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CpG Island Methylation in Familial Colorectal Cancer Patients Not Fulfilling the Amsterdam Criteria

To determine the role of methylation in colorectal cancer patients with a family history, we enrolled 25 colorectal cancer patients with a family history of colorectal cancer but without a mutation in the *hMLH1* and *hMSH2* genes. Thirty patients with sporadic colorectal cancer were included as control. The methylation status of *COX2*, *MGMT*, *hMLH1*, *TIMP3*, *p16,* and *MINT2* in normal mucosa and tumor were assessed using methylation-specific PCR. In patients with a family history, the methylation frequency ranged from 4.0% for *TIMP3* to 44.4% for *MGMT*, whereas, in patients with sporadic colorectal cancer, it ranged from 6.7% for *TIMP3* to 50.0% for *p16*. Nine of the 25 patients with family history (36.0%) were classified as methylation-prone, and nine of the 30 patients with sporadic cancers (30.0%) were as methylation-prone, making their methylation indices 0.19 and 0.16, respectively (p=0.522). As for the individual genes, the methylation rate of *MGMT* was higher in colorectal cancer patients with family history (44.0% vs. 13.0%, p=0.016), whereas the methylation rate of *p16* was higher in sporadic colorectal cancers (50.0% vs. 8.7%, p=0.046). While CpG island methylation of tumor suppressor genes may play a role in colorectal carcinogenesis, the genes involved may be different between tumors of patients with and without a family history of colorectal cancer.

Key Words : *Colorectal Neoplasms; Familial; Carcinogenesis; Methylation; Microsatellite Instability*

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INTRODUCTION

Colorectal cancer arises from a series of somatically inherited changes, including mutations and transmissible epigenetic events such as methylation of CpG islands (1), some or all of which produce a growth advantage relative to surrounding cells (2, 3). Among the epigenetic events involved in tumorigenesis are DNA methylation, genomic imprinting, and histone modification (4). Global decreases in 5-methylcytosine content have been associated with tumor formation, including that of colorectal cancers. Another molecular defect commonly occurring during neoplasia is de novo CpG island methylation (4-6). Recently, a distinct pathway of colorectal carcinogenesis was described, termed the CpG island methylation phenotype (CIMP) (7). This pathway was uncovered through a series of observations that included association between microsatellite instability (MSI) and hypermethylation of multiple genes, from the concordance between the methylation status of different genes unrelated to the gene function or chromosomal location, and from the bimodal distribution of methylation in a selected subset of genes.

Colorectal cancer is the third leading cause of cancer-related deaths worldwide (8). Almost 70% of all colorectal cancers are sporadic. Of the remaining 30%, a small number belong to the familial syndromes of hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP), whereas the vast majority show non-syndromatic familial susceptibility (9). The proportion of all cases considered familial depends in the definition. Of all patients with colorectal cancer, 11-16% have at least one first-degree relative with colorectal cancer (10, 11), but the proportion is much higher if second- or third-degree relatives are considered. In one study, 53% of colorectal cancer probands had a first-degree relative with cancer (11). In fact, first-degree relatives of patients affected by colorectal cancer, but who do not fulfill the criteria for FAP and HNPCC, have a more than 2-fold increased risk of developing tumors of the large intestine (12).

HNPCC is caused by germline mutations in the mismatch

repair (MMR) genes *hMLH1*, *hMSH2*, *hMSH6,* and *hPMS2*, with a penetrance of approximately 80% for colorectal cancer, 60% for endometrial cancer, and below 20% for other cancers (13). HNPCC is characterized by early onset, the development of neoplastic lesions in a variety of tissues, and MSI. The Amsterdam criteria for HNPCC are strictly defined and exclude most tumors suspected of being hereditary. In contrast, the Bethesda guidelines broaden the disease spectrum by including colorectal cancer families with specific accompanying cancers and clinicopathologic characteristics (13, 14). These latter criteria provide important clues for identifying genetic pathways associated with atypical phenotypes of hereditary colorectal cancer. Although the strict Amsterdam criteria could be used to detect underlying molecular events in hereditary colorectal cancers, only 50% (range, 30-80%) of cases involve a detectable germline mutation in an MMR gene (15). Therefore, by restricting the definition of HNPCC to tumors containing an MMR germline mutation, only about 2.5% of colorectal cancers are thought to be caused by HNPCC. Thus, the carcinogenesis pathways and molecular events in familial cancers with no detectable germline mutations and/or not fulfilling HNPCC criteria, remain to be determined.

