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Altered Gene Expression of Folate Enzymes in Adjacent Mucosa Is Associated with Outcome of Colorectal Cancer Patients

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ABSTRACT

Purpose: **The purpose of this study was to analyze whether gene expression levels of folate enzymes in adjacent mucosa were associated with outcome of colorectal cancer patients.**

Experimental Design: **Real-time PCR was used to quantify expression levels of folate-associated genes including the *reduced folate carrier (RFC-1), folylpolyglutamate synthase (FPGS), γ-glutamyl hydrolase (GGH),* and *thymidylate synthase (TS)* in tumor tissue and adjacent mucosa of patients with primary colorectal cancer ($n = 102$). Furthermore, reduced folates in the tissues were measured with a binding-assay method.**

Results: **Mean gene expression levels of *RFC-1, FPGS, GGH,* and *TS* were significantly higher in tumor biopsies compared with mucosa. Univariate and multivariate analyses showed that the *FPGS* gene expression level in mucosa, but not in tumor, was a prognostic parameter independent of the clinicopathological factors with regard to survival. Patients with high *FPGS* levels (>0.92) in mucosa also showed significantly higher total folate concentrations ($P = 0.03$) and gene expression levels of *RFC-1* ($P < 0.01$), *GGH* ($P < 0.01$), and *TS* ($P = 0.04$) compared with patients with low *FPGS* levels. The total reduced folate concentration correlated with the gene expression levels of *RFC-1* and *FPGS* but not with *TS* or *GGH*.**

Conclusion: **Our results suggest that normal-appearing colonic mucosa adjacent to primary colon cancer can show altered gene expression levels of *FPGS* that may have bearing on the development of aggressive metastatic behavior of the tumor and on tumor-specific survival.**

INTRODUCTION

For four decades 5-FU[5] has been the chemotherapy of choice for treatment of colorectal cancer. For 2 decades modulation of 5-FU with LV (folinic acid) has been standard. New drugs include irinotecan and oxaliplatin (1), which used in combination with 5-FU and LV can give response rates of >50% in patients with metastatic colorectal cancer. However, although improvements have been made, many patients are treated with chemotherapy without any proven clinical effects registered. Thus, there is a need to identify predictive and prognostic factors for tumor response to avoid unnecessary treatments. Also, there has been considerable interest in the study of folate metabolism regulation in tissues and tumors in the neoplastic process, because folates serve as one-carbon donors in the synthesis of purines and of thymidine, and are essential for normal cell growth and replication (2). Cells have a tightly regulated cellular uptake process to maintain sufficient levels of intracellular folates. Epidemiological studies have shown an association between folate deficiency and premalignant/malignant changes in epithelial tissues, including the colon mucosa (3). Previous studies have also demonstrated significantly lower colonic mucosa folate concentrations in patients having adenomatous polyps compared with those having hyperplastic polyps despite inapparent differences in serum folate levels (4). Meenan *et al.* (5) have shown that folate concentrations of colon carcinoma cells and adjacent normal-appearing cells differ significantly.

There are several reports showing that folate deficiency increases the risk of cancer development by induction of an imbalance in the DNA precursors and by altering DNA methylation (6–10). Folate deficiency may also cause excessive uracil misincorporation into human DNA in place of thymine leading to transient nicks and breaks during DNA repair (9).

Folate metabolism is responsible for conversion of homocysteine to methionine, important in the biosynthesis of *S*-adenosylmethionine (Fig. 2), that in turn is responsible for the methylation of CpG islands by DNA methyltransferases (11).

RFC-1, is the major transporter of folates and antifolates in the cells (12). Intracellularly, reduced folate monoglutamates are converted to polyglutamates by the enzyme FPGS (EC 6.3.2.17; Ref. 2). This enzyme, which has cytosolic and mitochondrial forms, adds up to seven glutamates to folate mono-

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[5] The abbreviation used are: 5-FU, 5-fluorouracil; FPGS, folylpolyglutamate synthase; GGH, γ-glutamyl hydrolase; RFC-1, reduced folate carrier; TS, thymidylate synthase; methylene-THF; 5,10-methylene-tetrahydrofolate; THF, tetrahydrofolate; FdUMP, fluoro deoxyuridine monophosphate; TNM, Tumor-Node-Metastasis; LV, leucovorin.

