Administration approval for CRC prevention, is a potent suppressor of angiogenesis and colonic polyp formation in familial adenomatous polyposis (FAP) animal models and in patients with FAP (8-10). However, the influence of COX2 expression levels in survival of patients with CRC has been relatively understudied.

A second molecular marker with potential prognostic value is thymidylate synthetase (*TYMS*) that catalyzes the methylation of deoxyuridylate to deoxythymidylate using 5,10-methylenetetrahydrofolate as a cofactor (11, 12). A

third prognostic marker is thymidine phosphorylase (*TYMP*), also known as platelet-derived endothelial cell growth factor (13). TYMP is a key enzyme with a dual role as it contributes to the metabolic processing of pyrimidine nucleotides but also promotes neovascularization (14).

A fourth marker is dihydropyrimidine dehydrogenase (DPYD), a pyrimidine catabolic enzyme and the initial and rate-limiting factor in uracil and thymidine catabolism. It is also responsible for the metabolism of 5-FU into its inactive metabolites (15). Finally, topoisomerase I (*TOPO1*) plays an important role in regulating DNA topology and DNA re-ligation (16). Irinotecan, a TOPO1 inhibitor recently evaluated in the context of adjuvant therapy in CRC, induces tumor cell death *via* stabilization of DNA–TOPO1 complexes and double-stranded DNA breaks (17).

A common characteristic of the biomolecules described above is their contribution to processes that are pivotal for tumor survival and progression, such as angiogenesis, inflammation, pyrimidine metabolism and nucleic acid homeostasis. Our study aimed to explore the prognostic value of the mRNA/protein expression patterns of PTGS2/COX2, TYMS, TYMP, DPYD and TOPO1 by means of quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and immunohistochemistry, respectively in a cohort of 96 patients with CRC, most of whom were treated with 5-FU-based adjuvant chemotherapy. In addition, in order to validate their potential prognostic value with another methodology, microarray gene expression profiling was performed on 30 fresh-frozen CRC tumor tissue samples obtained from a separate cohort of patients treated in a similar manner. The associations of the above factors with age, gender, primary site, lymph node positivity and histological grade were also investigated. Comparison of the markers assessed by different methods was also performed.

Patients and Methods

In total, 96 formalin-fixed paraffin-embedded (FFPE) and 30 fresh-frozen tumor tissue samples were retrieved for biomarker analysis. In the two cohorts of patients with CRC, 61.5% and 40.0%, respectively were treated with 5-FU-based adjuvant chemotherapy, except for one patient in each cohort that received capecitabine monotherapy. In the first cohort with FFPE tumor tissue samples, 27.1% of the patients received a combination of 5-FU/folinic acid, while 18.8% of the patients were treated with FOLFOX and 13.5% received FOLFIRI. In the second cohort with fresh-frozen tumor tissue samples, 10.0% of the patients received a combination of 5-FU/folinic acid, while 23.3% of the patients were treated with FOLFOX.

Tissue microarray (TMA) construction. Two TMA blocks, containing 283 and 300 cores, respectively, were constructed with a manual microarrayer (Beecher Instruments, Sun Prairie, WI, USA) to include five cores (0.6 mm in diameter) from each CRC case, randomly obtained from both the central part and the invasive front of the tumor. In each TMA block, 14 cores in total, from thyroid, skin and tonsil tissue were included as external controls for immunostaining, block orientation and alignment.

Immunohistochemistry. For the immunohistochemical assessment, 4-μm sections from the TMAs or, in some cases, from the original tissue blocks, were mounted on positively-charged slides and stained using antibodies against COX2 (clone 4H12, dilution 1:300; Novocastra™, Leica Biosystems, Newcastle, UK), TYMS (clone TS 106, dilution 1:100; Neomarkers, Fremont, CA, USA), TYMP (clone PGF-44C, dilution 1:100; Invitrogen, Camarillo, CA, USA) and Topo-I (clone 1D6, dilution 1:100; Novocastra™, Leica Biosystems). After de-paraffinization and rehydration of tissue sections, antigen unmasking was carried-out. The detection systems used were Envision (DAKO, Glostrup, Denmark) for TYMP and Super SensitiveTM Non-Biotin HRP Detection System (BioGenex Laboratories, San Ramon, CA, USA) for the remaining antibodies. Slides were counterstained with Mayer's hematoxylin. The thyroid and skin tissue cores served as negative immunohistochemical controls for the TYMS and TOPO1, while the tonsil tissue as a positive control for TOPO1 and TYMP. Skin tissue was used as control for COX2, while a known positive CRC case and an ovarian carcinoma case were used as external positive immunohistochemical controls for TYMS and TOPO1, respectively. DPYD protein expression was not evaluated due to non-availability of an antibody.
Evaluation of immunohistochemical staining. The percentage of stained cells and the intensity of the immunostaining within each specimen were evaluated according to previously proposed/established criteria with slight modifications (18, 19). The intensity score ranged from 0 to 3 (0=no staining, 1=weakly positive, 2=moderately positive and 3=strongly positive staining). The staining pattern score, based on the percentage of positive tumor cells, ranged from 0-3 (0=0 to 5%, 1=6 to 25%, 2=26% to 50% and 3=51% to 100%). The overall estimation (the sum of the two scores) was classified into two categories as high or low. Cases with a total score of at least 4, were considered to be positive or high expressing tumors, whereas cases with a total score of 0-3 were grouped together and were considered to be negative or low-expressing tumors. The localization of staining in each cell compartment was also indicated.

