SPANDIDOS PUBLICATIONS

Do thymidylate synthase gene promoter polymorphism and the C/G single nucleotide polymorphism predict effectiveness of adjuvant 5-fluorouracil-based chemotherapy in stage III colonic adenocarcinoma?

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Abstract. Since 5-fluorouracil (5-FU)-based chemotherapy has become standard adjuvant treatment for patients with node-positive colonic adenocarcinoma, there has arisen the need for predictive factors. Thymidylate synthase (TS) is a major target of 5-FU's action, and high TS expression in carcinoma cells could reduce its cytostatic effect. Both, a 28-base pair repeat polymorphism and a cytosine vs. guanine single nucleotide polymorphism in the promoter region of the TS gene are known to modulate its expression. All patients with a single, non-metachronous node-positive colonic adenocarcinoma who underwent a potentially curative resection at this institution in the years 1994-2002, and who received adjuvant 5-FU (n=95) were included in this study. Ninety-four of the 95 patients were successfully genotyped: 70 patients were classified as TS gene low-expressors (2R-2R, 2R-3C and 3C-3C), and 24 patients were classified as high-expressors (2R-3G, 3C-3G and 3G-3G). Contrary to the hypothesis, Kaplan-Meier survival analysis did not reveal any differences between the groups (power of 0.8 to detect an absolute survival difference >30%). In a Cox model, venous angioinvasion and the infiltrative pattern of tumour invasion were strong adverse factors. These results argue against a practical role for the TS gene repeat polymorphism or the C/G single nucleotide polymorphism as a predictive factor. However, by careful histopathological examination a high-risk group of node-positive patients can be defined that could be candidates for studies of alternative (more aggressive) adjuvant treatment.

Introduction

Surgery remains the mainstay in the treatment of colonic adenocarcinoma. However, large prospective randomized clinical studies have shown that roughly 20% of the patients in Union Contré le Cancer (UICC) TNM stage III benefit from post-operative adjuvant chemotherapy with 5-fluorouracil (5-FU) (1). Accordingly, there is consensus that 5-FU-based chemotherapy should be given to these patients (2), and for more than a decade this has been standard practice.

While UICC TNM stage as a prognostic factor is used to stratify patients to adjuvant chemotherapy, there is an urgent need for predictive factors that allow an assessment of the effectiveness of adjuvant 5-FU. If predicted to be ineffective, patients could be spared the negative side-effects of the treatment and would be candidates for trials of alternative regimens.

Thymidylate synthase (TS) catalyses the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), and this reaction provides the sole *de novo* source of thymidylate for DNA replication and DNA repair (3). The 5-FU metabolite FdUMP binds to TS to form a stable complex, making TS the main target of 5-FU. On the rationale that high TS expression would render colorectal carcinoma cells more resistant to 5-FU, retrospective clinicopathological studies have addressed whether differences in the levels of TS expression by the tumour cells would translate into a different clinical course for patients treated with 5-FU. Most of these studies were made by TS immunostaining and scoring on archived tumour materials, and the results have been conflicting, although in a recent meta-analysis a moderate negative predictive effect has been shown for TS overexpression (4). Methodologically, technical variations of immunostaining, particularly if performed on archived material not collected prospectively *ad hoc*, as well as the subjectivity of scoring are major limitations of such types of studies and might account for some of the variability.

The TS gene contains a repeat polymorphism and a single nucleotide polymorphism in the 5'-untranslated region (5,6). The repeat polymorphism consists of a 28-base pair nucleotide tandem repeat in a duplicate (designated 2R) or in a triplet (designated 3R) array, and this polymorphism can easily and objectively be assayed by PCR on genomic DNA. The single nucleotide polymorphism is a cytosine to guanine (C/G) difference at the 12th nucleotide of the second repeat of 3R, and it is assayed readily by a restriction fragment length polymorphism (RFLP). Both, the repeat polymorphism and the