glutamates. In contrast to monoglutamates, polyglutamates are retained inside the cells (13) and are considered to be better substrates for most cellular enzymes (14, 15). The enzyme GGH (EC 3.4.22.12) catalyzes the degradation of inter- and intracellular polyglutamates (16). GGH is an acidic lysosomal protein, which is also secreted on the outside of the cells. The secretion of GGH is considered to be important in the process of degradation of extracellular folate polyglutamates to transportable monoglutamates, which can accumulate in the cells (17). Folate polyglutamates regulate the reaction rate of the key enzyme metabolism and allow channeling of the substrates between enzymes. In the synthesis of dTMP, the cofactor methylene-THF methylates dUMP. The enzyme responsible for the methylation step is TS (EC 2.1.1.45), which is considered to be rate-limiting in the synthesis of pyrimidine nucleotides (18, 19). TS is also the main target for 5-FU, and high levels of methylene-THF are needed to achieve maximal TS-inhibition. The development of drug resistance to TS-inhibiting drugs may be caused by a decrease or absence of the FPGS protein (20) or by an increase in *GGH* expression (21). Several reports have suggested that a high expression of *TS* in the colorectal cancer is associated with a poor tumor-specific survival of cancer patients even after radical surgery. In the present study, real-time PCR was used to quantify the expression levels of the folate-associated genes *RFC-1*, *FPGS*, *GGH*, and *TS* in tumor tissue and in adjacent normal-appearing mucosa of patients with colorectal carcinomas ($n = 102$). Furthermore, the folate concentration in the tissues was measured by the TS-FdUMP binding-assay method. Of interest, associations are found between expression levels of genes of folate metabolism in normal mucosa and 5-year tumor-specific survival of patients with colorectal cancer.

MATERIALS AND METHODS

Patients and Study Design. Tumor and paired mucosa samples were obtained from 102 colorectal carcinoma patients undergoing primary tumor resection at the Sahlgrenska University Hospital/Östra during the period between 1994 and 2000. The ethic committee of Göteborg approved the study, and informed consent was obtained from each of the patients. The samples were excised fresh from operative specimens. Adjacent, normal-appearing mucosa were taken at a distance of ~10 cm from the tumors. The biopsies were snap-frozen in liquid nitrogen and stored at −70°C until used. Surgical and pathological records were reviewed for Dukes' stage, tumor differentiation grade, age, gender, and tumor localization. The growth pattern and the grade of differentiation were classified by pathologists as recommended by the WHO (22). The malignant tumors were classified according to Dukes' stage (23), as modified by others (24, 25), into the pathological stages Dukes' A (TNM stage I), Duke's B (TNM stage II, Dukes' C (TNM stage III), and Dukes' D (TNM stage IV; Ref. 26). Clinical characteristics of the patients are presented in Table 1. Tumor-specific survival was calculated from the time of surgery to the date of death because of cancer disease. Information concerning lymph node status for 1 Dukes' D patient could not be obtained. Four of the deceased patients had died of causes unrelated to colorectal cancer (these patients were censored).

Table 1 Clinical characteristics of the patients

Variable	No. of patients (%)
All patients	102
Gender	
Male	50 (49)
Female	52 (51)
Tumor site	
Colon	56 (55)
Rectum	46 (45)
Dukes' stage	
A	8 (8)
B	42 (41)
C	33 (32)
D	19 (19)
Histology	
Well differentiated	3 (3)
Moderately differentiated	65 (64)
Poorly differentiated	34 (33)
Age	
>77	40 (39)
≤77	62 (61)

