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Folate Levels and Polymorphisms in the Genes *MTHFR*, *MTR*, and *TS* in Colorectal Cancer

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ABSTRACT

AIM: The aim of the study was to explore and describe the effect of polymorphisms in folate-associated genes regarding the levels of different folate forms and their distribution in tumors and mucosa in patients with colorectal cancer.

MATERIALS AND METHODS: Tumor and mucosa tissues from 53 patients with colorectal cancer were analyzed. The concentrations of tetrahydrofolate (THF), 5-methylTHF, and 5,10-methyleneTHF were measured by liquid chromatography—mass spectrometry. Genotyping of polymorphisms in the folate-associated genes methylenetetrahydrofolate reductase (*MTHFR*, *C677T*), methionine synthase (*MTR*, *A2756G*), and thymidylate synthase (*TS*, 5'-TSER 28 bp tandem repeat and 3'-TSUTR 6 bp deletion/insertion), were done by real-time polymerase chain reaction. Folate levels and distributions were determined in the total patient cohort and after subgrouping by genotypes.

RESULTS: The total folate level, as well as the *THF* and 5,10-methyleneTHF levels, were significantly higher in the tumor compared with mucosa tissue ($P = 0.030$, 0.031, and 0.015, respectively). The individual variation in folate levels in both tumor and mucosa were larger than the variation found when the patients were subgrouped by the gene polymorphisms. No significant differences in the mean concentration of any folate in the mucosa or tumor tissue were found in relation to the analyzed polymorphisms. The percentage level of 5,10-methyleneTHF in tumors was highest in patients with the *MTHFR* 677 CC genotype, and lowest in patients with the *TT* genotype ($P = 0.033$). A significantly lower percentage level of the 5,10-methyleneTHF level was found in tumors of patients with the 5'-TSER 3R/3R genotype ($P = 0.0031$).

CONCLUSION: A significant difference was found between the percentage level of 5,10-methyleneTHF in tumor tissues in relation to the *MTHFR* C677T and 5'-TSER 28 bp repeat polymorphisms. However, no differences were found in the actual tissue folate levels, or in their distribution, in relation to the polymorphisms in the *MTHFR*, *MTR*, or *TS* genes. These findings could be of importance for further research in the field by explaining some of the difficulties of obtaining reproducible and uniform results when using a few selected polymorphisms as predictive markers.

KEYWORDS: colorectal cancer, folate levels, gene polymorphisms

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Introduction

Folates are forms of the water-soluble vitamin, B_9. The name comes from the Latin word "folium", meaning "leaf", as leafy vegetables such as spinach are a principal source of the vitamin, although in Western diets, fortified cereals and breads may be a larger dietary source.[1] Folates exist in various forms and are fundamental to the cell. Their role is to serve as one-carbon donors in the synthesis of purines and thymidine.[2] Folate deficiency has been associated with various conditions including cancer development. For example, it may cause excessive uracil misincorporation into human deoxyribonucleic acid (DNA) in place of thymine, leading to transient nicks and breaks during DNA repair. An imbalance of folate metabolism is suggested to affect several cellular processes, as folates are vital for DNA synthesis and repair, as well as for gene regulation by DNA-methylation.[3]

The intracellular folate metabolism is regulated by several enzymes including methylenetetrahydrofolate reductase

(MTHFR; EC 1.5.1.20), methionine synthase (MTR; EC 2.1.1.13), and thymidylate synthase (TS; EC 2.1.1.45) (Fig. 1). The activity of each enzyme can vary due to different genetic variations, which are often referred to as functional polymorphisms. The variations and their effect on enzyme efficacy have been studied in both preclinical and clinical settings.[4,5] There are many retrospective reports linking a specific polymorphism or even a genotype to clinical findings, ranging from cancer risk to chemotherapy-related side effects.[6,7] A common hypothesis is that the activity of the specific enzyme would affect the metabolism of folates, and chemotherapy such as antifolates or 5-fluorouracil (5-FU), to the extent of affecting prognosis and drug efficacy.

