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The Association Between Methylenetetrahydrofolate Reductase Polymorphism and Promoter Methylation in Proximal Colon Cancer

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Abstract. *Background: Methylenetetrahydrofolate reductase (MTHFR) plays a critical role in folate metabolism, which is an important pathway of the methyl donor for DNA methylation. The MTHFR gene has genetic variants (C667T and A1298C), which cause reduced enzyme activity. Impaired folate metabolism by these genetic variants of MTHFR could change the methylation pattern of DNA including promoter hypermethylation, which has been frequently observed in cancer. In this study, we compared the MTHFR genotypes and haplotype to the features of colorectal cancer focusing on the promoter methylation of tumor DNA. Materials and Methods: Genomic DNA was isolated from 194 colorectal cancer tissues and subjected to MTHFR genotyping by PCR-based restriction fragment length polymorphism analysis. The MTHFR haplotype was determined by combination of C667T and A1298C genotype and classified into 2 groups, high (H-haplotype) or low (L-haplotype) enzymatic activity of MTHFR. The methylation level of tumor suppressor genes (CDKN2A, hMLH1, ARF and TIMP3) was measured by a fluorescence-based, real-time methylation specific PCR method. Results: There was no significant association of the clinicopathological features with either C667T genotype, A1298C genotype or haplotype of MTHFR. The methylation level of CDKN2A was higher in cancer with the L-haplotype of MTHFR than in that with the H-haplotype when cancers of proximal origin were considered ($p=0.029$). hMLH1 methylation also tended to be higher in proximal colon cancers of MTHFR L-haplotype ($p=0.059$). In addition, the proximal colon cancers showing CpG island methylator phenotype (CIMP) were significantly more frequent in L-haplotype than in H-haplotype. Conclusion: These results suggest that the haplotype with low enzymatic activity of MTHFR is linked with promoter hypermethylation and consequently modifies the risk of CIMP(+) proximal colon cancer development in the Japanese people. The relationship between MTHFR polymorphism and DNA methylation in the Japanese is contrary to the previous results in Caucasians. Further study is needed focusing on ethnic variations in the relationships among MTHFR polymorphism, DNA methylation and the development of CIMP(+) colorectal cancer.*

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A number of epidemiological and experimental studies suggest the existence of a link between folate consumption and cancer predisposition (1-3). Although it is generally accepted that low folate intake is a risk factor for cancer development, the role of folate metabolism in carcinogenesis is not fully understood. One of the mechanisms may be involvement of folate metabolism in the DNA methylation pathway. Impairment of the folate metabolism can induce both global hypomethylation and promoter hypermethylation, which are commonly observed in a large variety of cancers (4-6). Global hypomethylation causes genomic instability and results in cancer development through gene aberration. However, promoter hypermethylation leads to silencing of tumor suppressor genes and is involved in carcinogenesis.

Methylenetetrahydrofolate reductase (MTHFR) catalyzes the reduction of methelenetetrahydrofolate to 5-methyltetrahydrofolate, a major form of circulating folate. Therefore, MTHFR is a critical enzyme in folate metabolism and its dysfunction may be implicated in cancer development. MTHFR polymorphism (C667T, alanine-to-valine) is known to be associated with its enzyme activity, affecting the folate metabolism coupling with the synthesis from homocysteine to methionine (7). Homozygous variant TT shows 30% activity of the enzyme compared to the CC genotype and is associated with colorectal cancer risk although the odds ratio

differs depending on dietary habit (8-10). This association of MTHFR genotype with cancer predisposition also can be attributable to the mechanism described above in which DNA methylation is a major player.

We hypothesized that there is a link between promoter methylation of tumor suppressor genes and the MTHFR genotype in colorectal cancer, assuming that the MTHFR genotype is involved in cancer development through aberrant DNA methylation. To test this hypothesis, we analyzed two MTHFR genotypes, C667T and A1298C, in 194 colorectal cancers and compared them for clinicopathological features and promoter methylation of CDKN2A, hMLH1, ARF and TIMP3.