In this study, familial colorectal cancer was defined broadly to include colorectal cancer patients who have at least one first-degree relative with colorectal cancer. Although these cancers may have a familial tendency clinically, their heredity has not been determined yet. Molecular profiles, including promoter methylation, have not been thoroughly examined in these types of tumors. To broaden our understanding of carcinogenesis in these colorectal cancers, and to determine the effect of methylation in familial colon tumors not fulfilling the Amsterdam criteria, we assayed the methylation status of the promoter region of several tumor-related genes.

MATERIALS AND METHODS

Characteristics of patients and specimens

We enrolled 25 colorectal cancer patients with a family history of colorectal cancer, defined as at least one first-degree relative with colorectal cancer, but who did not meet the strict Amsterdam criteria I and II for HNPCC. None of these patients was known to have germline mutations in *hMLH1* and *hMSH2*, indicative of HNPCC in the previous study (16). Family history was obtained from answers to a questionnaire and from an interview with a physician at the colorectal cancer clinic of Asan Medical Center. Patients with a vague family history and those with polyposis were excluded. As a control group, we enrolled 30 patients with sporadic colorectal cancers and no family history of colon tumors, including adenomas and HNPCC-related tumors in first- and second-degree relatives. This study was approved by the institutional review board of the hospital, and written informed consent was obtained from all the participants prior to the study entry.

The colorectal cancers with family history tended to be located in colon rather than in rectum, and there was no difference in other clinicopathologic variables between two groups (Table 1). All 55 tumors were confirmed as adenocarcinomas upon histologic examination. After resection, tumor and non-neoplastic colonic mucosa (mucosa) were obtained and stored at -80°C until DNA extraction.

Sodium bisulphite modification and methylation-specific PCR (MSP)

MSP distinguishes unmethylated from methylated alleles based on sequence alterations produced by treatment of DNA with bisulphite, which converts unmethylated, but not methylated, cytosine to uracil, followed by polymerase chain reaction (PCR) using primers specific to methylated or unmethylated DNA (17). The methylation status of 6 genes was analysed by MSP: *COX2*, *MGMT*, *hMLH1*, *TIMP3*, *p16*, and *MINT2*. One microgram of genomic DNA was denatured

Table 1. Clinicopathologic features of patients with a family history of colorectal cancers and those with sporadic colorectal cancers

Variables	Familial CRC	Sporadic CRC	*p* value
Sex			
Male	15	16	0.62
Female	10	14	
Age (yr)			
≤60	13	14	0.69
>60	12	16	
Location			
Colon	16	8	0.007
Rectum	9	22	
Size (cm)			
≤5	10	21	0.03
>5	15	9	
T			
1	1	1	0.85
2	5	8	
3	19	21	
N			
0	18	15	0.17
1-2	7	15	
Serum CEA (ng/mL)			
≤6	16	22	0.46
>6	9	8	
Differentiation			
WD, MD	22	28	0.51
PD, MUC	3	2	
Total	25	30	

CRC, colorectal cancer; CEA, carcinoembryonic antigen; WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated; MUC, mucinous.