RNA extraction and gene expression assessment. Out of the originally retrieved 96 paraffin blocks, 89 were evaluated as eligible for mRNA expression analysis based on histological review and tumor tissue abundance. RNA was extracted from macrodissected (>50% tumor cell content) FFPE sections with Trizol-LS (Invitrogen/Life Technologies, Paisley, UK), measured in a UV spectrophotometer and reverse transcribed (2-4 μg/reaction) with random hexamers and Superscript III followed by incubation with RNase H for excess RNA removal (all reagents from Invitrogen), according to the manufacturer's instructions. Exon spanning, premade TaqMan® Gene Expression Assays (Applied Biosystems, Antisel, Athens, Greece) were used to assess the relative expression of *PTGS2* (Hs00153133_m1; NM_000963.1; ex 506; 75bp), *TYMS* (Hs00426591_m1; NM_001071.2; ex 6-7; 87bp), *TYMP* (Hs00157317_m1; NM_001113755.1 ex 4-5; NM_001113756.1 ex 3-4; NM_001953.3 ex 4-5; 95bp), *DPYD* (Hs00559279_m1; NM_000110.3; ex 1-2; 74bp) and *TOPO1* (Hs00243257_m1; NM_003286.2; ex 13-14; 101bp) in comparison to a housekeeping gene [beta-glucuronidase (*GUSB*): Hs99999908_m1;

NM_000181.3; ex 11-12; 81bp]. The latter also served as an endogenous control for the evaluation of amplifiable template adequacy. Samples were assessed twice in 20 μl reactions, in separate runs along with no-template controls for 40 cycles under standard conditions in an ABI7500 real time PCR system and analyzed with SDS v1.4 software (Applied Biosystems, Antisel). Criteria for considering samples eligible for analysis were: (a) amplifiable cDNA control in both duplicates, with *GUSB* cycle threshold (CT) values <36; and (b) evaluation of sample adequacy and PCR efficiency in consecutive runs: absolute difference of dCT ($CT_{target} - CT_{GUSB}$) values for the same sample <0.5. Average CTs for each eligible sample were used for analysis. Relative quantification (RQ) was assessed in an analog manner as (40–dCT) (20-22). Based on these criteria, 84 out of the 89 samples (94.4%) were deemed eligible for *PTGS2, TYMS, TYMP* and *DPYD* mRNA analysis and 76 (85.6%) for *TOPO1* mRNA analysis.

Microarray gene expression profiling. Tumor tissue samples from 30 patients, collected at the time of surgery, were immediately frozen in liquid nitrogen and stored at –80˚C, until processing. RNA isolation from fresh-frozen tissue samples was carried-out using RNAeasy Mini Kits, (Qiagen, Hilden, Germany) and biotinylated cRNA targets were prepared using the standard Affymetrix Protocol. Expression profiling was performed using Human Genome U133 Plus 2.0 Arrays, containing a total of 22,000 probe sets (Affymetrix, Santa Clara, CA, USA).

Statistical analysis. Overall survival (OS) was defined as the time from the initiation of adjuvant chemotherapy to the date of death from any cause. Disease-free survival (DFS) was calculated as the time from adjuvant chemotherapy initiation to the date of verified disease recurrence or the date of death from any cause. Surviving patients were censored at the date of last contact.

The prognostic significance of immunohistochemical markers in OS and DFS was assessed by univariate Cox regression analysis at predefined cut-offs, while for mRNA markers, the median of the mRNA distribution was used as a cut-off. In addition, normalized mRNA expression values were used in the analysis as a continuous variable to evaluate prognostic significance. Time-to-event distributions were estimated using Kaplan–Meier curves.

Associations of immunohistochemically-detected protein expression status and relative mRNA expression levels with selected clinicopathological characteristics were performed using the χ^2 test. In order to compare the distribution of each mRNA marker with its protein expression status, the Mann–Whitney test for continuous variables was used. The associations between the expression of the different biomarkers were examined using the Fisher's exact or the $\chi 2$ test, as well as the Spearman's rank correlation R. For all correlations, the level of statistical significance was set at α=0.05.

The Cox proportional hazards model was used to assess the relationship of clinicopathological parameters and the examined biomarkers with OS and DFS. In the multivariate Cox regression analysis, a backward selection procedure with a removal criterion of p>0.10 based on likelihood ratio test was performed to identify significant variables among the following: age (≥65 *vs*. <65 years), gender (female *vs*. male), histological grade (III *vs*. I-II), primary site (rectal *vs*. colon), T classification (T3-T4 *vs*. T1-T2), N classification (N+ *vs*. N0), tumor stage (III *vs*. I-II), negative *vs*. positive protein expressions of COX2, TYMS, TYMP and TOPO1, and low *vs*. high mRNA expressions of *PTGS2, TYMS, TYMP, DPYD* and *TOPO1*.

Statistical analysis was conducted using SPPS software for Windows (version 15; SPSS Inc, Chicago, IL, USA) and JMP software (version 8.0.2; SAS Institute Inc., Cary, NC, USA). Statistical analysis of the microarray gene expression profiling data was performed using the BRB-ArrayTools software package, developed by Dr. Richard Simon and the BRB-ArrayTools Development Team (http://linus.nci.nih.gov/BRB-ArrayTools.html). Gene set analysis (GSA) was used to assess whether the expression profiles of patients with infiltrated lymph nodes were enriched for biological themes or functional groups of genes (23). Unsupervised hierarchical clustering was performed using the average linkage algorithm.

Results

Patient and tumor characteristics (FFPE tumor tissue sample cohort). Patient information on age, gender, tumor primary site, lymph node status, stage, histological grade, treatment and survival are presented in Table I. The median age at diagnosis was 70 (range=32 to 90) years, with the primary site being the colon in 55 patients and the rectum in 41. More than half of the carcinomas (55.2%) displayed grade II tumor cell differentiation and were associated with a negative lymph node status (59.4%).

Fifty-nine patients (61.5%) received adjuvant chemotherapy, as shown in Table I, with a median of six cycles (range 1-12). In total, 5-FU/folinic acid chemotherapy was given to 26 patients (27.1%), 31 patients were given 5-FU combined with other agents, and one patient was given capecitabine monotherapy.