Materials and Methods

Materials. A total of 194 tumor tissue samples were obtained by surgical resection from patients with primary colorectal adenocarcinoma. The patients comprised 116 males and 78 females, ranging in age from 33 to 93 years, with a mean age of 65.9 years. Approximately 2g of surgically resected tissues were frozen immediately in liquid nitrogen and stored at -80˚C until DNA isolation. The remaining section of the sample was fixed with formalin and used for further histological examination to confirm the diagnosis postoperatively. All histological examinations were performed after staining with H&E. We defined the location of tumor (proximal or distal) according to whether the lesion originated proximal or distal to the splenic flexure. Approval for this project was obtained from the Kanazawa University School of Medicine Ethics Committee, Japan.

MTHFR genotyping. Genomic DNA was isolated by the standard method of proteinase K digestion and phenol-chloroform extraction. The MTHFR genotypes, C667T and A1298C, were examined in all specimens using the PCR-restriction fragment length polymorphism method described previously (11, 12).

Bisulfite treatment. DNA was subjected to bisulfite treatment as described previously (13). Briefly, DNA was denatured using NaOH and modified by sodium bisulfite. Then DNA samples were purified using Wizard DNA purification resin (Promega, Madison, WI, USA) and precipitated with ethanol. DNA was resuspended in 5mM Tris (pH 8.0) and stored at -30˚C.

Real-time methylation specific PCR. Methylation analysis was performed using the fluorescence-based, real-time methylation specific PCR assay, MethyLight, as described previously (14). The sets of primers and probes used to specifically amplify the bisulfite-convert DNA in the promoter regions of CDKN2A, hMLH1, ARF and TIMP3 were designed previously (15). The specificities of the reactions for methylated DNA were confirmed separately using human sperm DNA (unmethylated) and SssI (New England Biolabs, Beverly, MA, USA) - treated sperm DNA (methylated). The percentage of fully methylated molecules at a specific locus was calculated by dividing the GENE: ACTβ ratio of a sample by the GENE: ACTβ ratio of SssI-treated sperm DNA and multiplying by 100. We used the abbreviation PMR (percentage of methylated reference) to indicate this measurement as described previously (15).

Statistical analysis. The results of the methylation analysis are expressed as medians and ranges. Associations of clinicopathological variables with MTHFR genotypes, haplotype or CpG island methylator phenotype (CIMP) were tested either by two-sided *t*-test, ANOVA or Chi-square test. Comparisons of methylation levels with genotypes or haplotype were made by Kruskal-Wallis analysis or Mann-Whitney *U*-test. $p<0.05$ was considered significant.

Results

MTHFR genotypes and clinicopathological features. MTHFR genotypes were successfully analyzed in all the 194 colorectal cancers. The frequency of the C667T genotype was as follows: CC, 91 (46.9%); CT, 77 (39.7%); TT, 26 (13.4%). The relationship between the genotype of C667T and clinicopathological features is summarized in Table I. There was no relationship between the C667T genotype and the variables. The incidence of A1298C was as follows: AA, 130 (67.0%); AC, 60 (30.9%); CC, 4 (2.1%). The A1298C genotype of AC and CC was combined in further analysis since the 1298CC genotype was quite rare. The relationship between the genotype of A1298C and clinicopathological features is summarized in Table II. Patients with 1298AA genotype tend to be younger than those with the AC or CC genotype ($p=0.054$). There were no relationships observed between the A1298C genotype and other clinicopathological factors.

Sequence disequilibrium was consistently observed in the current study between C667T and A1298C as reported previously (16). Since the double hetero-genotype (667CT and 1298AC) is suggested as a haplotype with reduced enzymatic activity (17) as well as homozygous variant (667TT or 1298CC), we stratified the patients according to haplotype of MTHFR as indicated in Table III. One hundred and forty-three patients were defined as the haplotype with high enzymatic activity of MTHFR (H-haplotype) and 51 patients as low enzymatic activity (L-haplotype). The relationship between the haplotype and clinicopathological features is summarized in Table IV. There was no significant relationship between MTHFR haplotype and any of the variables.