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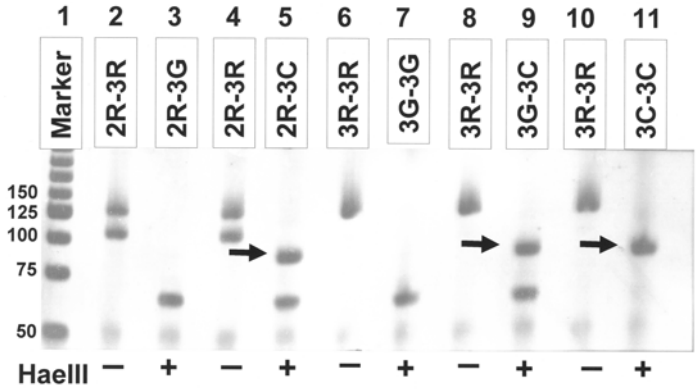

Figure 1. Example of RFLP. Fragments of 135 bp, and 107 bp were amplified from genomic DNA corresponding to the 3R and the 2R repeat polymorphism, respectively. These are seen in samples not digested with HaeIII (lanes 2, 4, 6, 8 and 10). For 3R alleles containing cytosine rather than guanine at the 12th nucleotide of the second repeat, digestion with HaeIII produces a 94-bp fragment (arrowed in lanes 5, 9 and 11). Typing was performed by comparison of electrophoretic patterns without and with HaeIII digestions. Bottom line (HaeIII) indicates if digestions were performed (+) or not performed (-).

C/G single nucleotide polymorphism, *in vitro*, were observed to associate with different levels of TS protein in cells, and this is ascribed to different translational efficiency (6-8). However, clinicopathological studies of their potential role as predictive factors have been limited, particularly in the adjuvant setting.

This study was designed to test the repeat polymorphism and the C/G single nucleotide polymorphism in the TS gene promoter region as objectively assessable predictive factors in the adjuvant setting. To this end, in a simulated prospective approach, all patients with stage III colonic adenocarcinoma potentially resected for cure (R0) during the years 1994-2002 who had received adjuvant 5-FU were identified (n=95). After PCR/RFLP typing, survival analyses were made on these patients.

Materials and methods

Patient selection and clinical data. Patients for this retrospective clinicopathological study were selected from all patients who during the years 1994-2002 received surgery for a single, non-metachronous colonic adenocarcinoma at the Department of Surgery, University of Rostock, or in the years 1997-1999 at the Südstadtklinikum, Rostock. All patients' colorectal carcinoma resection specimens had been sent to the Institute of Pathology for histopathological examination. Patients were included if the following inclusion criteria were met: i) no chemotherapy prior to the operation; ii) patients underwent a full surgical resection with formal lymphadenectomy; iii) by histopathological work-up the tumour proved to be a node-positive invasive colonic adenocarcinoma (ordinary type or mucinous) without involvement of resection margins (R0); iv) clinical staging examinations (surgical exploration, abdominal imaging, chest-X-ray) had excluded distant metastases at the time of operation (M0); v) patients had completed postoperative 5-fluorouracil-based chemotherapy as adjuvant treatment (6 monthly cycles); and vi) no death from perioperative complications. Thus, all patients were TNM stage III, potentially resected for cure (R0). These data were compiled from the colorectal carcinoma database at the Institute of Pathology, by review of the clinical charts, and by interview with the referring physicians or oncologists.

By these criteria a series of 95 patients were accrued. Sixty-nine of these patients operated on in the years 1994-1999 had been reported on in a published clinicopathological study on tumour-infiltrating lymphocytes (9), 26 additional patients operated on in the years 2000-2002 were added to these. Forty-six patients were male, 49 patients were female, patients' ages ranged from 32 to 82 years (median 65 years, mean 62.4 years; 10 patients older than 75 years).

During December 2005 until July 2006 a systematic effort was made to obtain or update follow-up information for all these patients. Information on vital status was obtained from the clinical charts, from the local authorities who ascertained the patient was still registered living at the home address listed in the clinical charts, and/or from the oncologists who had administered the adjuvant chemotherapy, or from the referring physicians. Death of disease was the clinical end-point of this study. By this approach, follow-up information of current date could be obtained for all patients but three. These three patients had moved, after surviving for 54, 60 and 91 months, respectively, each without evidence of metastatic disease at that time, as evidenced by scheduled clinical follow-up examination.