While the overall folate levels in blood have been described,[8] the composition of specific folate forms in colorectal tumor and mucosa tissues has not yet been characterized. The aim of the study was to determine the levels of different folates in colorectal tumor and mucosa tissues, and to note their distribution, as related to common polymorphisms in three folate-associated genes.

Materials and Methods

The study was conducted at a university hospital. Blood samples, as well as mucosa and tumor tissue samples, were routinely collected from patients with colorectal cancer at the time of surgery. Matching macroscopically normal colorectal mucosa samples were taken 10 cm from the tumor. Research nurses gathered and stored the material in a standardized way. Fifty-three patients with colorectal cancer were included in the study. The patients were randomly picked and the clinical data were blinded to the sampler. Clinical data such as demography and pathology were retrieved. All patients gave their written informed consent, and the local ethics committee approved the routine, as well as the use of samples for this study.

Genotyping. Genomic DNA was extracted from fresh—frozen blood using a QIAamp® DNA Mini Kit according to the manufacturer's instructions (Qiagen, Limburg, the Netherlands). Genotype analyses of the *MTHFR* C677T (rs1801133, assay number C_8714009_10) and *MTR* A2756G (rs1805087, assay number C_12005959_10) polymorphisms were run on an ABI PRISM® 7900HT sequence-detection system (Applied Biosystems, Inc, Foster City, CA, USA) using real-time polymerase chain reaction (PCR) and TaqMan chemistry. The single nucleotide assays (Applied Biosystems, Inc) and the TaqMan PCR master mix (Applied Biosystems, Inc) were aliquoted into a 384-well plate using a liquid-handling Biomek FX robot (Beckman Coulter, Inc, Brea, CA, USA). Reactions were characterized by comparing the threshold cycle (C_T) values, as described by the manufacturer.

To analyze the 5'-TSER 28 bp tandem repeat polymorphism, the following primers were used: (forward) 5'AAAAGGCGCGCGGAAGGGGTCCT3' and (reverse)

