

ORIGINAL ARTICLE

Prognostic significance of numeric aberrations of genes for thymidylate synthase, thymidine phosphorylase and dihydrofolate reductase in colorectal cancer

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Abstract

Background. Most human cancer cells have structural aberrations of chromosomal regions leading to loss or gain of gene specific alleles. This study aimed to assess the range of gene copies per nucleus of thymidylate synthase (TYMS), thymidine phosphorylase (TP) and dihydrofolate reductase (DHFR) in colorectal cancer, and to evaluate its prognostic significance following adjuvant chemotherapy, since these enzymes are closely related to efficacy of 5-fluorouracil (5FU). **Patients and methods.** Consecutive patients (n = 314), who were completely resected for colorectal cancer stages II–IV and adjuvantly treated with 5-FU were retrospectively evaluated. Paraffin embedded tumor specimens were assessed for gene copies per nucleus of TYMS, TP and DHFR by fluorescence in situ hybridisation (FISH) using specific peptide nucleic acid probes. Outcome according to gene copies per nucleus above and below the median were compared. Also TYMS expression, assessed by immunohistochemistry, was associated with TYMS copies per nucleus. **Results.** The number of gene copies per nucleus were 1.7 (0.7–2.8), 1.8 (0.9–3.1) and 1.8 (1.1–2.7) median (range) for TYMS, TP and DHFR, respectively. TYMS expression was directly associated with TYMS genes per nucleus ($p = 0.05$). Cox multivariate analysis, adjusted for the prognostic impact of disease stage, vascular tumor invasion, and bowel obstruction at resection, revealed that high TYMS gene copy number was associated with significantly higher risk of recurrence (HR = 1.6; 95%CI 1.1–2.2; $p = 0.02$) and death (HR = 1.6; 95%CI 1.1–2.3; $p = 0.01$). No significant differences in outcome appeared according to TP and DHFR gene ratios. **Conclusion.** Aberration of TYMS gene is of significance to expression of TYMS, which may influence the biology and 5-FU sensitivity of colorectal cancer. This may be utilized in the allocation of patients for treatment approaches and for decision on follow-up programs.

5-fluorouracil (5-FU) is used for adjuvant and palliative chemotherapy of gastrointestinal cancer.

A number of enzymes have significance for the efficacy of 5-FU. Cytotoxicity of 5-FU is mediated through irreversible blocking of thymidylate synthase (TS). Being a prodrug 5-FU requires activation by thymidine phosphorylase (TP). Dihydrofolate reductase (DHFR) provides reduced 5,10-methylenetetrahydrofolate cosubstrate for increased TS inhibition.

The chemotherapy is complicated by wide inter-patient variability in antitumor efficacy and host toxicity. Determinants for some of this variation may be differing activity of TYMS [1–3], TP [4–7], and

DHFR [8] deriving from germline genetic characteristics such as polymorph tandem repeat and single nucleotide polymorphism (SNP) in the promoter enhancer region of TYMS [9], and individual differences in TP protein expression [4], and short sequence deletions and SNP for DHFR [10].

In addition, tumor-specific somatic aberrations in TYMS, TP and DHFR genes may be of significance to tumor biology and sensitivity to chemotherapy. Most human cancer cells show aneuploidy, with structural aberrations of chromosomal regions [11]. Loss of entire chromosomes may result from mitotic non-disjunction due to segregation of sister chromatides, whereas deletion or translocation with

recombination between non-homologous chromosomes may account for loss of segments. Conversely, serial multiplication of a homologous chromosome, bridge-breakage-fusion cycles, generating large intrachromosomal repeats and amplified DNA sequences borne on extra chromosomal double minutes, may lead to multiplication of the harbored genes [11]. At the molecular level the genomic instability is reflected in loss or gain of gene specific alleles [11].

Compared to the adjacent normal mucosa colorectal tumor cells display significant differences in *TYMS* [12] gene heterogeneity or *TP* [13], and *DHFR* [14] gene expression. The significance of tumor specific aberrant gene copy numbers to explain for varying protein expression and outcome of 5-FU chemotherapy, however, remains to be clarified.