Total RNA-Extraction and Real-Time Quantitative PCR. Total RNA was isolated according to Chomczynski and Sacchi (27) and reverse-transcribed according to Horikoshi *et al.* (28). Quantitative PCR was performed using the Sequence Detector ABI Prism 7700 (Applied Biosystems). The housekeeping gene β-*actin* was used as an endogenous control to compensate for the variation in RNA amount and to check for the efficiency of the reverse transcription reaction. The fluorescent dye carboxyfluorescein (emission maximum 518 nm) was used as a reporter dye for the genes *RFC-1*, *FPGS*, *TS*, and *GGH*, whereas the reporter dye VIC (emission maximum 552 nm) was used for β-*actin*. The quencher dye 6-carboxytetramethylrhodamine (emission maximum 582 nm) was attached to the 3′-end of each forward primer. The reporter dye signal was measured against the internal reference 6-carboxy-X-rhodamine signal (emission maximum 610 nm) to normalize for non-PCR-related fluorescence. The PCR primers and the TaqMan fluorogenic probes were designed using the Primer Express software program (29). Applied Biosystems performed the synthesis and purification of the fluorogenic probes. The primer sets for β-*actin*, *RFC-1*, FPGS, GGH, and TS were chosen to lie within two different exons to avoid amplification of genomic DNA or unspliced transcripts. The following primer and probe sequences were used: *RFC-1*, probe: 5′-CCC GGT CCG CAA GCA GTT CCA-3′, forward primer: 5′-TCA AGA CCA TCA TCA CTT TCA TTG T-3′, reverse primer: 5′-AGG ATC AGG AAG TAC ACG GAG TAT AAC-3′; *FPGS*, probe: 5′-CAG CTG TGT CTC CAT GCC CCC CTA C-3′, forward primer: 5′-GGC TGG AGG AGA CCA AGG AT-3′, reverse primer: 5′-CAT GAG TGT CAG GAA GCG GA-3′; *GGH*, probe: 5′-ACC CCA CGG CGA CAC CGC-3′, forward primer: 5′-GCG AGC CTC GAG CTG TCT A-3′, reverse primer: 5′-AAT ATT CCG ATG ATG GGC TTC TT-3′; *TS*, probe: 5′-TAT TCG GCA TGC AGG CGC GC-3′, forward primer: 5′-GGG AAT TCA TCT CTC AGG CTG T-3′, reverse primer: 5′-CAC CGG CAC CCT GTC G-3′; and β-*actin*, probe: 5′-CCT GAA CCC CAA GGC CAA CCG-3′, forward primer: 5′-CGT GCT GCT GAC CGA GG-3′,

Table 2 Relative gene expression levels of *RFC-1*, *FPGS*, *GGH*, and *TS* in the colorectal mucosa and corresponding carcinomas

	RFC-1 Mean ± SD	*FPGS* Mean ± SD	*GGH* Mean ± SD	*TS* Mean ± SD	*FPGS/GGH* Mean ± SD
Mucosa ($n = 102$)	0.16 ± 0.16	0.87 ± 0.58	5.1 ± 6.9	2.0 ± 2.5	0.45 ± 1.3
Colorectal carcinoma ($n = 102$)	0.40 ± 0.35	1.3 ± 1.5	28 ± 72	4.9 ± 9.6	0.23 ± 0.52
Wilcoxon (signed-rank)	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

reverse primer: 5′-GAA GGT CTC AAA CAT GAT CTG GGT-3′.

Multiplex PCR was performed as follows: 25 μl of the TaqMan Universal PCR Master mix, composed of PCR buffer, $MgCl_2$, deoxynucleoside triphosphate, AmpErase UNG enzyme, and AmpliTaq Gold DNA polymerase were mixed with 7.5 μl of the target primers (final concentration 300 nM) and probe (final concentration 200 nM), and 7.5 μl of β-actin primers and probe (final concentration 100 nM). Forty μl of the reaction mixture were added to the PCR tubes containing 10 μl of the cDNA sample. The activation of the uracil-DNA N-glycosylase enzyme was done by incubation for 2 min at 50°C and 10 min at 95°C. Thermal cycling proceeded with 40 cycles of 95°C for 15 s and 58°C for 1 min. All of the samples were amplified simultaneously in triplicate in a one assay-run. The quantitative data were calculated according to the instructions given by Applied Biosystems (30).