Histopathological work-up of the resection specimens. After overnight-fixation in buffered formalin (10%), the dissection and reporting of the resection specimens was performed in a standardized fashion by two histopathologists (F.P, M.B.), and all slides were reviewed by the first author at the beginning of the study. The tumour was blocked generously (about one paraffin-block per centimetre diameter), and specific care was taken to sample thoroughly the deep-invasive margin, and areas with possible serosal involvement or tumour perforation. Tumours were typed and graded according to the WHO criteria, and staging was completed according to the UICC TNM classification (6th edition) (10). To maximize the lymph node harvest, the pericolic fat was cleared overnight in acetone. Lymph node numbers examined were: median 19; mean 21.6; range 7-58; 5 cases with <12 lymph nodes examined.

In addition, venous angioinvasions were recorded, and step-sections and elastic stains were made in doubtful cases;

Clinicopathological features and their associations with the TS gene promoter polymorphism and the C/G single nucleotide polymorphism.

	Total[a]	2R-2R	2R-3C	2R-3G	3C-3C	3C-3G	3G-3G
Histological type							
Ordinary adenocarcinoma	77	24	27	9	9	7	1
Mucinous	18[a]	3	5	4	2	1	2
Grade (WHO)							
G1	7	1	2	2	0	1	1
G2	62[a]	21	16	8	8	6	2
G3	26	5	14	3	3	1	0
Depth of invasion (pT)							
Mural (pT1/2)	8	3	3	0	1	1	0
Extramural (pT3)	48[a]	13	17	8	7	0	2
Serosal involvement (pT4)	39	11	12	5	3	7	1
Nodal involvement (pN)							
pN1	58[a]	16	18	8	7	6	2
pN2	37	11	14	5	4	2	1
Lymphatic permeation (L)							
L0	55[a]	15	17	6	7	6	3
L1	40	12	15	7	4	2	0
Venous angioinvasion (V)							
V0	71[a]	17	23	11	8	8	3
V1	24	10	9	2	3	0	0
Invasive margin							
Expansive	63[a]	17	22	10	9	1	3
Infiltrative	32	10	10	3	2	7	0

[a]TS gene repeat polymorphism and C/G single nucleotide polymorphism typing was technically not feasible for one mucinous adenocarcinoma G2, pT3, pN1, L0, V0, expansive margin.

venous angioinvasion was scored positive only if the presence of tumour within the lumen of one or more veins could be demonstrated unequivocally. Lymphatic permeation was recorded, and the invasive front of the tumours was classified as expansive or infiltrative according to Jass' criteria (11).

Analysis of the thymidylate synthase repeat polymorphisms and the C/G single nucleotide polymorphism

DNA isolation. One section (10 μm) of paraffin-embedded tumour material was dewaxed and incubated for 12 h at 56˚C in 100 μl of a digestion solution containing 10 mM Tris-HCl (pH 7.2); 0.1 mM EDTA, 0.5% Tween and 0.4 mg proteinase K. After heating to 95˚C to inactivate the enzyme, 2 μl of the digestion solution was used for PCR.

PCR amplification and restriction fragment length polymorphism. PCR amplification of the polymorphism containing region of the TS gene promoter was carried out with forward primer: 5'GCGGAAGGGGTCCTGCCA, and backward primer: 5'TCCGAGCCGGCCACAGGCAT. PCR was performed in a 25-μl reaction volume containing 0.2 μmol/l of each primer, 200 μmol/l dNTPs, 1X PCR buffer (Qiagen, Hilden, Germany), 1X Q-Solution (Qiagen), and 1 unit of Taq polymerase (Qiagen). The 35 amplification cycles were 94˚C for 40 sec, 62˚C for 60 sec, and 72˚C for 40 sec. For typing the TS gene repeat polymorphism, 10 μl of the PCR product were run on an agarose gel (4%). To assay for the C/G single nucleotide polymorphism contained in the second repeat of cases with a 3R allele, 15 μl of the PCR product of cases classified 2R-3R or 3R-3R was digested with the restriction endonuclease HaeIII, followed by electrophoresis in a polyacrylamide gel (8%) and silver staining (Fig. 1). Restriction fragment lengths are 66-bp, 37-bp, 28-bp for the 3G type polymorphism, and 94-bp, 37-bp, and 10-bp for the 3C type.