This retrospective explorative study evaluated the prognostic significance of numerical variation of *TYMS*, *TP* and *DHFR* genes in paraffin embedded tumor specimens from 314 consecutive patients, who were completely resected for colorectal cancer stages II–IV and subsequently received adjuvant chemotherapy with 5-FU (Mayo regimen). Using a panel of new probes that spanned the entire promoter/enhancer and encoding regions, target genes were quantified by fluorescence in situ hybridisation (FISH). The FISH technology combines the advantage of providing an account of gene specific copy numbers in morphologically identified tumor cell nuclei.

Patients and methods

Patients and chemotherapy

Consecutive patients completely resected for colon or rectal carcinomas of stages II–IV, who received adjuvant chemotherapy at the Department of Oncology, Rigshospitalet, Copenhagen University Hospital, Denmark, from February 1996 to December 2003, were evaluated [3,15].

The adjuvant treatment was according to the Mayo regimen, including bolus infusion of 5-FU (425 mg/m²) and isovorin (10 mg/m²) for 5 days, repeated every 4 weeks, for 6 courses. Data on clinical and pathological characteristics and chemotherapy were obtained from surgical, pathological and oncological records. Data on recurrence and survival were followed-up until March 2006 (censoring date) using the National Central Registry on hospital admissions and death recordings. The research ethics committee has approved the study (KF 01-201/03, KF 01-286965).

Tumor specimens

Archival tumor specimens were collected from the primary pathology departments, serving the surgical departments at the hospitals that referred patients to Rigshospitalet for oncological treatment.

From each tissue block one section was stained by routine hematoxylin and eosin for assessing tumor type and degree of cellular differentiation. From each specimen 1 mm cylinders were punched out and collected in multitumor specimen blocks each containing 40 samples. Tissue from normal kidneys was included in arrays serving as reading frame and controls (n = 10). Sections of 4 µm were cut from the arrays and prepared for in situ hybridization.

Thymidylate synthase immunohistochemistry

Sections of 4 µm thickness were deparaffinized in xylene and rehydrated. Antigen retrieval was performed with the slides immersed in citrate buffer in a microwave oven at 95°C for 15 minutes. Endogenous peroxidase was quenched by incubation with 0.3% hydrogen peroxide in methanol. After washing in phosphate buffered saline (PBS), they were placed in 20% normal goat serum in PBS for 20 minutes to block non-specific staining.

The sections were incubated with polyclonal antibodies raised against TS (Dako, Denmark) at a dilution of 1:50 for 1 hour at room temperature. Subsequently, visualization was performed using the Envision Duallink technique (Dako, Denmark) according to the manufacturer's instruction and counterstaining with Mayer's haematoxylin.

Staining intensity was assessed semiquantitatively using the scale 0: no staining, 1: weak staining, 2: moderate staining, and 3: intense staining.

Fluorescence in situ hybridisation (FISH)

For assessment of changes in gene copy numbers, FISH probes were developed by Dako (patent application WO2006128463) [16] for *TYMS*, *TP* and *DHFR*. These probes consisted of a texas red labelled sequence, which spans the relevant gene sequence for *TYMS* (18p11.32), *TP* (22q13) and *DHFR* (5q12–14). Internal controls consisted of a fluorescein isothiocyanate (FITC) labelled sequence targeted at the corresponding a-satellite of the centromere (*TYMS*) or a sequence from the opposite arm of the chromosome (*DHFR* and *TP*). The probes were used with the histology FISH accessory kit (K5599, Dako, Denmark) and Hybridizer® (S2451, Dako, Denmark) following the manufacturers

instructions. Briefly, after deparaffinization and re-hydration the slides were placed in pretreatment solution at 95°C for 10 minutes, cooled for 20 minutes washed for 2 × 5 minutes in wash buffer. The sections were treated with pepsin at 37°C on the hybridizer for 7 minutes and washed in buffer. After the probe mixture was applied, the slides were sealed with a coverslip and rubber cement and placed on the hybridizer. Denaturation occurred at 95°C for 5 minutes followed by overnight hybridisation at 45°C. Coverslips were removed and the slides were placed in stringent wash buffer at 65°C for 10 minutes followed by buffer washes, dehydration and mounting in fluorescence mounting media containing 4,6-diamidino-2-phenylindole (DAPI).