Determination of Folate by the FdUMP-Binding Assay. The FdUMP binding assay was used for measuring the folate levels in the mucosa ($n = 45$). It was not possible to analyze >45 tissue samples, because the method requires a minimum of 100 mg tissue. Total reduced folate and methylene-THF concentrations were analyzed using the Priest methodology (31), slightly modified by us (32). Briefly, the tissues were homogenized at 4°C in a 10-fold homogenization buffer for 30 s. The homogenization buffer contained 550 mg of CMP, 420 mg of sodium fluoride, 28 μl of 14.2 M β-mercaptoethanol, 1g of sodium ascorbate, and 200 mg of BSA diluted to 100 ml with 0.18 M Tris-HCL buffer (pH 7.4). The folate assay mix contained 50 μl of tissue homogenate, 50 μl of H^3-FdUMP (800,000 dpm), 25 μl of TS (4 pmol), and 25 μl of buffer with or without formaldehyde (6 mM final concentration). Addition of formaldehyde yielded the total concentration of folates, whereas no formaldehyde addition yielded the methylene-THF concentration. After incubation of the folate sample tubes for 10 min at 37°C, 1 ml of 3% charcoal was added, followed by vortex mixing. The charcoal was first washed with acid, and coated and activated with dextrane and BSA. The samples were centrifuged at 4°C for 15 min, thereafter 0.8 ml of the supernatant was mixed with 10 ml of scintillation liquid and counted. The methylene-THF and total folate concentrations were obtained as nmol/gram tissue (wet weight). THF levels were obtained by subtracting the methylene-THF concentration from the total folate concentration.

Statistical Analyses. The clinicopathological variables used in this study were the following: Dukes' stage, differentiation grade, age, gender, lymph node metastasis, and tumor location. The obtained data were analyzed by statistical modeling using the commercial software JMP (33). Unless otherwise stated, the data were presented as means and SDs. To compare sets of continuous parameters measured in the same tumor tissues, the Spearman's correlation coefficient (r) and the Wilcoxon signed-rank test were used. The correlation between folate concentration and *FPGS*, *GGH*, *RFC-1*, and *TS* gene expression was measured. The cutoff point of the age, *FPGS*, *TS*, *GGH*, and *RFC-1* gene expression with respect to survival was chosen according to largest χ^2 in the log-rank test, and the significance level was evaluated by 10,000 permutations. Overall survival curves were constructed using the Kaplan-Meier's method (34). The statistical significance of the difference in survival of the groups was calculated using the log-rank test. Relative risk was assessed by univariate and multivariate Cox proportional hazard model. Statistical values of $P \leq 0.05$ were considered to be significant. Unless explicitly stated no correction for multiple testing was done.

RESULTS

Clinical Characteristics of the Patients. As shown in Table 1, the median age of the patients was 74 (range, 35–87) years. Fifty patients were male, and 52 were female. Among the 102 patients, 56 patients had colon cancer, and 46 had rectal carcinoma. Of the primary carcinomas, 3 were highly, 65 were moderately, and 34 were poorly differentiated. Primary tumor stage was Dukes' A in 8 patients, B in 42 patients, C in 33 patients, and D in 19 patients. At the closure of the study, 41 of the 102 patients (40%) had died, whereas 23 (24%) had lived for >5 years. The median follow-up time was 918 (range, 6–1,819) days.

Gene Expression Levels and Folate Concentrations in Colorectal Mucosa and Carcinomas. The relative gene expression levels of *RFC-1*, *FPGS*, *GGH*, and *TS* in the mucosa and in carcinomas are presented in Table 2. As shown, significantly higher expression levels were found in tumors as compared with mucosa. The ratio *FPGS:GGH*, representing the folate turnover in tissues, was significantly lower in colorectal carcinomas as compared with mucosa. In mucosa, a significant correlation (0.47; $P < 0.0001$; $n = 102$) was found between *FPGS* and *GGH* gene expression levels. Furthermore, in mucosa the total folate concentration correlated with the gene expression levels of *RFC-1* (0.3; $P = 0.05$; $n = 45$) and *FPGS* (0.3; $P = 0.05$; $n = 45$). However, no correlation was found between total folates and the *TS* or *GGH* gene expression levels.