With a median follow-up of 82.7 (range=16-95) months, 21 relapses (21.9%) and 30 deaths (31.2%) were recorded. The 3-year OS rate was 84.2%, while the 3-year DFS rate was 78.1%.

Patient and treatment information (fresh-frozen tumor tissue sample cohort). Regarding the 30 patients with fresh-frozen tumor tissue samples, patient and treatment information and tumor characteristics are presented in Table II. Their median age at diagnosis was 71 (range=32-84) years, with the primary site being the colon in 16 patients and the rectum in 14. Half of the carcinomas (55.7%) displayed grade II tumor cell differentiation and more than half (60.0%) were associated with a negative lymph node status.

With a median follow-up of 66.6 months (range 1-85 months), 10 relapses (33.3%) and six deaths (20.0%) were recorded. The 3-year OS rate was 76.9%, while the 3-year DFS rate was 67.9%.

Protein expression of markers. The immunohistochemical analysis revealed high protein expression levels for COX2, TYMS and TOPO1 in 68.4%, 74.3% and 74.6% of the tumors, respectively. Conversely, TYMP protein expression levels in 75.3% of the cases were characterized as low or negative.

Representative protein expression patterns of the different markers are displayed in Figure 1. Significant associations were observed between TYMS and COX2, TYMS and TOPO1 protein expression, as well as between TOPO1 and COX2 (Table III).

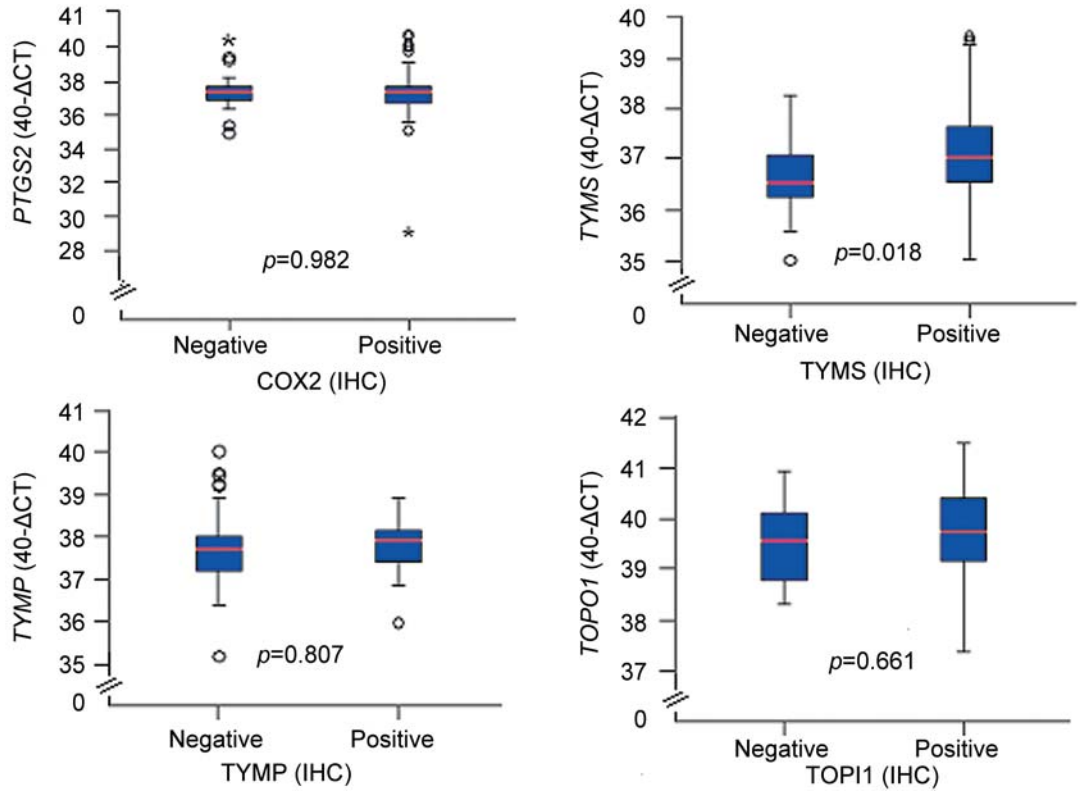

Figure 2. *Association of mRNA expression with corresponding protein expression for all markers [prostaglandin synthase 2 (PTGS2), cyclooxygenase 2 (COX2), thymidylate synthetase (TYMS), thymidine phosphorylase (TYMP), topoisomerase I (TOPO1)]. Comparisons were made using Mann-Whitney tests. Regarding the box-plots, the upper and lower bars represent the maximum and minimum values, respectively. The upper and lower ends of the blue boxes represent the third and first quartiles, respectively, while the red line represents the median. The circles are the outliers that are more than 1.5-times the inter-quartile range, while the asterisks are the outliers that are more than 3-times the inter-quartile range.*

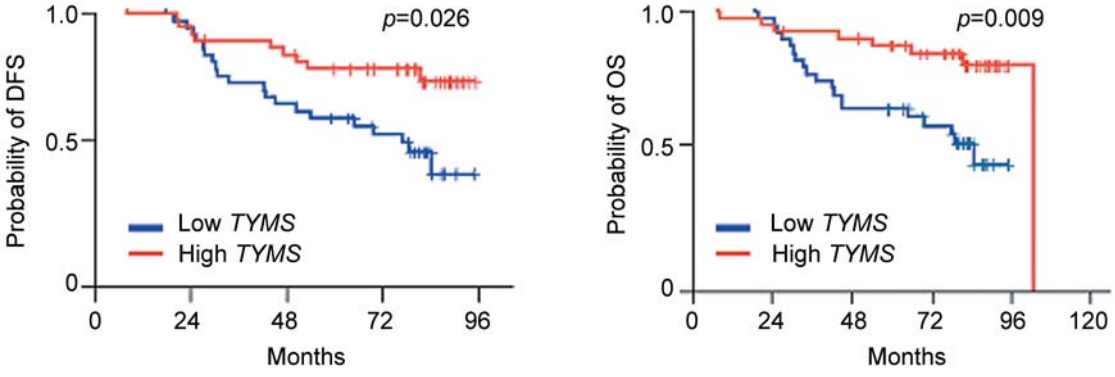

Figure 3. *Disease-free survival (left) and overall survival (right) according to thymidylate synthetase (TYMS) mRNA expression. Comparisons were made using log-rank tests.*

mRNA expression of markers. For all examined mRNA markers, the distribution of RQ values was unimodal, the median cut-off was, therefore, used in order to distinguish tumors expressing high and low numbers of transcripts of the corresponding genes. There were significant but weak correlations among all markers (Spearman's rho: 0.243-0.573), except for TOPO1, for which no significant correlations were observed (Table IV).