Evaluation of FISH

Probe signals were assessed using a Leica fluorescence microscope (Leica Imaging System Ltd, Cambridge, UK) equipped with a 100 × oil immersion objective and a 10 × ocular (magnification: 1000 fold). Filter cubes were either single bandpass filters for DAPI (A, P31000, Chroma Technology Corp., Rockingham, Vermont, US), FITC (L5, 513849, Chroma) and Texas Red (HQ-TRITC, 41002B, Chroma) or FITC/Texas Red dual bandpass filters (XF-53, Omega Optical Inc. Brattleboro, Vermont, US or P51006, Chroma).

FISH analyses were performed blinded to all clinical informations. Results were recorded in a data report form, listing the number of red signals for the gene specific probe and the number of green signals for the corresponding chromosome specific probe. Signals were counted in 60 nuclei with identifiable boundaries or in enough nuclei to ensure 60 red signals. For evaluation of ploidy status, the ratio of gene to chromosome FISH signals was calculated from the total number of red signals divided by number of green signals counted in each tumor.

The rational for the cut off points eventually adopted were based on the medians of FISH signals per nucleus for TYMS, TP and DHFR and corresponding centromeres in tumors and normal kidney tissue controls being 1.8, though without obvious biological well founded significance.

Statistical analysis

Association between TYMS, TP and DHFR gene status and clinical and pathological parameters were investigated using non-parametric tests.

Survival time was calculated from the time point of surgery. The primary endpoints were relapse free

survival (RFS), defined as time to relapse of primary disease or to death, whichever occurred first, and death from any cause for overall survival (OS). The survival curves were depicted according to the Kaplan-Meier product-limit methodology and compared using the log-rank test. Multivariate survival analysis was conducted using Cox proportional hazards models with backward selection of covariates to evaluate the association of RFS and OS to clinical, pathological, and genetic characteristics. The prognostic value of a given characteristic was quantified by the hazard ratio (HR).

Two-sided $p \leq 0.05$ was regarded statistically significant. Statistics was performed with Statistica software (Statsoft Inc. Tulsa, OK, USA).

Results

FISH analyses

The study included tumor specimens from 328 patients, of which 314 (96%) were assessable by FISH analysis for at least one target gene. The FISH analyses succeeded for TYMS, TP and DHFR genes in 303 (92%), 300 (91%), and 216 (66%) cases, respectively. In the remaining cases the FISH analyses failed for technical reasons, with blurring of the probe signals.

DHFR, TP and TYMS gene aberrations and clinicopathology

In colorectal tumor tissue the median (range) gene copies per cell were 1.7 (0.7–2.8) for TYMS, 1.8 (0.9–3.1) for TP and 1.8 (1.1–2.7) for DHFR, respectively. FISH signals for target genes and corresponding centromeres per tumor cell were correlated for TYMS ($r=0.57$), TP ($r=0.54$) and DHFR ($r=0.36$).

Distributions of gene copy number per tumor cell below and above the median TYMS (0.7–1.7 versus 1.8–2.8), TP (0.9–1.7 versus 1.8–3.1) and DHFR (1.1–1.7 versus 1.8–2.7) according to clinical and pathological parameters are shown in Table I. Significantly lower TYMS gene copies occurred in rectal tumor cells ($p=0.01$). Also vascular tumor invasion was associated with low number of TP genes per cell ($p=0.04$). No other significant association of clinical and pathological parameters with allele imbalance appeared.