Determination of Cutoff Values for Age, *FPGS*, *TS*, *GGH*, and *RFC-1* in the Mucosa Adjacent to Cancer with Respect to the Outcome. To determine whether there was any prognostic significance connected to differences in folate enzyme expression levels we determined the cutoff value by the maximal χ^2. The best cutoff values for age and for *FPGS* was found to be 77 years ($P = 0.007$ adjusted to $P = 0.04$ after 10,000 permutations) and 0.92 ($P = 0.002$ adjusted to $P = 0.02$ after 10,000 permutations) with respect to the tumor-specific

Table 3 Cox univariate and multivariate analyses demonstrating the influence of the clinicopathological and the dichotomized *FPGS*, *TS*, *GGH*, and *RFC-1* gene expression parameters on the survival of the patients with colorectal cancer

Clinicopathological variables/gene expression level		No. of patients	Univariate hazard ratio (CI)[a]	P^b	Multivariate hazard ratio[c] (CI)	$P^{b,c}$
Dukes' stage	A	8	1	<0.0001	1	<0.0001
	B	42	2.1 (0.39–38) [B–A]		2.2 (0.33–44) [B–A]	
	C	33	2.5 (1.1–5.9) [C–B]		1.4 (0.17–8.0) [C–B]	
	D	19	8.4 (3.8–19) [D–C]		20 (7.1–64) [D–C]	
Histological type	Moderate/well	68	1		1	
	Poor	34	2.0 (1.1–3.7)	0.03	1.1 (0.75–1.6)	0.65
Gender	Female	52	1		1	
	Male	50	1.4 (0.75–2.6)	0.30	1.2 (0.60–2.7)	0.56
Tumor location	Colon	56	1		1	
	Rectum	46	1.2 (0.62–2.1)	0.65	1.4 (0.66–3.1)	0.37
Age (yr)	≤77	62	1		1	
	>77	40	2.3 (1.2–4.3)	0.0086	3.0 (1.4–6.5)	0.004
Lymph node metastasis	Negative	54	1		1	
	Positive	47	3.8 (2.0–7.6)	0.0001	1.3 (0.27–9.6)	0.75
FPGS level in mucosa	>0.92	34	1		1	
	≤0.92	68	3.6 (1.6–9.6)	0.0009	3.2 (1.3–9.5)	0.013
TS level in mucosa	≥1.5	42	1		1	
	<1.5	60	1.9 (0.96–4.2)	0.06	1.2 (0.55–2.9)	0.65
GGH level in mucosa	≥5.5	26	1		1	
	<5.5	76	2.0 (0.95–4.9)	0.07	0.87 (0.34–2.4)	0.78
RFC-1 level in mucosa	≥0.14	45	1		1	
	<0.14	55	2.0 (1.1–4.2)	0.03	0.92 (0.39–2.3)	0.86

[a] CI, 95% confidence interval.
[b] *P* by likelihood ratio tests.
[c] Multivariate analysis was performed by using the clinicopathological parameters (Dukes' stage, gender, tumor location, age, lymph node metastasis, and differentiation grade) FPGS, TS, GGH, and RFC-1 expression parameter.

survival time. Patient groups with high and low levels of *FPGS* were denoted $FPGS^{high}$ and $FPGS^{low}$, respectively. The best cutoff values for *TS*, *GGH*, and *RFC-1* were found to be 1.5, 5.5, and 0.14, but after 10,000 permutations none of them were found to be significant in the mucosa with respect to survival.

Determination of Prognostic Clinicopathological Factors and Prognostic Marker. As expected, 5-year tumor-specific survival of the patients gradually decreased from those with lesions classified as Dukes' A (75%), Dukes' B (67%), and Dukes' C (37%), to those with Dukes' D (0%). To determine whether any of the analyzed clinicopathological factors (Dukes' stage, age, gender, tumor location, lymph node metastasis, and differentiation grade) or folate-associated genes (*FPGS*, *RFC-1*, *TS*, and *GGH*) could predict the tumor-specific survival of the patients, univariate and multivariate analyses were performed using the Cox proportional hazard models (Table 3). The relationship between the gene expression level of *FPGS* in the mucosa and the clinical outcome of the colorectal patients was demonstrated using Kaplan-Meier survival curves (Fig. 1). A significantly longer tumor-specific survival time was found in patients having a high level of *FPGS* expression: the 5-year survival rate was 75% for patients of group $FPGS^{high}$ and 35% for patients of group $FPGS^{low}$. The multivariate analysis demonstrated that Dukes' stage and age were the only independent clinicopathological parameters (Table 3). The *FPGS* expression level was the only marker found to be a prognostic parameter in the mucosa independent of each of the clinicopathological factors and expression level with regard to tumor-specific survival. The relative risk of dying for Dukes' B and C patients of group $FPGS^{low}$ independent of age, differentiation grade, and lymph node metastasis was 5.9 (1.7–38; $P = 0.003$) compared with patients of group $FPGS^{high}$.