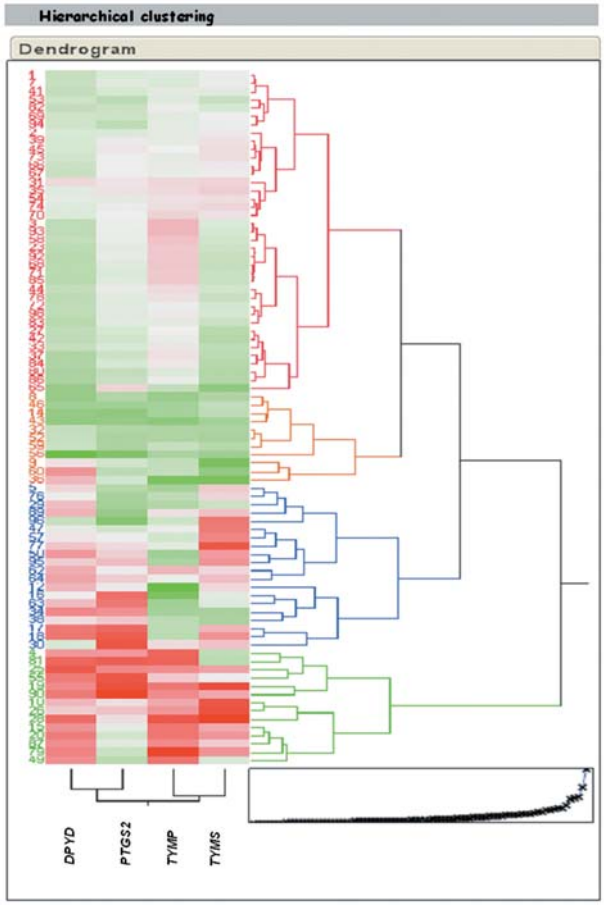

Figure 4. *Hierarchical clustering based on mRNA expression of prostaglandin synthase 2 (PTGS2), thymidylate synthetase (TYMS), thymidine phosphorylase (TYMP) and dihydropyrimidine dehydrogenase (DPYD) identified four groups of patients (red, orange, blue and green) in the second patient cohort (fresh-frozen tumor tissue cohort).*

Association of relative mRNA expression values with corresponding COX2, TYMS, TYMP and TOPO1 protein expression status assessed by immunohistochemistry are shown in Figure 2. Increased *TYMS* mRNA expression was found significantly more frequently in tumors with high TYMS protein expression (p=0.018).

Associations of protein and mRNA expression markers with clinicopathological features. Analysis for the association of immunohistochemical markers with patient and tumor characteristics (Table V) revealed a significant correlation between TYMP protein expression and T classification, gender and stage (p=0.04, p=0.041 and p=0.011 respectively). *TOPO1* mRNA expression (Table VI) was positively associated with stage (p=0.002) and lymph node infiltration (p=0.004).

Associations of clinicopathological features and protein and mRNA expression of markers with survival outcomes. Univariate Cox regression analysis of clinicopathological parameters in relation to DFS and OS showed a significant association between age ≥65 years and tumor stage III with worse OS. Additionally significant associations were found among the presence of positive lymph nodes and advanced tumor grade and stage. Univariate Cox regression analysis for protein and mRNA expression of markers in relation to DFS and OS are shown in Table VII. Out of all assessed markers, a statistically significant association was found only for *TYMS*. More specifically, patients with high *TYMS* mRNA expression were found to have a significantly lower risk for relapse and death (Wald's p=0.030 and p=0.015, respectively). Kaplan–Meier curves for DFS and OS according to TYMS mRNA expression are shown in Figure 3.

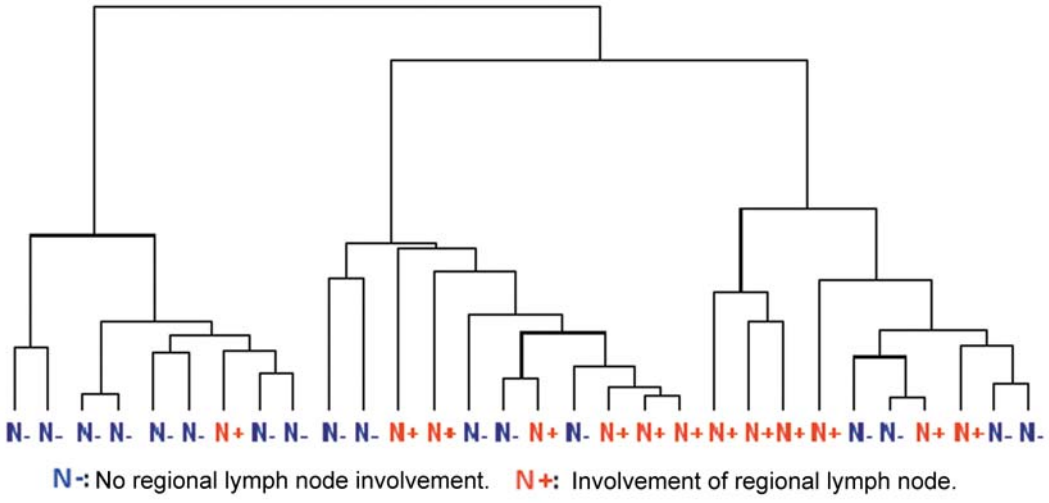

Figure 5. *Supervised hierarchical clustering based on prostaglandin synthase 2 (PTGS2), thymidylate synthetase (TYMS), thymidine phosphorylase (TYMP) and dihydropyrimidine dehydrogenase (DPYD) mRNA expression, as determined with U133 Plus 2.0 chips, according to regional lymph node involvement. The cluster on the right is enriched with patients with infiltrated lymph nodes (χ^2 test, $p<0.05$).*