TYMS gene aberration and protein expression

Distributions of TYMS expression (immunohistochemical score) according to number of TYMS

Table I. Distribution of thymidylate synthase (TYMS), thymidine phosphorylase (TP) and dihydrofolate reductase (DHFR) allele/nucleus according to clinical and pathological characteristics in 314 colorectal cancer patients.

	TYMS (low; high) % n = 303	P	TP (low; high) % n = 300	P	DHFR (low; high) % n = 216	P
Gender						
Male	(24; 27)	0.4	(25; 26)	1.0	(26; 25)	0.8
Female	(26; 23)		(25; 24)		(24; 25)	
Age						
<70	(42; 38)	0.09	(41; 39)	0.4	(39; 39)	0.9
≥70	(8; 12)		(9; 11)		(11; 11)	
Tumor site						
Colon	(38; 44)	0.01	(40; 42)	0.6	(40; 44)	0.2
Rectum	(12; 6)		(10; 8)		(10; 6)	
Stage						
II	(5; 6)	0.9	(5; 6)	0.1	(6; 6)	
III	(41; 39)		(39; 40)		(40; 38)	
IV	(4; 5)		(6; 4)		(4; 6)	
Differentiation grade						
Well	(15; 17)	0.8	(15; 15)	0.9	(13; 17)	
Intermediary	(21; 18)		(20; 21)		(24; 19)	
Poor	(14; 15)		(14; 15)		(13; 14)	
Perineural invasion						
+	(9; 10)	0.8	(8; 9)	0.9	(8; 10)	0.8
–	(27; 26)		(26; 28)		(26; 25)	
not assessed	(14; 15)		(16; 13)		(16; 15)	0.2
Vascular tumor invasion						
L0, V0	(27; 27)	0.6	(24; 29)	0.04	(26; 26)	0.7
L1, V1	(12; 10)		(13; 8)		(10; 12)	
Lx, Vx	(11; 14)		(13; 13)		(14; 12)	
Intestinal rupture at resection						
+	(3; 5)	0.2	(3; 5)	0.3	(6; 4)	0.9
–	(47; 45)		(46; 45)		(44; 46)	
Bowel obstruction before resection						
+	(5; 8)	0.2	(6; 7)	0.8	(6; 8)	0.4
–	(45; 42)		(44; 43)		(44; 42)	

alleles/nucleus in cancer cells being below (≤ 1.7) or above (≥ 1.8) the median were statistically significantly different ($p = 0.05$) (Figure 1).

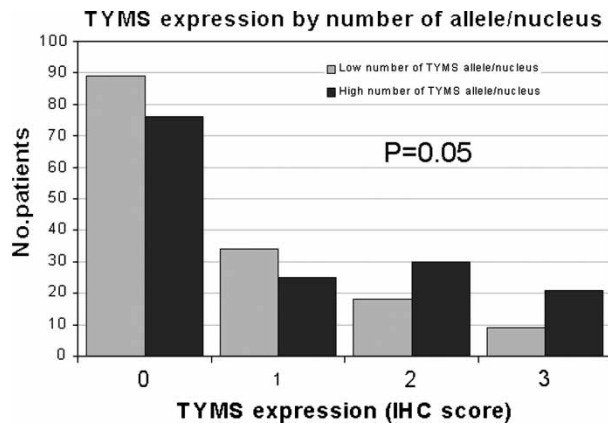


Figure 1. Distribution of thymidylate synthase expression score (0–3) assessed by immunohistochemistry according to number of TYMS allele/nucleus in colorectal cancer cells ($n = 302$).

DHFR, TP and TYMS gene aberrations and association with outcome

Univariate analyses of outcome according to number of TYMS, TP and DHFR gene to tumor cell nucleus, and disease stage in the 314 adjuvantly treated colorectal cancer patients are shown in Table II.

High number of TYMS gene per nucleus, as compared to low number, was associated with significantly higher risk of recurrence (HR = 1.5; 95%CI 1.1–2.2; $p = 0.02$) and death (HR = 1.6; 95%CI 1.1–2.3; $p = 0.02$) (Table II, Figure 2). The association of TYMS gene per nucleus with outcome applied to stage III disease, with hazard ratios of 1.7 (95%CI 1.1–2.6; $p = 0.01$) and 1.8 (95%CI 1.1–2.7; $p = 0.01$) for recurrence and death, respectively.