Statistical data analysis. All data were stored in a personal computer-based data bank implemented with the Statistical Package for the Social Sciences software (SPSS, version 12.0). Kaplan-Meier curves were generated for univariate survival analyses, and the log-rank test was used for significance testing. Cox analysis was used for multivariate regression in a stepwise forward procedure with the level of significance set to <0.05.

Results

Clinicopathological data. This retrospective clinicopathological study is based on a consecutive series of 95 patients

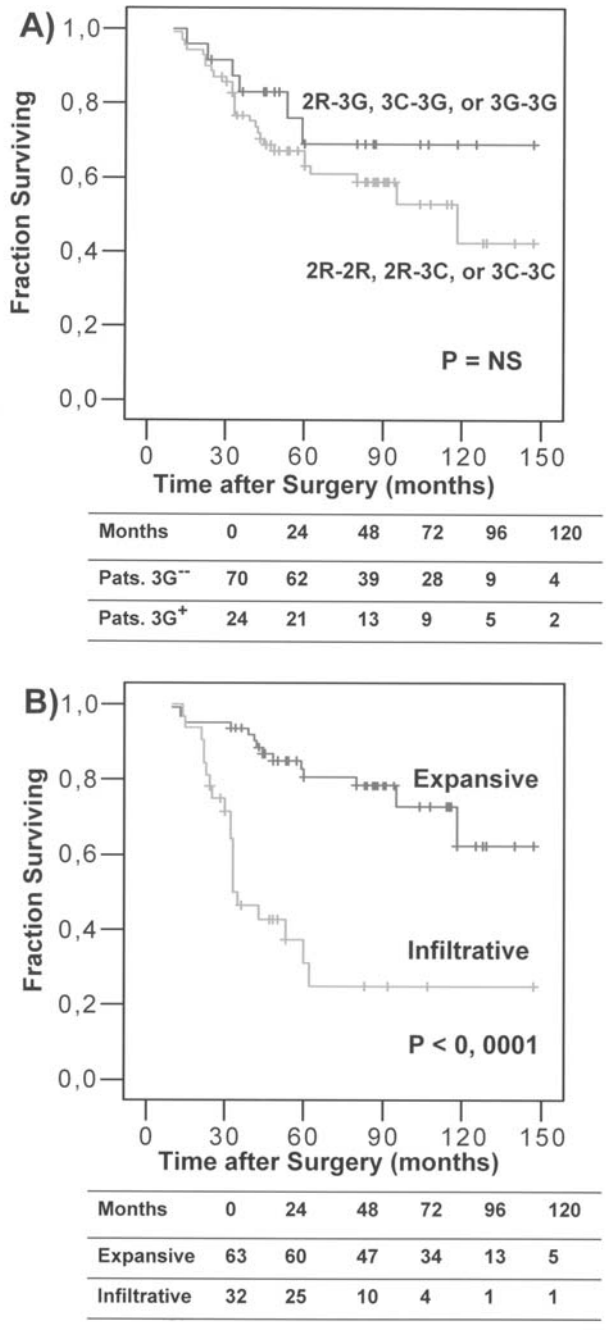

Figure 2. Kaplan-Meier survival curves for patients with death of disease as clinical end-point. Stratification in A was made according to the high- or low-expressor status, classification based on presence or absence of the 3G type single nucleotide polymorphism. In B stratification was made according to the type of the invasive margin (expansive vs. infiltrative).

with colonic adenocarcinoma UICC TNM stage III who underwent a potentially curative resection, and received a standard regimen of 5-FU-based adjuvant chemotherapy. Details of the patients and their tumours are listed in Table I.

Long-term follow-up information was obtained for all patients (median follow-up 54 months, mean follow-up 63.5 months, range 10-147). During follow-up, 34 patients succumbed to metastatic disease.