Table III. *Associations between protein expression of examined markers.*

	COX2, N (%)			TYMP, N (%)			TOPO1, N (%)		
	Negative	Positive	*p*-Value	Negative	Positive	*p*-Value	Negative	Positive	*p*-Value
TYMS									
Negative	16 (66.7)	4 (6.9)	<0.001	13 (20.6)	8 (42.1)	0.076	12 (63.2)	9 (14.8)	**<0.001**
Positive	8 (33.3)	54 (93.1)		50 (79.4)	11 (57.9)		7 (36.8)	52 (85.2)	
TYMP									
Negative	16 (64.0)	46 (82.1)	0.093						
Positive	9 (36.0)	10 (17.9)							
TOPO1									
Negative	12 (46.2)	7 (13.2)	**0.002**	14 (23.0)	6 (31.6)	0.54			
Positive	14 (53.8)	46 (86.8)		47 (77.0)	13 (68.4)				

COX2, Cyclooxygenase 2; TYMS, thymidylate synthetase; TYMP, thymidine phosphorylase; DPYD, dihydropyrimidine dehydrogenase; TOPO1, topoisomerase I. Comparisons were made using Fisher's exact test. Significant *p*-values are shown in bold.

Multivariate analysis of all mRNA markers and selected clinicopathological parameters are shown in Table VIII. Age of 65 years or more and grade III histology were associated with increased risk for death (Wald's $p=0.019$ and $p=0.035$, respectively), while positive lymph nodes and grade III histology were associated with increased risk for relapse ($p=0.024$ and $p=0.031$, respectively). In addition, a trend for a lower risk for death was shown for patients with high *TYMS* mRNA expression (Wald's $p=0.083$), while patients with high *PTGS2* mRNA expression showed a trend for a lower risk for relapse ($p=0.064$).

Microarray gene expression profiling. The findings from the FFPE sample cohort were compared against those of a distinct dataset from 30 fresh-frozen CRC tumor tissue samples that had been profiled for gene expression with the Affymetrix U133 Plus 2.0 array. The microarray data showed that *PTGS2, TYMS, TYMP, DPYD* and *TOPO1* mRNA expressions were not associated with DFS or OS. Unsupervised hierarchical clustering, based on mRNA expression of the first four genes, identified four clusters (Figure 4). Since these clusters did not appear to be of prognostic value, we investigated whether they were associated with other factors, such as lymph node infiltration. Using supervised hierarchical clustering, according to the involvement of regional lymph nodes, our 30 patients were separated into two clusters. One of the clusters was enriched with patients with infiltrated lymph nodes (χ^2 test, $p<0.05$), suggesting that these genes might have an impact on a tumor's ability to metastasize (Figure 5).

Discussion

As far as we are aware, for the first time, our study investigated mRNA expression of *PTGS2, TYMS, TYMP, DPYD* and *TOPO1* and protein expression of COX2, TYMS, TYMP and TOPO1 in relation to the outcome of patients with stage I-III CRC.

Table IV. *Spearman's correlation among mRNA markers.*

		PTGS2	*TYMS*	*TYMP*	*DPYD*
PTGS2	Spearman's (rho)		0.387	0.265	0.482
	p-Value		**<0.001**	**0.017**	**<0.001**
TYMS	Spearman's (rho)			0.352	0.573
	p-Value			**0.001**	**<0.001**
TYMP	Spearman's (rho)				0.243
	p-Value				**0.022**
TOPO1	Spearman's (rho)	–0.142	0.034	0.132	–0.031
	p-Value	0.20	0.76	0.25	0.78

PTGS2, Prostaglandin synthase 2; *TYMS*, thymidylate synthetase; *TYMP*, thymidine phosphorylase; *DPYD*, dihydropyrimidine dehydrogenase; *TOPO1*, topoisomerase I. Significant *p*-values are shown in bold.

COX2 protein overexpression is considered an early event in colorectal carcinogenesis that may increase the metastatic potential of malignant cells and thus their survival (24, 25). One study indicated that COX2 may promote its effects by silencing tumor-suppressor and DNA repair genes *via* its action on CpG island methylation (26). Our study investigated the association of COX2 protein expression with outcome in CRC patients treated with 5-FU-based chemotherapy in the adjuvant setting and detected COX2 protein expression in the tumor tissue of most patients. Most physiological tissues do not express constitutively COX2 but several studies have shown COX2 protein expression in CRC tumor tissues and suggested a possible therapeutic role of non-steroidal anti-inflammatory drugs for this disease (27-30). A prospective study in a stage I-III patient population, evaluating regular aspirin use after CRC diagnosis, indicated a lower risk of CRC-specific mortality among participants

Table V. *Associations of protein expression markers with clinicopathological features.*