No significant differences in outcome appeared according to TP and DHFR gene copy number and disease stages (Table II, Figure 2).

Multivariate analysis of associations of outcome with number of TYMS, TP and DHFR genes to

Table II. Univariate analyses of outcome according to TYMS, TP and DHFR allele/nucleus and disease stage in 314 colorectal cancer patients adjuvantly treated with 5-FU.

	Recurrence free survival					Overall survival			
	No.	Events	Hazard ratio (95% CI)		P	Events	Hazard ratio (95% CI)		P
TYMS high/low*									
II, III, IV	303	124	1.5	1.1–2.2	0.02	118	1.6	1.1–2.3	0.02
II	32	12	1.4	0.4–4.9	0.6	11	1.2	0.3–4.2	0.8
III	242	91	1.7	1.1–2.6	0.01	88	1.8	1.1–2.7	0.01
IV	29	21	0.8	0.3–2.0	0.7	19	0.8	0.3–1.9	0.5
TP high/low*									
II, III, IV	300	121	0.9	0.6–1.3	0.6	115	1	0.7–1.4	0.9
II	33	13	0.6	0.2–1.8	0.3	12	0.7	0.2–2.5	0.6
III	238	87	1.1	0.7–1.7	0.7	84	1.2	0.7–1.8	0.5
IV	29	21	0.7	0.3–1.8	0.5	19	0.6	0.2–1.6	0.3
DHFR high/low*									
II, III, IV	216	92	0.8	0.5–1.3	0.4	89	0.8	0.6–1.3	0.4
II	27	11	0.9	0.3–2.9	0.8	10	1.2	0.3–4.1	0.8
III	168	67	0.9	0.5–1.5	0.6	65	0.9	0.5–1.4	0.6
IV	21	14	0.4	0.1–1.3	0.1	14	0.5	0.2–1.4	0.2

*Hazard ratio of high relative to low allele/nucleus.