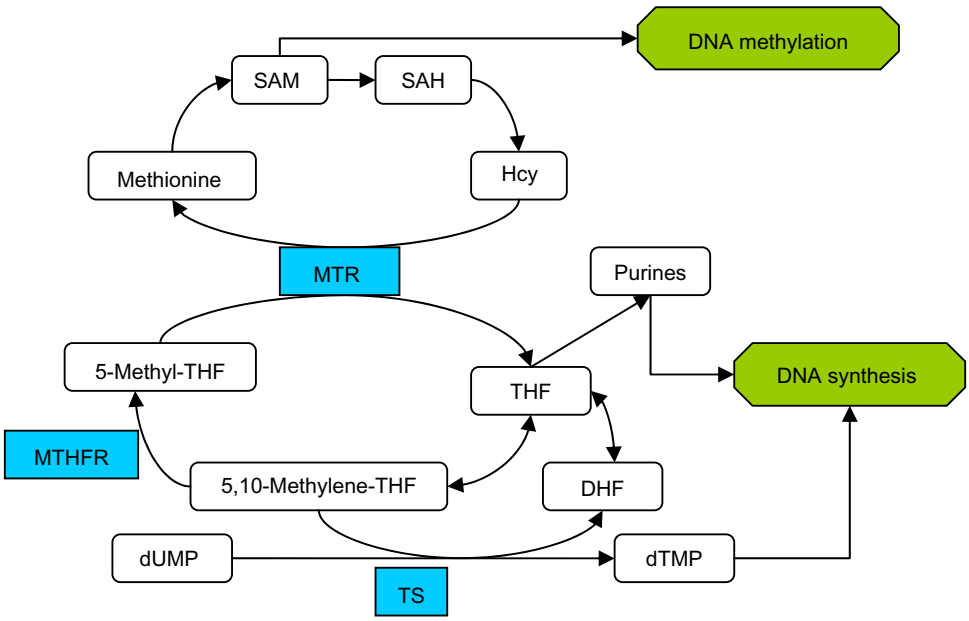

Figure 1. A simplified overview of folate metabolism showing the enzyme steps catalyzed by MTHFR, MTR, and TS.
Notes: Within the cells, folate polyglutamates are converted to 5,10-methyleneTHF, which is required as a methyl donor in the synthesis of dTMP from dUMP. The reaction requires the catalytic activity of the enzyme TS. In addition, 5,10-MethyleneTHF is also the precursor of metabolically active 5-methyl-THF, utilized in the remethylation of the amino acid HCy to methionine. This reaction is catalyzed by MTR. Endogenous methionine is then catabolized to produce the universal methyl donor SAM. The conversion of 5,10-methylene-THF to 5-methyl-THF is dependent on the enzyme MTHFR.
Abbreviations: SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; HCy, homocysteine; DHF, dihydrofolate; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate.

5'TCCGAGCCGGCCACAGGCAT3'. The 3'-TSUTR 6 bp deletion/insertion TTAAAG polymorphism was analyzed, as described by Ren et al,[4] using the following primers: (forward) 5'GACGAATGCAGAACACTTCT3' and (reverse) 5'AATCTGAGGGAGCTGAGTAAC3'. The PCR reaction was carried out as described in a previous study by Iacopetta et al.[5] The labeled PCR fragments were run on an ABI 3730 DNA analyzer. The 5'-TSER 28 bp tandem repeats were classified as (2R/2R), (2R/3R), or (3R/3R). The 3'-TSUTR polymorphism was classified as (0 bp/0 bp), (0 bp/6 bp), or (6 bp/6 bp). All genotype analyses were performed by the Genomics Core Facility (Gothenburg, Sweden).

Folate analyses. A liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS) method was used to evaluate levels of the folate derivatives, tetrahydrofolate (THF), 5-methylTHF, and 5,10-methyleneTHF in tumor tissue and adjacent mucosa separately.[9] In brief, the folate extraction method involved homogenization, heat treatment, and folate conjugate treatment to hydrolyze polyglutamyl folates to monoglutamyl folates. Tomudex was used as an internal standard. Before analysis on LC-MS/MS, simple and fast sample purification with ultrafiltration (molecular weight cut-off membrane, 10 kDa) and centrifugation for 30 minutes at 21,500 × g, 20°C, was performed. The solution at the bottom of the centrifugation tube was used for LC-MS/MS analysis. Folates were detected and quantified using positive electrospray. Calibration graphs were constructed by plotting the peak area ratio of each compound to internal standard against concentration. The standards and samples were processed using the QuanLynx quantitative processing tool in MassLynx (Waters, Corporation, Milford, MA, USA). The levels of THF, 5-methylTHF, and 5,10-methyleneTHF in each sample were expressed as pmol/g wet weight (pmol/g_{ww}). The levels of the different folate forms, as well as a total folate value (equaling the sum of the THF, 5-methylTHF, and 5,10-methyleneTHF concentrations) were determined for the entire patient cohort and for the cohorts that were subgrouped by each polymorphism. The proportion of the separate folate forms was calculated as a percentage of the total folate amount.

Statistics. JMP 10.0/SAS software (SAS Institute, Inc, Cary, NC, USA) was used for the statistical analysis. The statistical analysis consisted of descriptive statistics with mean or median values and measures of dispersion, as appropriate. The folate levels were assessed by appropriate polymorphisms by nonparametric Kruskal–Wallis or Wilcoxon signed rank tests. The significance level was set at 95%.

Results

All patients were operated for colorectal cancer and all tumors were adenocarcinomas. The median age of the patients was 71 years with TNM cancer stages varying from 1–4. The patients' characteristics are summarized in Table 1. The distribution and levels of THF, 5-methylTHF, and 5,10-methyleneTHF in the tumor and mucosa tissues are presented in Table 2. As shown, there was a large individual variation in the folate levels in both tumor and mucosa tissue. The total folate level as well as the THF and 5,10-methyleneTHF levels were significantly higher in the tumor tissue when compared with the mucosa tissue. However, there was no significant difference in the 5-methylTHF levels.