MTHFR haplotype is associated with promoter methylation and CIMP in proximal colon cancer. To test the hypothesis that there is a link between promoter methylation of tumor suppressor genes and the MTHFR genotype in colorectal cancer, we measured the methylation level of the promoter region in CDKN2A, hMLH1, ARF and TIMP3. The median values and ranges of methylation in each gene were as follows: CDKN2A, median 0.40, range 0.00-468.59; hMLH1, median 0.00, range 0.00-463.03; ARF, median 0.00, range 0.00-719.93; TIMP3, median 0.69, range 0.00-452.97. There was no relationship between the MTHFR genotypes and methylation level of the genes when all cases were

Table I. *The relationship between MTHFR C677T genotype and clinicopathological features.*

			MTHFR C677T genotype			
		Total	CC	CT	TT	*P* value
Number		194	91	77	26	
Age		66±13	66±12	67±14	63±10	0.414
Gender	Male	116	58	42	16	0.472
	Female	78	33	35	10	
Site	Proximal	68	28	29	11	0.457
	Distal	126	63	48	15	
UICC[a]	I	17	8	6	3	
stage	II	70	37	24	9	0.344
	III	78	34	37	7	
	IV	29	12	10	7	
Histology[b]	Well	86	41	35	10	
	Moderate	90	43	33	14	
	Poorly	11	4	5	2	0.874
	Muc[c]	6	3	3	0	
	Sig[d]	1	0	1	0	

[a]Tumor staging was performed according to the International Union Against Cancer (UICC) TNM classification.
[b]Histology of adenocarcinoma was sub-classified into well-, moderate- and poorly-differentiated adenocarcinoma according to their grading.
[c]Muc; Mucinous adenocarcinoma.
[d]Sig; Signet-ring cell carcinoma.

Table II. *The relationship between MTHFR A1298C genotype and clinicopathological features.*

			MTHFR A1298C genotype		
		Total	AA	AC or CC	*P* value
Number		194	130	64	
Age		66±13	65±13	68±11	0.054
Gender	Male	116	81	35	0.309
	Female	78	49	29	
Site	Proximal	68	44	24	0.616
	Distal	126	86	40	
UICC stage	I	17	8	9	
	II	70	47	23	0.306
	III	78	54	24	
	IV	29	21	8	
Histology	Well	86	59	27	
	Moderate	90	60	30	
	Poorly	11	7	4	0.838
	Muc	6	3	3	
	Sig	1	1	0	

included in the analysis (data not shown). We then explored this relationship in the sub-group stratified by tumor site (proximal or distal) since the methylation is suggested to have a greater link with cancer development in the proximal colon (18). The results showed that the methylation level of CDKN2A was significantly higher in proximal colon cancers

Table III. *Haplotype definition of MTHFR according to both C667T and A1298C genotype.*

		A1298C		
		AA	AC	CC
C667T	CC	H (49)	H (39)	L (3)
	CT	H (55)	L (21)	L (1)
	TT	L (26)	L (0)	L (0)

H and L indicate high(H)- and low(L)-haplotype with regard to enzymatic activity of MTHFR respectively. Number of patients is indicated within parentheses.

Table IV. *The relationship between MTHFR haplotype and clinicopathological features.*

			MTHFR haplotype		
		Total	H	L	*P* value
Number		194	143	51	
Age		66±13	66±13	66±11	0.938
Gender	Male	116	86	30	0.869
	Female	78	57	21	
Site	Proximal	68	46	22	0.159
	Distal	126	97	29	
UICC stage	I	17	10	7	
	II	70	51	19	0.173
	III	78	63	15	
	IV	29	19	10	
Histology	Well	86	65	21	
	Moderate	90	64	26	
	Poorly	11	8	3	0.891
	Muc	6	5	1	
	Sig	1	1	0	

of MTHFR L-haplotype than those of H-haplotype ($p=0.029$, Table V). hMLH1 methylation also tended to be higher in proximal colon cancers of MTHFR L-haplotype ($p=0.059$). In addition, the methylation levels of both ARF and TIMP3 were higher in cancer of L-haplotype than in those of H-haplotype, although there was no statistical significance. There was no relationship between the MTHFR haplotype and the methylation of genes in distally located cancers (data not shown).