Stratification of the Patients by High and Low *FPGS* Levels in the Mucosa. The patients were dichotomized according to the *FPGS* gene expression levels in the mucosa and

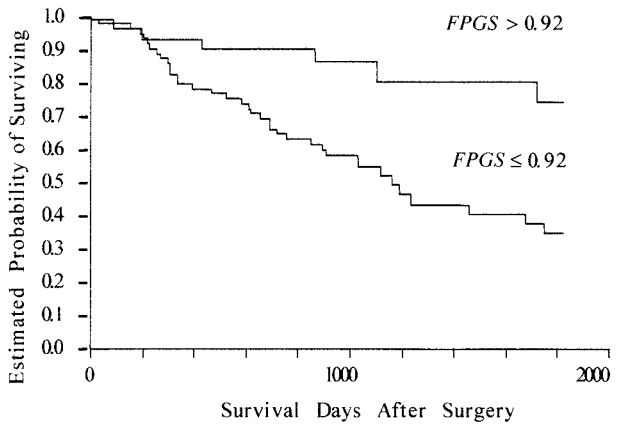

Fig. 1 Tumor-specific survival curves according to high (>0.92) and low (≤0.92) *FPGS* gene expression levels in the mucosa of the patients with colorectal cancer. Patients with high levels of *FPGS* ($n = 34$) had a significantly better outcome ($P = 0.002$ by log-rank analysis) compared with patients having low levels of *FPGS* ($n = 68$). Survival was estimated by use of the Kaplan-Meier method.

Table 4 The average gene expression levels of *RFC-1*, *FPGS*, *GGH*, and *TS* relative to β-*actin*, the *FPGS:GGH* ratio, and the methylene-THF, THF, and total folate concentrations in the mucosa as stratified by high (>0.92) and low (≤0.92) *FPGS* gene expression levels

	Stratification by *FPGS*				
	n	>0.92 Mean ± SD	*n*	≤0.92 Mean ± SD	P^a
RFC-1 gene expression	34	0.2 ± 0.2	68	0.1 ± 0.1	<0.01
FPGS gene expression	34	1.5 ± 0.6	68	0.6 ± 0.2	<0.01
GGH gene expression	34	8.7 ± 10	68	3.3 ± 2.4	<0.01
TS gene expression	34	3.2 ± 3.5	68	1.4 ± 1.4	0.04
FPGS/GGH	34	0.8 ± 2.2	68	0.3 ± 0.3	0.07
Methylene-THF, nmol/g	14	1.7 ± 1.5	31	0.9 ± 0.7	0.08
THF, nmol/g	14	1.4 ± 1.0	31	0.9 ± 0.6	0.3
Total folates, nmol/g	14	3.0 ± 1.9	31	1.8 ± 1.1	0.03

[a] *P* by Wilcoxon/Kruskal-Wallis tests (rank sums), 2-sample test.

were then additionally subgrouped according to the gene expression levels of *RFC-1*, *GGH*, and *TS*, the *FPGS:GGH* ratio, and the folate levels (Table 4). As shown, significantly higher gene expression levels of *RFC-1*, *GGH*, and *TS* were detected in the mucosa of group $FPGS^{high}$ as compared with those of group $FPGS^{low}$. Patients of group $FPGS^{high}$ also had a significantly higher total folate concentration in the mucosa.

DISCUSSION

The ability of the cells to add polyglutamates to folates is a requisite for their growth and viability. In dividing cells, folates are readily converted to polyglutamated forms by the enzyme FPGS. Polyglutamated folates have a high affinity for TS, an essential enzyme needed for DNA synthesis. A decrease in polyglutamation will result in a folate deficiency in the cells, which, in turn, may affect the DNA synthesis, DNA repair, and the methylation status of the cells. RFC-1 is the major transporter of folates into the cells (12), and the enzyme GGH catalyzes the degradation of inter- and intracellular polyglutamates (16).