The genotype frequencies of the different polymorphisms, as well as the folate concentrations, were grouped by genotypes and are presented in Tables 3–6. Folate forms that represent the substrates and products of the MTHFR and MTR enzymes were analyzed according to the genotypes MTHFR C677T and MTR A2756G, respectively, whereas 5,10-methyleneTHF (ie, the cofactor that binds to TS) and THF (the precursor of 5,10-methyleneTHF) were analyzed according to the 5'-TSER 28 bp and 3'-TSUTR 6 bp deletion/insertion genotypes.

The results showed that there were no statistically significant differences in the mean concentration of any folate in the

Table 1. Patient characteristics.

Median age, years (range)	73 (30–92)
Sex	
Male	28
Female	25
Tumor location	
Colon	39
Rectum	14
Tumor differentiation[a]	
High	4
Medium	35
Low	13
Median assessed lymph nodes (range)	18 (3–33)
Median positive lymph nodes (range)	1 (0–13)

Note: [a]Tumor differentiation data missing for one patient.

Table 2. Folate levels in tumor and mucosa.

	TUMOR MEAN ± SD (RANGE)	MUCOSA MEAN ± SD (RANGE)	*P*-VALUE[B]
Total folate amount[a]	2705 ± 2006 (280–10870)	2149 ± 1206 (540–5697)	0.012
THF[a]	1154 ± 809 (120–4400)	891 ± 427 (364–2153)	0.014
5-methylTHF[a]	552 ± 568 (40–2250)	528 ± 436 (65–2200)	0.31
5,10-methyleneTHF[a]	985 ± 714 (24–3454)	768 ± 507 (41–2683)	0.0045

Notes: [a]The folate concentration equals pmol/g wet weight tissue. [b]*P*-value was calculated by Wilcoxon signed rank test.

Table 3. Percentage[a] levels of 5,10-methyleneTHF and 5-methylTHF by the MTHFR C677T polymorphism.

MTHFR C677T		5,10-METHYLENETHF (% ± SD)		5-METHYLTHF (% ± SD)	
GENOTYPE	N	TUMOR	MUCOSA	TUMOR	MUCOSA
CC	30	39.1 ± 12.9	33.9 ± 12.8	18.2 ± 12.0	21.7 ± 11.0
CT	18	35.6 ± 13.3	34.4 ± 12.7	19.7 ± 10.4	22.2 ± 9.4
TT	4	20.8 ± 9.7	31.6 ± 7.6	26.2 ± 12.1	26.2 ± 9.5
P-value[b]		0.033	0.93	0.31	0.71

Note: [a]In relation to the total folate concentration. [b]*P* by Kruskal—Wallis tests.

mucosa or tumor tissue in relation to the analyzed polymorphisms (data not shown). When comparing the folate levels expressed as the mean percentage of the total folate amount, the level of 5,10-methyleneTHF in tumors was found to differ according to the MTHFR C677T polymorphism. The level was highest in patients with the MTHFR CC genotype, and lowest in patients with the TT genotype (Table 3; $P = 0.033$). Furthermore, a significantly lower percentage level of 5,10-methyleneTHF was found in the tumors of patients with the TS-5ER 3R/3R genotype (Table 5; $P = 0.0031$). No difference in any of the folate levels in relation to the analyzed polymorphisms was found in the colorectal mucosa.