Frequencies and associations of the TS gene repeat polymorphism and the C/G single nucleotide polymorphism. PCR amplifications of DNA from the paraffin-embedded material was straightforward for all but one case, a mucinous adenocarcinoma that did not amplify even after repeated attempts. A typical gel with fragments from the PCRs after digestion with HaeIII used for TS promoter repeat polymorphism typing is shown in Fig. 1. Classifications of the patients according to their combined TS gene repeat polymorphism and G/C single nucleotide polymorphism patterns yielded 27 patients typed 2R-2R (28.7%), 32 patients typed 2R-3C (34.0%), 13 patients typed 2R-3G (13.8%), 11 patients typed 3C-3C (11.7%), 8 patients typed 3C-3G (8.5%), and 3 patients typed 3G-3G (3.2%). Since the 3R-3R repeat polymorphism, and the C/G single nucleotide polymorphism are known to enhance TS gene expression, the types were grouped into high-expressor classes vs. low-expressor classes, viz. 3R-3R vs. others, and positive vs. negative for the C/G single nucleotide polymorphism. Thus, 22 of 94 patients (23.4%) were classified as high-expressors on the basis of the 3R-3R repeat polymorphism, and 24 of 94 patients (24.5%) were classified as high-expressors on the basis of having the 3G single nucleotide polymorphism. These classifications and their associations with clinicopathological data are cross-tabulated in Table I.

Table II. Results from the Cox multivariate regression analysis.

	HR	95% CI	P-value
Infiltrative invasive margin	2.26	1.59-3.22	<0.000
Venous angioinvasion (V1)	2.209	2.46-3.00	<0.04

Survival analyses. Univariate survival analyses revealed that neither the 3R-3R repeat polymorphism, nor the C/G single nucleotide polymorphism were associated with a statistically different clinical course (example of a Kaplan-Meier curve shown in Fig. 2A). Therefore, in this series the hypothesis of a predictive impact of molecular differences in the TS gene promoter was tested negative.

As histopathological prognostic factors, extent of nodal involvement (pN2 vs. pN1), serosal involvement (pT4b vs. others), lymphatic spread (L1 vs. L0), venous angioinvasion (V1 vs. V0), and infiltrative pattern of growth, were observed to have a strong negative impact on survival (example of a Kaplan-Meier curve in Fig. 2B), but histological tumour type (ordinary adenocarcinoma vs. mucinous carcinoma), and WHO grading did not. Subsequently, all factors that were significant in univariate analysis were entered into a stepwise forward Cox regression analysis. The resulting Cox model selected infiltrative growth pattern, and venous angioinvasion as independent prognostic parameters (Table II).

Discussion

This retrospective clinicopathological study was undertaken to test a potential role of the TS gene repeat polymorphism and the C/G single nucleotide polymorphism as predictive

Summary of clinicopathologic studies addressing the predictive effect of the TS gene repeat polymorphism and/or single nucleotide polymorphism as predictive factors for 5-FU-based chemotherapy.

Refs.	Country	Patients	Location	Stage	R-type[a]	SNP[b]	Response[c]	Survival
Palliative treatment								
(12)	USA	50	Colorectal	IV	2-2: 22/50 2-3: 20/55 3-3: 22/50	ND[d]	2-2: 4/8 2-3: 3/20 3-3: 2/22	ND
(13)	USA	24	Colorectal	IV	2-2: 4/24 2-3: 12/24 3-3: 8/24	ND	2-2: 3/4 2-3: 1/12 3-3: 2/8	ND
(14)	Spain	89	Colorectal	IV	2-2: 17/89 2-3: 37/89 3-3: 35/89	Low-expressor[e]: 49/89 High-expressor: 40/89	Low-expressor: 32/49 High-expressor: 17/40	Better survival for low-expressors by Kaplan-Meier analysis
(15)	USA	30	Colorectal	IV	2-2: 3/30 3-3: 5/30 2-3: 10/22	ND	2-3: 33/30 2-2: 10/13 3-3: 1/5	Better survival for low-expressors by Kaplan-Meier analysis
(16)	Denmark	88	Colorectal	IV	2-2: 21/88 2-3: 46/88 3-3: 21/88	ND	2-2: 5/21 2-3: 13/46 3-3: 11/21	ND
Adjuvant treatment								
(17)	Australia	221	Colorectal	III (117 5-FU)	2-2: 62/221 2-3: 101/221 3-3: 46/221	ND	NA[f]	Better survival for low-expressors by Kaplan-Meier analysis
(18)	Japan	135	Colorectal	I-III[g]	2-2: 11/135 2-3: 32/135 3-3: 85/135 3-5: 7/135	ND	NA	Kaplan-Meier analysis without survival differences
(8)	Japan	111[h]	Colorectal	II/III (64 5-FU)	2-2: 10/252 2-3: 68/252 3-3: 174/252	Low-expressor[e]: 88/252 High-expressor: 164/252	NA	Better survival for low-expressors by Kaplan-Meier analysis
Own	Germany	94	Colon	III	2-2: 27/94 2-3: 45/94 3-3: 22/94	Low-expressor[e]: 70/94 High-expressor: 24/94	NA	Kaplan-Meier analyses without survival differences