with NaOH and modified with sodium bisulphite. The DNA samples were purified using Wizard DNA purification resin (Promega, Madison, WI, U.S.A.) and resuspended in ddH_2O. PCR was performed using a mixture containing 10 × PCR buffer (16.6 mM ammonium sulphate, 67 mM Tris (pH 8.8), 6.7 mM $MgCl_2$, and 10 mM 2-mercaptoethanol), 1.25 mM of each dNTP, 300 ng of each primer, and bisulphite-modified DNA (50 ng) in a final volume of 50 μL. Following a hot start at 95°C for 5 min, 1.25 units of *Taq* polymerase (Promega) were added, and amplification was carried out in a Hybaid OmniGene temperature cycler (Hybaid, Middlesex, United Kingdom) using previously reported primer sequences and PCR conditions (18). Control PCR reactions lacking genomic DNA were performed for each set of reactions. Ten microliters of each PCR reaction product were electrophoresed on nondenaturing 6% polyacrylamide gels, which were stained with ethidium bromide and visualized under UV illumination (Fig. 1). Bisulphite genomic sequencing of representative MSP samples for each gene was performed, validating the adequacy of the bisulphite modification and indicating that all of the cytosines at non-CpG sites were converted to thymines. All of the sequenced MSP products showed extensive methylation of CpG sites within the primer sequences.

Grouping of adenomas and carcinomas by promoter methylation status

Tumors were classified as methylation-resistant (MR) if fewer than two loci were methylated or methylation-prone (MP) if two or more loci were methylated. Each tumor and group were represented by a methylation index (number of loci methylated/number of loci evaluated).

MSI

MSI status was determined by PCR using primers to amplify the five microsatellite markers recommended by the National Cancer Institute (Bethesda, MD., U.S.A.), i.e., *BAT25*, *BAT26*, *D17S250*, *D5S346*, and *D2S123* (13). Denaturation of the PCR products, gel electrophoresis, and silver staining were performed as described. MSI was scored as positive when there was a definite shift of PCR product in tumor DNA compared with normal mucosal DNA. All MSI-positive loci were confirmed on duplicate examinations. Tumors with MSI in at least two loci were classified as high-frequency MSI (MSI-H), tumors with MSI at one locus were classified as low frequency MSI (MSI-L), and tumors with MSI at no locus were classified as microsatellite stable (MSS).

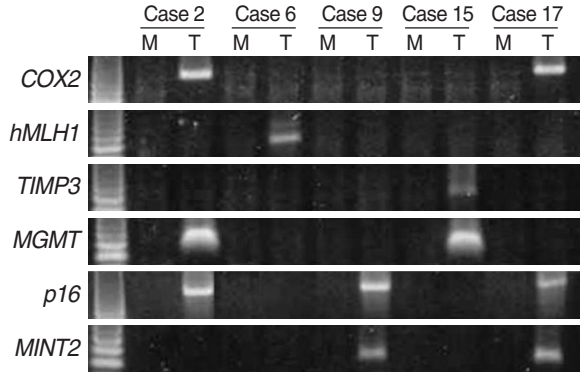

Fig. 1. Methylation status of *COX2*, *MGMT*, *hMLH1*, *TIMP3*, *p16*, and *MINT2* using methylation-specific polymerase chain reaction (MSP) in sporadic colorectal cancers. The samples examined are indicated above each gel. M and T indicate normal mucosa and tumor tissue, respectively.

Statistical analysis

The interactions between methylation and clinicopathologic parameters in two groups were evaluated with chi-square tests and Fisher's exact tests. All *p* values were two-sided, and a *p* value less than 0.05 was considered statistically significant. Calculations were performed using the SPSS program (Version 12.0, Chicago, IL, U.S.A.).