Discussion

One reason for the medical interest in folate metabolism is its association with chemotherapy. One of the most commonly used drugs in colorectal cancer, in both adjuvant and palliative treatment, is 5-FU, which targets the enzyme TS.[10] However, the treatment carries risks of resulting in toxic reactions.[11] Therefore, clinical and scientific interest has been directed at finding factors that could forecast the response to the intended treatment, and thus enable a tailored therapy. Differences in folate metabolism between individuals, which are due to various enzyme activities caused by genetic polymorphisms, have been suggested as the possible explanation for the difference in the response and side effects observed between patients who are treated with 5-FU or antifolates.[12–15] Several polymorphisms in the genes involved in folate metabolism have been suggested as both prognostic and predictive factors in colorectal cancer.[5,16] Among the most studied ones are those of the genes *MTHFR*, *MTR*, and *TS*.[14,15,17] For example, MTHFR enzyme activity for individuals with the MTHFR 677 CC genotype is set at 100%, and declines to 70% among those with the CT genotype, and to around 30% for the TT homozygote.[19] This difference in enzyme activity might affect the levels of 5,10-methyleneTHF in terms of its effects on the responses associated with using 5-FU or antifolate-based treatment for colorectal cancer.[20]

Table 4. Percentage[a] levels of THF and 5-methylTHF by the MTR A2756G polymorphism.

MTR A2756G		THF (% ± SD)		5-METHYLTHF (% ± SD)	
GENOTYPE	N	TUMOR	MUCOSA	TUMOR	MUCOSA
AA	31	42.7 ± 12.8	43.6 ± 11.2	19.4 ± 12.1	21.0 ± 8.6
AG	18	44.6 ± 11.3	44.4 ± 14.0	19.4 ± 10.5	24.4 ± 12.1
GG	3	45.2 ± 14.1	38.7 ± 16.0	21.7 ± 12.4	25.1 ± 14.0
P-value[b]		0.99	0.78	0.83	0.54

Notes: [a]In relation to the total folate concentration. [b]*P* by Kruskal—Wallis tests.

The results of the present study showed that the folate level was significantly higher in tumor tissues when compared with mucosa tissues. The higher folate content may be caused by a higher activity of folate receptor α (FRα), which is a membrane-bound protein that transports folates into the cells. FRα is usually selectively expressed at a high level in malignant epithelial cells and rarely expressed in normal cells.[21] The results further showed that the 5,10-methyleneTHF levels in tumor tissue, which was expressed as percentage of the total folate amount, was highest in patients with the MTHFR CC genotype and lowest in patients with a TT genotype. These results are in agreement with those of our previous study where the 5,10-methyleneTHF content was determined by the 5-fluoro-2'deoxyuridine 5'-monophosphate binding assay.[7] The significantly lower percentage level of 5,10-methyleneTHF found in the tumors of patients with the TS-5ER 3R/3R genotype, as compared to those with the 2R/2R or 2R/3R genotypes, is a novel finding that may be explained by the expected higher expression rate of TS messenger ribonucleic acid in 3R/3R individuals.[22] A higher transcription level would lead to increased levels of TS protein that binds free 5,10-methyleneTHF, thus lowering the level of this particular cofactor.

Although several studies have been conducted in the search for predictive factors in colorectal cancer, there has been no real implementable breakthrough, and none of the suggested factors (such as *TS* or *MTHFR* polymorphisms) are yet in clinical use. This has been noted and discussed in several papers, including in the publication by Tejpar et al.[18] The large individual variation in the mean folate levels in both tumor and mucosa tissues found in the present study provides one explanation as to why the results are so difficult to reproduce and implement in a clinical setting. Further complicating the interpretation of the data is the possibility that decreased enzyme activity would be compensated for by increased activity of other enzymes involved in the same folate pathway. As discussed in a study by Odin et al,[7] high levels of folates might compensate for unfavorable genotypes. This could lead to an overestimation of the clinical relevance of genetic polymorphisms.

The present study is, to our knowledge, one of the first to describe the actual and percentage levels of the various folate forms in correlation with functional polymorphisms in the genes *MTHFR*, *MTR*, and *TS* in colorectal cancer. There is a

Table 5. Percentage[a] levels of 5,10-methyleneTHF and THF by the 5'-TSER 28 bp polymorphism.