[a]R-type, type of repeat polymorphism. Numbers of patients/total numbers of patients in the study. [b]SNP, C/G single nucleotide polymorphism. [c]Regression of metastatic disease in studies of patients receiving palliative treatment. Numbers of patients/total numbers of patients in the study. [d]ND, no data given. [e]Low-expressors: 2-2, 2-3C, 3C-3C. High-expressors: 2-3G, 3C-3G, 3G-3G. [f]NA, not applicable. [g]Sixteen, 61 and 58 patients in UICC TNM stages I, II or III, respectively. [h]Only 111 patients included in the survival analysis, selected from 258 by criteria not cited. Genotypes for patients included in the survival analysis not given in the publication.

factors in the 5-FU-based adjuvant treatment of colonic adenocarcinoma. To eliminate bias as much as possible in a retrospective study, strict inclusion criteria were defined for patient selection. Using these criteria, a prospective design was simulated, and long-term follow-up data could be obtained for all patients. The hypothesis at the base of the study was to

test, if among all the patients treated with adjuvant 5-FU those with a TS gene low-expressor genotype would benefit more from the treatment than high-expressors. In other words, the study was designed to test if the beneficial effect of approximately 20% absolute survival difference effected by 5-FU overall for node-positive colon cancer patients (1) would reside preferentially in the stratum with TS low-expressor genotype. If this were the case, the survival benefit of TS gene low-expressors, in fact, should exceed the 20% survival difference for the group of UICC TNM stage III patients as a whole. Thus, the numbers of patients included in this study should allow the detection of differences (for discussion of the power see below). The molecular analysis of the TS gene promoter rather than assaying for TS protein by immunohistochemistry *in situ* was chosen in order to circumvent the problematic variations incurred in carrying out and scoring immunostains.

Using published protocols, the PCR-based repeat polymorphism typing and the RFLP to assay for the C/G single nucleotide polymorphism were straightforward to perform on the archived materials for all cases but one, and the interpretations were unequivocal. Classifying patients in high- vs. low-expressor types on the basis of these analyses led to a fraction of 23.4 and 24.5% high-expressors (for the 3R-3R, and the C/G single nucleotide-positive type, respectively). These frequencies are in an order observed in previous publications in populations of Caucasian extraction (Table III), and the groups resulting from these classifications are of meaningful sizes for survival analyses. Thus, as suggested by previous studies, size of strata and methodology make TS genotyping an attractive potential predictive factor.

However, the survival analysis both for the promoter repeat polymorphism and the C/G single nucleotide polymorphism did not reveal any difference in the Kaplan-Meier survival curves. These results are at variance with some published clinicopathological studies, all retrospective, that tested the predictive value of the TS gene repeat polymorphism and/or the C/G single nucleotide polymorphism. An overview of the published clinicopathological studies is given in Table III.

Five of the published studies were restricted to patients with metastatic disease who received palliative treatment with 5-FU-based chemotherapy (12-16). In four of these studies, better treatment responses (determined by partial or complete remissions) were observed for low-expressors. In three of these studies, patients were classified as low-expressors by the 2R-2R or 2R-3R genotype, and in one study by the presence of cytosine at the C/G single nucleotide polymorphism locus. However, in one of the palliative treatment studies (the second largest series) the reverse was observed, with even better responses for the group of high-expressors.