The phenotype with activated methylation over a number of genes in the promoter region has been proposed as CIMP (19). Although there has been no consensus definition for CIMP yet, we defined CIMP as a cancer with higher methylation level than 10 PMR in the promoter region of at least 2 genes among CDKN2A, hMLH1, ARF and TIMP3. Under this definition the proximal colon cancers showing CIMP were significantly more frequent in L-haplotype than in H-haplotype ($p=0.049$, Table V). The characteristics of the cancer with CIMP are summarized in Table VI.

Table V. *The association of MTHFR haplotype with promoter methylation level and CIMP in proximal colon cancer.*

		H-haplotype	L-haplotype	*P* value
Methylation level				
	CDKN2A	0.37 (0.00-138.15)	2.22 (0.00-468.59)	0.029
	hMLH1	0.00 (0.00-228.72)	2.60 (0.00-463.03)	0.059
	ARF	0.00 (0.00-201.84)	0.00 (0.00-719.93)	0.434
	TIMP3	0.65 (0.00-259.13)	3.72 (0.00-452.97)	0.268
CIMP	(+)	7	8	0.049
	(-)	39	14	

Methylation level was expressed by the median (range)

Table VI. *The relationship between CIMP and clinicopathological features.*

			CIMP		
		Total	(+)	(-)	*P* value
Number		194	28	166	
Age		66±13	69±12	65±13	0.110
Gender	Male	116	17	99	0.915
	Female	78	11	67	
Site	Proximal	68	15	53	0.026
	Distal	126	13	113	
UICC stage	I	17	2	15	
	II	70	9	61	0.551
	III	78	15	63	
	IV	29	2	27	
Histology	Well	86	8	78	
	Moderate	90	12	78	
	Poorly	11	4	7	<0.001
	Muc	6	4	2	
	Sig	1	0	1	

Significant association of CIMP with proximal location of cancer and histological type (poorly-differentiated adenocarcinoma and mucinous adenocarcinoma) was observed. These features of CIMP(+) colorectal cancer were consistent with previous reports (20, 21) and support our definition of CIMP in the current study.

Discussion

In this study, we compared the MTHFR genotype and haplotype to the clinicopathological features and methylation status of CDKN2A, hMLH1, ARF and TIMP3 in 194 advanced colorectal cancers. We observed significant and borderline associations of the MTHFR haplotype with the methylation level of CDKN2A and with those of hMLH1, respectively, when cancers of proximal origin were considered. Both methylations were higher in cancer with the MTHFR L-haplotype than in that with the H-haplotype. The same relationship between MTHFR haplotype and promoter methylation of ARF and TIMP3 was observed, although there was no statistical significance. These consistent associations suggest that the mechanism of aberrant methylation is modified by the MTHFR haplotype, presumably through its relationship to the enzyme activity. Previous reports have demonstrated that homozygous variants, either MTHFR 667TT or 1298CC, and double heterozygous variant, both 667CT and 1298AC, result in a reduced enzyme activity of MTHFR (7, 17). These variants were defined as L-haplotype in the current study and others were defined as H-haplotype. Therefore, the results suggest that individuals who have low enzymatic activity of MTHFR are predisposed to aberrant hypermethylation of DNA. This suggestion was further supported by the observation that the MTHFR L-haplotype is more prevalent in CIMP(+) cancer of proximal origin than in those without CIMP.