The results of the present study showed that the mean gene expression levels of *RFC-1*, *FPGS*, *GGH*, and *TS* were significantly higher in the tumor biopsies compared with the mucosa. Thus, alterations of several key enzymes of the folate metabolism clearly have occurred in the tumor cells. However, the tumor tissue is known to be highly heterogeneous, and widely different gene expression results can be obtained depending on the biopsy excision site. The deviations in the gene expression levels in different parts of the tumor might make it hard to identify reliable prognostic markers. In fact, the more homogenous cell population of the mucosa adjacent to the tumor often seems to have a better prognostic value (35, 36).[6] To minimize the risk of tumor recurrence after surgical resection, it is of outermost importance to clarify at which distance from the tumor the mucosa can be regarded as normal. Several studies have shown that alterations in the normal-appearing transitional mucosa (defined as the mucosa surrounding the colorectal adenocarcinoma that shows altered mucous secretions) can be used as markers of premalignant changes that probably would not be detected by routine histological examination (37–40). Likewise, a gradual decrease in the folate level in the mucosa adjacent to adenomas, polyps, and colorectal carcinoma was detected by Kim *et al.* (4), and still other reports have shown the presence of localized folate deficiency within the rapidly proliferating epithelium such as the inflamed tissue (41). Gloria *et al.* found (42) significantly higher DNA hypomethylation and cell proliferation levels in rectal biopsies from patients with long-standing ulcerative colitis compared with samples from healthy controls. A long-lasting chronic inflammation and immune activation in the tissue may ultimately lead to a folate deficiency (43), which, in turn, may lead to mismatch repair deficiencies, chromosome imbalances, and alterations of the gene methylation patterns (44). Thus, the chronic inflammation may induce a multitude of alterations in the mucosa providing a prerequisite environment for neoplastic transformation (45, 46). It seems likely that depending on the cause of the inflammation, some patients may have a global folate deficiency affecting the whole gut, whereas other may suffer from infection and inflammation of smaller patches of the intestines.

Mucosa obtained at a distance of 10 cm from the tumor is usually regarded as normal and is often used as a "matched control" in gene expression analyses. The results of the present study suggest that mucosa even this far away from the tumor may be altered and, furthermore, might relate to the tumor-specific survival of colorectal patients. The *FPGS* gene expression levels in the mucosa were found to be an independent prognostic parameter, and patients with low *FPGS* had significantly shorter tumor-specific survival than patients with high levels. Patients with low gene expression of *FPGS* also had a significantly lower gene expression of *RFC-1* compared with patients of group $FPGS^{high}$ (Table 4). Also, the univariate analysis showed that *RFC-1* expression was a prognostic marker in the mucosa with regard to tumor-specific survival. Thus, a decreased transport and polyglutamation of folates within adjacent mucosa relate to survival. The gene expression levels of *RFC-1* and *FPGS* in the mucosa (and the tumors) were found to correlate with the total folate concentration, suggesting that the expression and activity of the *RFC-1* and *FPGS* genes were highly regulated by the concentration of the intracellular folates,

[6] Y. Wettergren, E. Odin, S. Nilsson, R. Willén, G. Carlsson, L. Larsson, and B. Gustavsson, Could lack of Expression of a DCC splice variant in the colonic, normal-appearing mucosa affect lymph node metastasis and survival of colorectal carcinoma patients? submitted for publication, 2003.

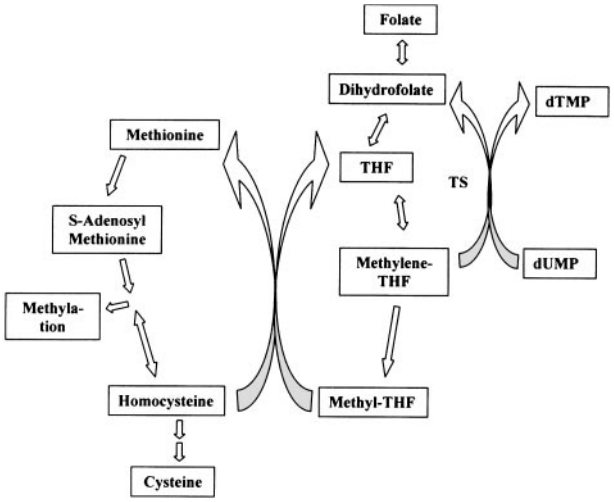

Fig. 2 Folate-dependent pathways leading to methionine and nucleotide biosynthesis. 5-Methyl-THF is an important cofactor in the synthesis of S-adenosylmethionine, which is the primary methyl donor. Impaired methionine biosynthesis may lead to both hypo- and hypermethylation, which, in turn, may affect the expression of several genes.