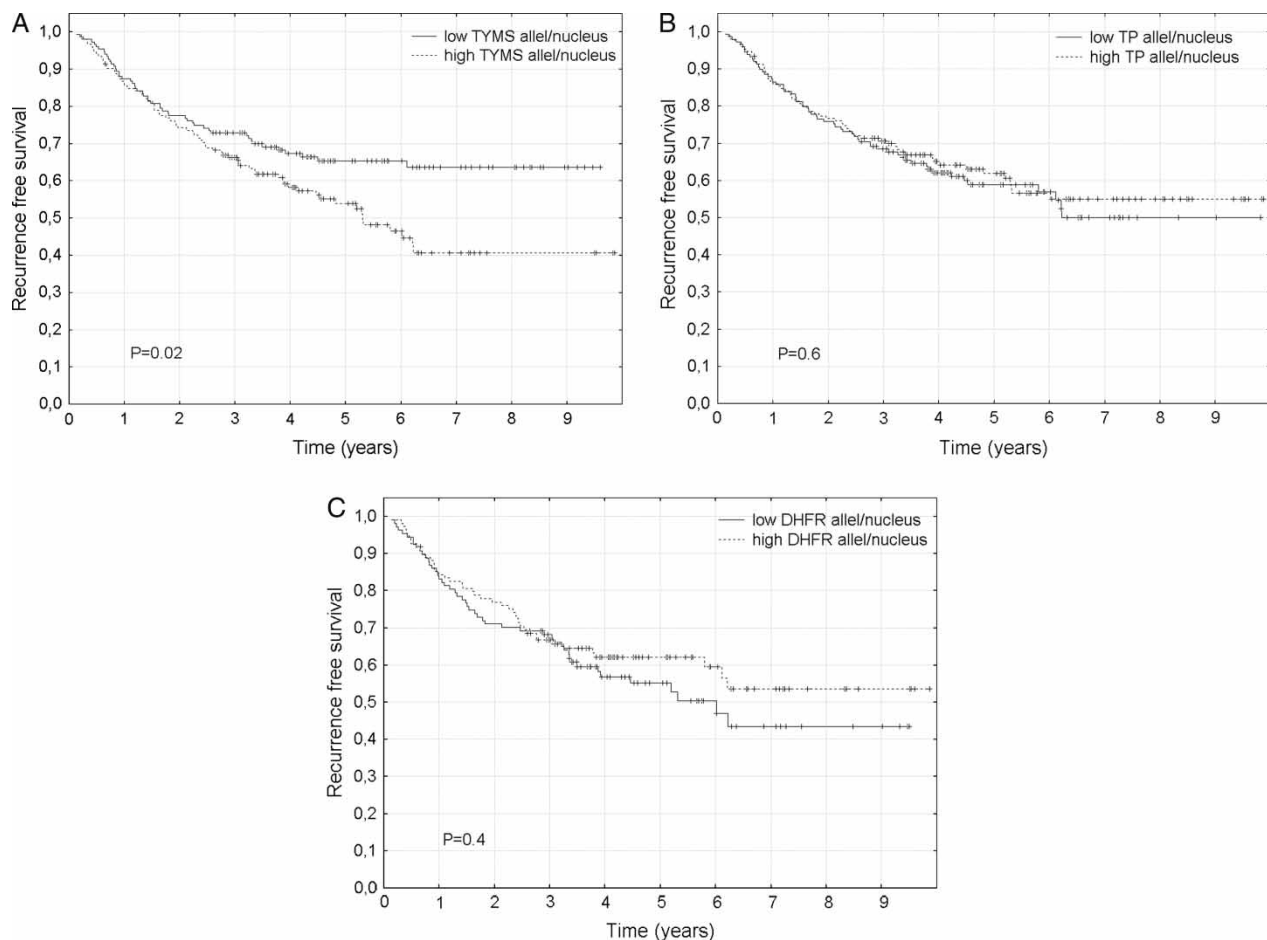


Figure 2. Recurrence free survival following adjuvant chemotherapy of colorectal cancer stage II–IV by number of allele/nucleus for thymidylate synthase (A), thymidine phosphorylase (B) and dihydrofolate reductase (C) in tumor cells. Censored data are indicated (+).

Table III. Multivariate analysis of outcome according to allele/nucleus of TYMS, TP, and DHFR adjusted for the independent prognostic variables stage, vascular tumor invasion and bowel obstruction.

	Recurrence free survival			Overall survival		
	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P
TYMS*	1.6	1.1–2.2	0.02	1.6	1.1–2.3	0.01
TP*	1.0	0.7–1.5	0.9	1.1	0.8–1.6	0.6
DHFR*	0.7	0.4–1.1	0.08	0.7	0.7–1.1	0.1

*high relative to low allele/nucleus.

tumor cell, adjusted for the prognostic impact of disease stage, vascular tumor invasion, and bowel obstruction at resection are displayed in Table III.

High TYMS gene per nucleus was associated with significantly higher risk of recurrence (HR = 1.6; 95%CI 1.1–2.2; $p = 0.02$) and death (HR = 1.6; 95%CI 1.1–2.3; $p = 0.01$). No significant differences in outcome appeared according to TP and DHFR gene number.

Discussion

The tumoral gene dosage for TYMS, TP and DHFR basically covered a biological continuum with at most 4-fold range of numerical variation. The correlation of FISH signals for target genes and corresponding centromeres implied that little (13–32%) of this variability could be explained by the association with number of centromere per nucleus. Which suggest that deletion or independent multiplication was the main underlying pathofysiologic mechanism, whereas aneusomy and co-segregation with entire gene bearing chromosomes to a lesser extent accounted for the variation of gene copy numbers.