	COX2, N (%)			TYMS, N (%)			TYMP, N (%)			TOPO1, N (%)		
	Negative	Positive	*p*-Value	Negative	Positive	*p*-Value	Negative	Positive	*p*-Value	Negative	Positive	*p*-Value
Age (at surgery)			0.16			0.77			0.54			0.54
<65 years	3 (11.5)	16 (27.1)		4 (19.0)	15 (23.4)		15 (23.4)	3 (15.0)		3 (15.8)	15 (24.6)	
≥65 years	23 (88.5)	43 (72.9)		17 (81.0)	49 (76.6)		49 (76.6)	17 (85.0)		17 (85.0)	47 (75.8)	
Gender			0.35			0.32			**0.041**			0.99
Male	16 (61.5)	29 (49.2)		13 (61.9)	31 (48.4)		37 (57.8)	6 (30.0)		11 (55.0)	2 (51.6)	
Female	10 (38.5)	30 (50.8)		8 (38.1)	33 (51.6)		27 (42.2)	14 (70.7)		9 (45.0)	30 (48.4)	
Primary site			0.49			0.31			0.19			0.070
Colon	16 (61.5)	31 (52.5)		14 (66.7)	33 (51.6)		33 (51.6)	14 (70.0)		15 (75.0)	31 (50.0)	
Rectal	10 (38.5)	28 (47.5)		7 (33.3)	31 (48.4)		31 (48.4)	6 (30.0)		5 (25.0)	31 (50.0)	
T Classification			0.13			0.75			**0.040**			0.17
T1-T2	2 (7.7)	13 (22.0)		3 (14.3)	12 (18.8)		8 (12.5)	7 (35.0)		1 (5.0)	13 (21.0)	
T3-T4	24 (92.3)	46 (78.0)		18 (85.7)	52 (81.3)		56 (87.5)	13 (65.0)		19 (95.0)	49 (79.0)	
N Classification			0.64			0.99			0.065			0.20
N0	14 (53.8)	35 (59.3)		13 (61.9)	38 (59.4)		36 (54.7)	16 (80.0)		14 (70.0)	33 (53.2)	
N+	12 (46.2)	24 (40.1)		8 (38.1)	26 (40.6)		29 (45.3)	4 (20.0)		6 (30.0)	29 (46.8)	
Histological grade			0.76			0.99			0.10			0.50
I-II	21 (80.8)	49 (83.1)		18 (85.7)	53 (82.8)		50 (78.1)	19 (95.0)		15 (75.0)	52 (83.9)	
III	5 (19.2)	10 (16.9)		3 (14.3)	11 (17.2)		14 (21.9)	1 (5.0)		5 (25.0)	10 (16.1)	
Stage*			0.43			0.60			**0.011**			0.056
I	2 (7.7)	11 (18.6)		2 (9.5)	11 (17.2)		6 (9.4)	7 (35.0)		1 (5.0)	11 (17.7)	
II	12 (46.2)	24 (40.7)		11 (52.4)	27 (42.2)		29 (45.3)	9 (42.0)		13 (65.0)	22 (35.5)	
III	12 (46.2)	24 (40.7)		8 (38.1)	26 (40.6)		29 (45.3)	4 (20.0)		6 (30.0)	29 (46.8)	

*AJCC, American joint committee in cancer, cancer staging 7th Edition; COX2, cyclooxygenase 2; TYMS, thymidylate synthetase; TYMP, thymidine phosphorylase; TOPO1, topoisomerase I. Significant *p*-values are shown in bold.

with primary tumors expressing COX2 (31). Our study found a trend for improved DFS (p=0.063), but not OS, in patients with high *PTGS2* mRNA expression. Similarly, another study has associated mRNA expression for *COX2* to longer survival (32). One study indicated that mRNA expression for *COX2* might be negatively associated with OS (33), while a second study found that *PTGS2* 8473T>C polymorphisms correlated positively with progression-free survival and OS in patients with recurrent CRC treated with XELOX chemotherapy (34).

The TYMP enzyme catalyzes the phosphorylation of thymidine to thymine, thus contributing to the regulation of the deoxythymidine monophosphate pool in cells (14). TYMP activity is thought to limit possible substrate toxicity and prevent replication errors during DNA synthesis (14). It is elevated in CRC and is expressed in tumor epithelial cells and in tumor stroma, where it stimulates tumor growth by promoting angiogenesis and evasion of apoptosis (14). In addition, TYMP is involved in the metabolism of 5-FU, as well as in the conversion of the oral prodrug, capecitabine, to 5-FU (14). In our study, the majority of patients had a low intratumoral TYMP expression and this was correlated with more advanced disease and T stage. In the literature, similar investigations have reported controversial results. Two studies had results that were either negative or comparable to ours. The first study, performed by ELISA, showed no differences in clinicopathological features between high and low TYMP protein expression levels (35). The second study, performed by quantitative PCR on microdissected tumor specimens, showed a significant reduction in *TYMP* mRNA with increasing N and T stages (36). The opposite findings were reported by two other studies. In the first one, using *in situ* reverse-transcription PCR, a higher tumoral *TYMP* expression was associated with a more advanced Dukes' stage (37). Similar results have been reported by another study applying immunohistochemistry that found a positive TYMP protein expression to be associated with a more advanced Dukes' stage, as well as with lymph node metastasis and extensive angiogenesis (38). In a recent study, the 5-year DFS rate was statistically significantly higher in patients with high TYMP/DPYD ratios compared to patients with low TYMP/DPYD ratios following adjuvant therapy with oral fluoropyrimidines (39). An earlier study showed that among patients undergoing curative resection, those with high stromal TYMP expression had a favorable prognosis (40).