5'-TSER 28 BP		5,10-METHYLENETHF (% ± SD)		THF (% ± SD)	
GENOTYPE	N	TUMOR	MUCOSA	TUMOR	MUCOSA
2R/2R	11	38.5 ± 7.8	32.5 ± 13.0	45.2 ± 10.3	47.1 ± 12.9
2R/3R	26	41.2 ± 13.9	34.8 ± 12.8	41.9 ± 12.5	44.8 ± 12.7
3R/3R	16	28.0 ± 12.1	34.1 ± 11.6	46.5 ± 14.2	39.3 ± 10.1
P-value[b]		0.0031	0.60	0.64	0.25

Notes: [a]In relation to the total folate concentration. [b]*P* by Kruskal—Wallis tests.

weakness of this study in that only 53 patients were assessed. However, there are data on folate concentrations in both the mucosa and tumor tissues of each patient, which strengthens the results. Furthermore, the study was performed at a single center, and all patients and tests have been carried out by the same staff using the same guidelines. Among the potential weaknesses of this study is the fact that we had to limit our study to three key enzymes due to resource limitations. There are, of course, interesting gene polymorphisms in other enzymes that have putative effects on folate composition and concentration at the tissue level.

In a previously published study, our group showed that there is an association between side effects and certain combinations of MTR A2756G and MTHFR C677T polymorphisms in patients with colorectal cancer treated with adjuvant 5-FU.[14] Those findings suggested a very complex correlation between the folate metabolism and the chemotherapy effects, and that it might be difficult to use a single polymorphism as the sole predictive factor. The results of the present study support our line of thought and suggest that the interaction between polymorphic genes involved in a biochemical pathway must be carefully analyzed and taken into account in order to properly evaluate its clinical impact. Large patient groups are needed in order to conduct such studies, which emphasizes the need of collaboration between different research groups. From a clinical point of view, it can be argued that it will be challenging to implement gene polymorphism data when making decisions about treatment options for individual patients.

Table 6. Percentage[a] levels of 5-methyleneTHF and THF by 3'-TSUTR 6 bp del/ins polymorphism.

3'-TSUTR DEL/INS		5,10-METHYLENETHF (% ± SD)		THF (% ± SD)	
GENOTYPE	N	TUMOR	MUCOSA	TUMOR	MUCOSA
0 bp/0 bp	5	30.5 ± 12.1	45.1 ± 12.7	48.2 ± 17.1	38.8 ± 14.2
0 bp/6 bp	27	36.0 ± 15.0	30.7 ± 11.9	43.6 ± 14.4	45.7 ± 12.0
6 bp/6 bp	21	39.0 ±11.6	35.8 ± 11.2	43.5 ± 8.9	42.1 ± 11.9
P-value[b]		0.36	0.056	0.81	0.33

Notes: [a]In relation to the total folate concentration. [b]*P* by Kruskal—Wallis tests.

Conclusion

The total folate level was higher in tumor tissue when compared to mucosa tissue. A significant difference was found between the percentage level of 5,10-methyleneTHF in the tumor tissue in relation to the MTHFR C677T and 5'-TSER 28 bp repeat polymorphisms. However, no differences were found in the actual tissue folate levels, or in their distribution, with respect to the polymorphisms in the *MTHFR*, *MTR*, or TS genes. These findings could be of importance for further research in the field, as they might explain some of the difficulties in obtaining reproducible and uniform results when using a few selected polymorphisms as predictive markers.

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Author Contributions

Conceived and designed the experiment: HT, YW, KD. Analysed the data: HT, EO, YW, KD. Wrote the first manuscript: HT, YW, KD. Contributed to the writing of the manuscript: HT, YW, EO, KD. Agree with the manuscript results and conclusion: HT, YW, EO, GC, KD. Jointly developed the structure and arguments for the paper: HT, YW, EO, GC, KD. Made critical revisions and approved final version: HT, YW, EO, GC, KD. All authors reviewed and approved of the final manuscript.

DISCLOSURES AND ETHICS

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

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