Concerning the studies testing adjuvant 5-FU, there are three published (8,17,18). The largest study by Iacopetta *et al* (17) reports on a well-selected, consecutive series of 221 patients of which 117 received 5-FU. Classifications of low- vs. high-expressor were made according to the TS gene repeat polymorphism (low-expressors defined by the 2R-2R or 2R-3R status), the C/G single nucleotide repeat polymorphism was not addressed in that study. Important to note, in their survival analyses Iacopetta *et al* made separate comparisons for low-expressors (n=163) and high-expressors (n=58). Within these two groups, stratification of patients was made according to having received adjuvant 5-FU or not. By this approach they observed better survival in the subset of low-expressors after adjuvant 5-FU compared to the subset of low-expressors not having had adjuvant chemotherapy, but such a difference was not seen for high-expressors. The principal drawback of this approach is that there may have been strong selection-bias: why did the compared group of patients not receive adjuvant 5-FU?

Tsuji *et al* (18) made a study of 135 patients in UICC TNM stages I-III (16, 61 and 58, respectively), all of whom received adjuvant 5-FU. Stratified by the TS gene repeat polymorphism, no survival differences were observed by these authors.

Kawakami and Watanabe were the first to report a negative predictive effect of the C/G single nucleotide polymorphism for adjuvant 5-FU (8). Clearly, that study has the merit of demonstrating high TS expression of the 3G-type promoter in transfection experiments, and it provided the rationale for classification of the 3G-type patients as high-expressors. However, the clinicopathological part in the paper by Kawakami and Watanabe is seriously flawed methodologically: of 258 patients typed for the C/G single nucleotide polymorphism, only 111 were selected for the survival analysis for reasons not cited, and only 64 of these 111 patients had received adjuvant 5-FU. Though numbers were not given with the Kaplan-Meier curves in the publication, by estimation on the basis of the overall frequencies listed, apparently the numbers in the groups are very low, below 20 patients.

The present study was made on a comparatively large, pathologically and clinically well-defined patient group with stringent inclusion criteria. Contrary to the study of Iacopetta *et al* (17), but similarly to that of Tsuji *et al* (18), in the present study the TS gene repeat polymorphism was not observed to be a predictive factor. Also, contrary to the study of Kawakami and Watanabe (8), the C/G single nucleotide polymorphism did not show up as a predictive factor, either.

An important point to regard in this sort of investigation must be to estimate the power since a survival difference might go undetected by chance. A calculation of the power attained in the present study can be made using the nomograms published by Altman (19). With the number of patients included in the present study, a power of 0.8 is maintained if the fraction of this study's low-expressors surviving follow-up would be above 70%, and the fraction of high-expressors surviving would be below 40% (the difference detected at $p<0.05$). For detecting smaller differences in survival, a larger cohort of patients would have had to be contracted. At first sight this would seem a desirable study. However, it must be recognized that if absolute survival differences are much smaller than 30%, the point of predicting effectiveness of 5-FU-based adjuvant chemotherapy in the oncologist's practice of treating patients with colonic adenocarcinoma largely would be lost. If differences were smaller, it would be unethical to withhold patients from a standard treatment that for the group as a whole (i.e., all stage III patients) is known to be significantly effective in the order of 20% absolute survival difference. Thus, from the point of view of a standard care setting in the treatment of colonic adenocarcinoma, the present results argue against a practical role of TS gene repeat

hism or the C/G single nucleotide polymorphism in ı of 5-FU effectiveness.

Another important aspect of the present study stems from the fact that histopathological work-up was standardized, remaining with two histopathologists with a long-standing special interest in the surgical pathology of colorectal carcinoma. Careful screening for venous angioinvasion, serosal involvement, and typing of the invasive margin revealed that node-positive patients with either of these features fare very poorly indeed, in spite of adjuvant treatment. These patients would be the first stratum to be recruited for future clinical trials of alternative (probably more aggressive) adjuvant treatment of colonic adenocarcinomas. Oncologists would be well advised to pay more attention to high-quality histopathology, and to include surgical pathologists in the team when delineating the conceptions of their trials.

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