a finding that has been confirmed by several investigators (47–49). In 1992, Barredo and Moran (50) presented a study showing that the expression of *FPGS*, which correlates with the FPGS enzymatic activity (51, 52), was regulated by at least two mechanisms in mammalian cells and tissues. One of the mechanisms was linked to proliferation, whereas the other was tissue-specific and controlled the enzyme levels after cellular differentiation. It was suggested that intracellular entrapment and a slow turnover of folylpolyglutamates occur in tissues with very low levels of FPGS where polyglutamate forms of folates predominate. In a study of HL-60 cells (53), the FPGS activity was shown to be a proliferative marker that declined during cellular maturation. A low intracellular folate concentration permits competition between different folate-dependent metabolic pathways. One such pathway is the conversion of homocysteine to methionine (Fig. 2). The folate status seems to be one of the most important nutritional factors regulating the total homocysteine levels, and a decrease in the plasma (and tissue) folate concentration is known to correlate with an increase in the plasma homocysteine concentration (54). As shown in Fig. 2, the remethylation of homocysteine to methionine requires acceptance of a methyl group from 5-methyl-THF.

The present study is to our knowledge the first one regarding *GGH* gene expression in colorectal tissues. The results showed that the *GGH* levels were much higher than those of *FPGS* in the malignant tumors as well as in the adjacent mucosa indicating that the turnover rate of the folates was high in these tissues. The variation in the *GGH* levels in colorectal carcinoma was much greater than the variation in *FPGS* and *RFC-1* levels suggesting that the degrading GGH enzyme might limit the availability of folates in the mucosa as well as in the growing tumor. Because GGH is known to be nonspecific, *i.e.*, hydrolyzes other targets than folylpolyglutamates, several important cellular processes may be affected by alterations of its activity. Barrueco *et al.* (55) showed that the enzyme GGH needs sulfhydryl groups for its activation, and evidence showing that high levels of cysteine are required to activate GGH in the lysosome has been presented. As shown in Fig. 2, homocysteine is transsulfurated to cysteine, which then is transported into the lysosome. The interesting relationship between homocysteine and GGH needs to be additionally studied.

Several studies have shown that TS is a prognostic marker for colorectal patients (56–58). A high *TS* gene expression in the tumor has been shown to be associated with a worse clinical outcome. This finding can be attributed to the higher cellular growth potential conferred by the high TS activity, leading to a more aggressive tumor. Normally, a cellular decrease in the folate and *FPGS* levels would give rise to a decreased *TS* gene expression, a diminished cell proliferation, and an increased differentiation. A continuously high *TS* level and cellular proliferation rate despite a folate deficiency in the tissue would, however, be detrimental, because it will alter the normal DNA replication, repair, and methylation. Thus, to summarize, we have found that mucosa samples with high *FPGS* levels also expressed high *RFC-1*, *GGH*, and *TS* levels, and also higher total folates levels (Table 4). This observation might have an impact on the capacity for the cell proliferation and regeneration of the mucosa.

In conclusion, the results of the present study suggest that *FPGS* gene expression in the normal-appearing mucosa adjacent to the tumor is a prognostic marker independent of clinicopathological parameters, including Dukes' stage, that could help in identifying patients having a high risk of tumor recurrence. A high *FPGS* gene expression level in the mucosa was found to be associated with a better tumor-specific survival of the patients than a low level. The low level of *FPGS* in the mucosa probably indicates a folate-deficient state that could increase the aggressiveness of the tumor and promote metastasis. Studies are now in progress to accurately evaluate the prognostic value of *FPGS* gene expression in the mucosa of colorectal carcinoma patients.

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