In our study TYMS protein expression was found in the great majority of patients and *TYMS* mRNA expression was related to improved survival outcomes. The findings on

Table VI. *Associations of mRNA expression markers with clinicopathological features.*

	PTGS2, N (%)			TYMS, N (%)			TYMP, N (%)			DPYD, N (%)			TOPO1, N (%)		
	Low	High	*p*-Value	Low	High	*p*-Value	Low	High	*p*-Value	Low	High	*p*-Value	Low	High	*p*-Value
Age (at surgery)			0.13			0.62			0.62			0.81			0.80
<65 years	7 (16.7)	14 (33.3)		9 (22.0)	12 (27.9)		9 (21.4)	12 (28.6)		11 (26.8)	10 (23.3)		9 (23.7)	11 (28.9)	
≥65 years	35 (83.3)	28 (66.7)		32 (78.0)	31 (72.1)		33 (78.6)	30 (71.4)		30 (73.2)	33 (76.7)		29 (76.3)	27 (71.1)	
Gender			0.82			0.054			0.51			0.28			0.82
Male	21 (50.0)	23 (54.8)		26 (63.4)	18 (41.9)		20 (47.6)	24 (57.1)		24 (58.5)	20 (46.5)		21 (55.3)	19 (50.0)	
Female	21 (50.0)	19 (45.2)		15 (36.6)	25 (58.1)		22 (52.4)	18 (42.9)		17 (41.5)	23 (53.5)		17 (44.7)	19 (50.0)	
Primary site			0.52			0.83			0.83			0.83			0.65
Colon	25 (59.5)	21 (50.0)		23 (56.1)	23 (53.5)		24 (57.1)	22 (52.4)		23 (56.1)	23 (53.5)		22 (57.9)	19 (50.0)	
Rectal	17 (40.5)	21 (50.0)		18 (43.9)	20 (46.5)		18 (42.9)	20 (47.6)		18 (43.9)	20 (46.5)		16 (42.1)	19 (50.0)	
T Classification			0.28			0.59			0.28			0.99			0.39
T1-T2	6 (14.3)	11 (26.2)		7 (17.1)	10 (23.3)		6 (14.3)	11 (26.2)		8 (19.5)	9 (20.9)		10 (26.3)	6 (15.8)	
T3-T4	36 (85.7)	31 (73.8)		34 (83.9)	33 (76.7)		36 (85.7)	31 (73.8)		33 (80.5)	34 (79.1)		28 (73.7)	32 (84.2)	
N Classification			0.99			0.50			0.66			0.19			**0.004**
N0	26 (61.9)	25 (59.5)		23 (56.1)	28 (65.1)		24 (57.1)	27 (64.3)		28 (68.3)	23 (53.5)		17 (44.7)	30 (78.9)	
N+	16 (38.1)	17 (40.5)		18 (43.9)	15 (34.9)		18 (42.9)	15 (35.3)		13 (31.7)	20 (46.5)		21 (55.3)	8 (21.1)	
Histological grade			0.41			0.09			0.17			0.78			0.76
I-II	36 (85.7)	32 (76.2)		30 (73.2)	38 (88.4)		37 (88.1)	31 (73.8)		34 (82.9)	34 (79.1)		30 (78.9)	32 (84.2)	
III	6 (14.3)	10 (23.8)		11 (26.8)	5 (11.6)		5 (11.9)	11 (26.2)		7 (17.1)	9 (20.9)		8 (21.1)	6 (15.8)	
Stage*			0.58			0.62			0.64			0.38			**0.002**
I	6 (14.3)	9 (21.4)		6 (14.6)	9 (20.9)		6 (14.3)	9 (21.4)		8 (19.5)	7 (16.3)		8 (21.1)	6 (15.8)	
II	20 (47.6)	16 (38.1)		17 (41.5)	19 (44.2)		18 (42.9)	18 (42.9)		20 (48.8)	16 (37.2)		9 (23.7)	24 (63.2)	
III	16 (38.1)	17 (40.5)		18 (43.9)	15 (34.9)		18 (42.9)	15 (35. 7)		13 (31.7)	20 (46.5)		21 (55.3)	9 (21.1)	

*AJCC, American joint committee in cancer, cancer staging 7th Edition; PTGS2, prostaglandin synthase 2; TYMS, thymidylate synthetase; TYMP, thymidine phosphorylase; DPYD, dihydropyrimidine dehydrogenase; TOPO1, topoisomerase I. Significant *p*-values are shown in bold.

TYMS protein and mRNA expression in CRC are conflicting. One meta-analysis concluded that patients with tumors expressing high levels of TYMS appeared to have a poorer OS compared to those with tumors expressing low levels (41). On the other hand, another meta-analysis concluded that TYMS is not associated with poorer survival and that it is inappropriate to regard TYMS expression as a prognostic factor for patients with stage II/III CRC treated with surgery and adjuvant chemotherapy (42). In yet another study, overexpression of TYMS in patients with lymph node infiltration was significantly associated with increased 5-year recurrence rate and decreased 5-year OS rate (43).

In agreement with our study, a report from the American Society of Clinical Oncology Conference 2013 noted that high TYMS protein and mRNA expression was associated with increased relapse-free survival and OS, notably when patients were treated with FOLFIRI (44). Similarly, another study also noted that patients with the highest level of TYMS expression had an improved clinical outcome following adjuvant 5-FU-based chemotherapy (45). A third study pointed out that patients bearing the *TYMS* 3RG allele have a tendency to have a better response and a longer OS. In multivariate analysis, this favorable genotype was a stronger survival predictor than performance status (46). A prospective study, applied *TYMS* genotyping to separate T_{3-4}, N_{0-2}, M_{0-1} rectal cancer patients into high- and low-risk groups. Neoadjuvant chemoradiotherapy was allocated, with low-risk patients receiving single agent 5-FU and high-risk patients 5-FU and irinotecan. Such strategy led to higher down-staging rates and pathological T_0 responses in high-risk patients receiving combination chemotherapy (47). The results of these studies indicate that further prospective trials on larger numbers of patients with CRC are required to clarify the role of *TYMS* mRNA and protein expression.