The cut-off points based on the medians of gene copies in tumors should be considered arbitrary, as there is no uniform thresholds for defining copy loss or amplification using the FISH technology. The indistinct criteria for categorizing the magnitude of gene specific copy number largely derive from the nuclear truncation that occurs during the sectioning process. Moreover, variable hybridization efficiency, interpretation of signal overlaps and nuclear borders, and interference from specimen autofluorescence, background probe staining, and necrotic tumor tissue are factors that might interfere with the specificity of the technique.

Within the range of TYMS gene aberration established, an association with varying protein expression and outcome was suggested. TYMS may have both prognostic and predictive significance in colorectal cancer with implications for the tumor biology and sensitivity to 5-FU. Evidence suggests

that high TYMS [17] expression may be associated with early spontaneous disease recurrence and death following complete resection, independent of chemotherapy, while also being related to improved outcome from adjuvant 5-FU chemotherapy [17,18]. Conversely, low TYMS [17–19] expression may be associated with low spontaneous recurrence rate and longer survival, while also indicating less benefit from adjuvant 5-FU chemotherapy [17,18]. In accordance with these findings, a previous study in the same patient cohort revealed that high TYMS protein expression was associated with better outcome following adjuvant 5-FU treatment [3].

There may be more explanations why this was not consistent with the relationship between TYMS genes per cell and outcome. While low gene copy number may restrict protein expression, wide ranges for correlation of these parameters appeared, stressing the role of posttranscriptional regulation of TYMS expression. In addition there are shortcomings to the FISH technology. Though it provides a quantitative estimate of gene specific copy number changes it cannot account for the allelic imbalance of polymorph alleles characteristic of this gene. Due to a 28-basepair repeat polymorphism in the promoter region, individuals heterozygous for TYMS can acquire the loss or amplification from either the dominant overriding triple repeats or the recessive double repeat allele in their tumors [12]. Arguing that the composite genotype, covering the profile of alleles quantitatively and qualitatively, needs to be considered for prognostic purposes.

TP has previously been evaluated as a prognostic marker in colorectal [4,6,20] and rectal [21,22] cancer. In the adjuvant [20], neoadjuvant [21,22] and palliative [4,6] setting of 5-FU based chemotherapy, either inverse [4,6,22], no [20] or direct [21] association of tumor expression of TP mRNA [4,6,22] or protein [20,21] with outcome have been found.

Whereas increased TP activity is anticipated to lead to stronger exposure to activated 5-FU, these conflicting reports should be considered in the context that dual functions have been ascribed to

this protein. Besides being a key enzyme in the activation of 5-FU and capecitabine, TP is identical to the angiogenic factor platelet-derived endothelial cell growth factor [13]. Although no association of TP gene copies and pathological parameters appeared in this study, occurrence of lymphatic and venous invasion, and depth of tumor invasion have previously been related to high TP expression [13]. As a result, the therapeutic benefit due to greater sensitivity to chemotherapy conferred by high TP catalytic activation of 5-FU may to some extent have been outweighed by a more malignant phenotype from marked tumor angiogenesis.

While amplified gene copy and expression of DHFR may enhance the effect of 5-FU, as increased availability of reduced folates synergistically exert tight binding of the active 5-FU metabolite FdUMP to TS [8] no such association was found. However, co-administration of folinic acid together with 5-FU, leading to equal availability of reduced folate, likely made this study inconclusive as regards the prognostic significance of the numerical variation of DHFR genes.

Gene aberration may also be a pathophysiological mechanism by which cancer cells acquire resistance to chemotherapy. Thus progression of colorectal cancer during 5-FU based chemotherapy was associated with increased number of DHFR gene copies [23]. Also induced TP gene expression [24] and TYMS copy number [23,25] have been associated with reduced sensitivity to 5-FU under experimental conditions.

In conclusion, a multi faceted technological approach including FISH analysis may provide a more comprehensive picture of the ways in which gene deregulation occurs in colorectal cancer and establish how this leads to specific tumor phenotypes. Characteristics of the heterogeneous genetic of colorectal cancer may contribute to a better understanding of the biology and prediction of 5-FU sensitivity.

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