The suggested association between MTHFR polymorphism and promoter methylation is somewhat contrary to previous reports with subjects from the Western population. Paz *et al.* reported lower levels of global 5-methyl cytosine in DNA from normal and tumor tissues of individuals harboring the MTHFR C677T variant (22). In addition, genomic DNA hypomethylation in the peripheral white blood cells of MTHFR 677TT individuals has been described (23, 24). These reports were consistent in that low enzymatic activity of MTHFR was linked to low DNA methylation, whereas our data suggest the link between the MTHFR L-haplotype, low enzymatic activity of MTHFR and higher DNA methylation. The inconsistent result in the current study may be caused by ethnic variation in the relationship between MTHFR polymorphism and folate intermediate (FI), methylenetetrahydrofolate and tetrahydrofolate, in colorectal cancer tissue. We reported that FI in colorectal cancer tissue with MTHFR 667CT genotype is higher than that with 667CC genotype in the Japanese people (11). In contrast, our research group observed that FI in colorectal cancer tissue with MTHFR 667TT genotype is significantly lower than that with 667CC or 667CT in an Australian population (25). The inverse association between the MTHFR polymorphism and FI can result in a contrary relationship between the MTHFR polymorphism and the status of aberrant methylation in cancer. Overall, ethnic variations are suggested in the relationships among MTHFR polymorphism, FI, promoter hypermethylation and colorectal cancer risk with CIMP. The ethnic variation may arise from differences in dietary habits and/or genetic variations in other polymorphism such as thymidylate synthase (26). Further study on the role of MTHFR polymorphism in cancer epidemiology should consider ethnic variations in gene-nutrition interference.

Recently, we observed a significant association between FI in colorectal cancer tissue and hypermethylation in the promoter region of hMLH1, ARF and TIMP3 (25). The FI was higher in cancer tissue showing hypermethylation in those three genes. Accordingly, we hypothesized that the MTHFR C677T variant is related to hypermethylation since our previous study found that FI in colorectal cancer tissue with MTHFR 667CT genotype is higher than that with 667CC genotype in the Japanese population (11). The current study, however, did not show any association between the MTHFR C677T variant and methylation level in the promoter region of CDKN2A, hMLH1, ARF and TIMP3. The lack of an association might be due to the influence of other genotypes of folate metabolizing enzymes including MTHFR A1298C, methionine synthase A2756G and repeat-length polymorphism of thymidylate synthase. Haplotype analysis of MTHFR indeed showed that the methylation level of CDKN2A was higher in cancer with the L-haplotype of MTHFR than in that with the H-haplotype. Since sequence disequilibrium is present between MTHFR C677T and A1298C (16), it is necessary to consider mutual interference of the MTHFR genotypes in a genotype-phenotype association study. Although our classification of MTHFR haplotype should be further validated, haplotype analysis of MTHFR may be indispensable in the future study.

It is not apparent at this moment why the MTHFR polymorphism is associated with promoter methylation only in proximal colon cancer. There are a number of lines of evidence to suggest that genetic and epigenetic changes differ between proximal and distal colon cancer (18, 27, 28). The involvement of promoter methylation in cancer development is more apparent in proximal colon cancer. Therefore, a relationship between MTHFR polymorphism and promoter methylation might be retained only in cancers of proximal origin. To better understand how MTHFR polymorphisms are involved in DNA methylation and colorectal cancer development, future studies conducted with normal colorectal mucosa as the material may be useful.

In conclusion, we found association of the MTHFR haplotype with the methylation level of CDKN2A promoter and the incidence of CIMP in proximal colon cancer. The results suggest that the haplotype with low enzymatic activity of MTHFR is linked with promoter hypermethylation and consequently modify the risk of CIMP(+) proximal colon cancer development in the Japanese. However, ethnic variations are suggested to occur with regard to how MTHFR polymorphisms are implicated in folate metabolism, promoter methylation and CIMP(+) colorectal cancer risk. Further molecular epidemiological study in MTHFR polymorphisms may consider the level of FI as a mediator and its ethnic variations.

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