DPYD plays a crucial role in the pharmacology of fluoropyrimidines as it inactivates 5-fluorouracil and its oral prodrug capecitabine. Clinical studies have suggested that genetic variations in *DPYD* increase the risk for 5-FU toxicity; however, there is no clear consensus about which variations are relevant predictors. Our study did not show any associations of *DPYD* mRNA levels with clinical features or patient outcome. However, another study showed that, among patients with Dukes' B and C disease treated with adjuvant chemotherapy, those having high *DPYD* mRNA expression had significantly shorter DFS and OS

Table VII. *Univariate Cox regression analysis for tumor markers according to disease-free survival and overall survival.*

	Disease-free survival			Overall survival		
	HR	95% CI	Wald's *p*-Value	HR	95% CI	Wald's *p*-Value
Protein expression						
COX2						
Negative	1			1		
Positive	0.85	0.42-1.75	0.66	0.80	0.36-1.78	0.59
TYMS						
Negative	1			1		
Positive	0.82	0.38-1.78	0.62	1.11	0.44-2.76	0.82
TYMP						
Negative	1			1		
Positive	0.67	0.27-1.62	0.37	0.76	0.29-2.03	0.59
TOPO1						
Negative	1			1		
Positive	1.20	0.52-2.77	0.66	0.81	0.34-1.94	0.64
mRNA expression*						
PTGS2						
Low	1			1		
High	0.56	0.27-1.10	0.09	0.55	0.26-1.18	0.12
TYMS						
Low	1			1		
High	0.46	0.23-0.94	**0.030**	0.37	0.17-0.82	**0.015**
TYMP						
Low	1			1		
High	1.193	0.58-2.23	0.70	1.08	0.52-2.25	0.82
DPYD						
Low	1			1		
High	0.97	0.49-1.92	0.95	1.08	0.52-2.25	0.82
TOPO1						
Low	1			1		
High	1.09	0.54-2.22	0.79	0.89	0.41-2.1.92	0.76

HR, Hazard ratio; CI, confidence interval; COX2, cyclooxygenase 2; TYMS, thymidylate synthetase; TYMP, thymidine phosphorylase; PTGS2, prostaglandin synthase 2; DPYD, dihydropyrimidine dehydrogenase; TOPO1, topoisomerase I. *For mRNA markers, continuous values were also examined for prognostic significance in terms of disease-free survival and overall survival. No significant associations were found using continuous values (Wald's *p*-value >0.05 for all markers). Significant *p*-values are shown in bold.

compared to patients with low *DPYD* expression (48). An additional study identified a haplotype containing no non-synonymous or splice-site polymorphisms in the *DPYD* gene of patients experiencing toxicity to 5-FU, indicating that additional important genetic variations may be located in non-coding gene regions (49). A third study showed that individual *DPYD* single-nucleotide polymorphisms were not associated with OS, whereas one haplotype was (50).

TOPO1 has recently been shown to correlate with survival and response to chemotherapy in patients with CRC. Our study indicated some interesting findings, with the majority of studied samples found to express TOPO1 protein. Previous studies have reported TOPO1 protein expression in 43-51% of CRC (19, 51). A statistically significant correlation was found between *TOPO1* mRNA levels and infiltrated lymph nodes and tumor stage. Our group previously carried-out a retrospective analysis of 498 cases treated with adjuvant chemotherapy showing that TOPO1 protein expression is an independent prognostic factor for survival (52). A recent study of 154 chemo-naive patients with stage III CRC showed that increased *TOPO1* gene copy number is a frequent occurrence and is significantly associated with longer OS (53). A smaller study also investigated the significance of TOPO1 protein expression in 62 patients with advanced disease receiving first-line 5-FU/irinotecan and concluded that TOPO1 protein expression had no influence on objective response, time to progression and OS (51).

Our analyses detected grade III and lymph node infiltration as important predictors of poor survival, as previously reported (54). Additionally, patients over 65 years of age had a worse survival, regardless of whether they were fit for treatment with adjuvant chemotherapy. Older age has previously been reported as a negative prognostic factor in

Table VIII. *Multivariate Cox regression analysis*.

	Disease-free survival			Overall survival		
	HR	95% CI	Wald's *p*-Value	HR	95% CI	Wald's *p*-Value
Age (at surgery)						
<65 years				1		
≥65 years				5.6	1.33-23.97	**0.019**
N Classification						
N0	1					
N+	2.22	1.11-4.43	**0.024**			
Histological grade						
I-II	1			1		
III	2.32	1.08-4.98	**0.031**	2.44	1.06-5.59	**0.035**
PTGS2						
Low	1					
High	0.48	0.24-1.04	0.064			
TYMS						
Low				1		
High				0.48	0.21-1.10	0.083

HR, Hazard ratio; CI, confidence interval; *PTGS2*, prostaglandin synthase 2; *TYMS*, thymidylate synthetase. Significant *p*-values are shown in bold.

patients with CRC, due to the increasing proportion of non-cancer deaths associated with increasing age (55). We also found a significant correlation between protein expression of COX2 and TOPO1, and COX2 and TYMS, suggesting a regulatory role for COX2 in pyrimidine and DNA synthesis.

Assessment of the genetic profile of CRC using microarray platforms might be a useful tool in determining groups of patients with different clinical outcomes, as well as those patients that would benefit from additional personalized treatment. Our microarray analysis based on the expression of four genes (*PTGS2, TYMS, TYMP* and *DPYD*) identified a tumor cluster with a propensity for lymph node infiltration. It is not known whether these four genes are directly implicated in metastatic dissemination and more aggressive disease biology, or whether they are surrogate markers for the former. However, these findings, as a means of identifying patients at risk for relapse, are questionable, and were not confirmed in our RT-PCR experiments. Despite the microarray findings, our results show that immunohistochemically-assessed COX2, TYMS, TYMP and TOPO1 protein expression levels have no independent prognostic significance for OS and DFS in the CRC cohort studied.

Limitations of our study are the small sample size, preventing definite conclusions, its retrospective nature and the fact that the stroma was not represented in the examined samples. As a result we cannot draw any safe conclusions in relation to outcome.

In conclusion, the results of our study indicating a prognostic role of *TYMS* mRNA expression in CRC and a cluster of genes to be associated with nodal metastases deserve further investigation.

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