RESULTS

Methylation in colorectal cancer patients with family history

We determined CpG island methylation at six loci and MSI in paired normal mucosa and tumor tissue from 25 colorectal cancer patients with family history. Of the 25 tumors, 16 (64.0%) showed promoter methylation of at least one gene, ranging to four genes. Seven tumors showed methylation at one gene, seven at two genes, one at three genes, and one at four genes. In contrast, of the 25 samples of normal colonic mucosa, 12 (48.0%) showed promoter methylation: eight samples at one gene, and four samples at two genes. When patients with a family history were categorized as having 0-1 (MR group) or ≥ 2 (MP group) methylated loci, we found that 36.0% of the tumors and 16.0% of the normal mucosa samples could be categorized as MP. The mean methylation index (the number of methylated loci divided by the total number of tested loci) was 0.11 (0-0.33) in normal mucosa and 0.19 (0-0.66) in tumors (p=0.078). A high proportion of tumors (44.0%) were methylated at the *MGMT* locus, whereas 20.0%, 20.0%, and 16.0% were methylated at the *COX2*, *hMLH1*, and *MINT2* loci, respectively. In contrast, only 8.0% of *p16* loci and 4.0% of *TIMP3* loci were methylated (Fig. 2). We found that the *COX2*, *MGMT,* and *p16* loci were methylated at 28.0%, 20.0%, and 16.0%, respectively, of normal mucosa samples of patients with a family history, whereas the *hMLH1*, *TIMP3*, and *MINT2* loci were not methylated in any of the normal mucosa samples. The frequency of methylation of the *COX2* and *p16* loci was higher in normal mucosa than in tumor, but the differ-

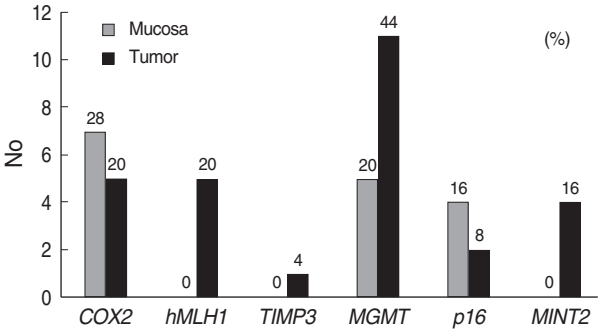

Fig. 2. Methylation status of *COX2*, *MGMT*, *hMLH1*, *TIMP3*, *p16*, and *MINT2* in normal mucosa and tumor tissues of patients with a family history of colorectal cancer. In tumors, the locus most frequently methylated was *MGMT* (44% of cases).

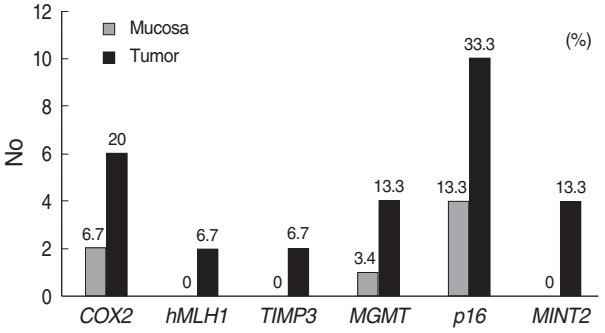

Fig. 3. Methylation status of *COX2, MGMT*, *hMLH1*, *TIMP3*, *p16*, and *MINT2* in normal mucosa and tumor tissue of patients with sporadic colorectal cancer. In tumors, the locus most frequently methylated was *p16* (33.3% of cases).

ence did not reach a statistical significance.

Methylation in sporadic colorectal cancer patients

We also assayed CpG island methylation at these six loci and MSI in paired tumors and normal mucosa from 30 patients with sporadic colorectal cancer. We found that 16 (53.0%) of the tumors showed promoter methylation of at least one gene, ranging to three genes. Seven tumors showed methylation at one locus, six at two loci, and one at three loci. In contrast, only 7 (23.0%) of the normal colonic mucosa samples showed promoter methylation, each at one gene. We found that 30.0% of the tumors, and none of the normal mucosa, could be classified as MP. The mean methylation index was 0.04 (0-0.17) in normal mucosa and 0.16 (0-0.5) in tumors (p<0.001). Methylation of the *p16* locus was observed in a high proportion (33.3%) of sporadic tumors cases, whereas methylation of the *C0X2*, *MGMT*, and *MINT2* loci were observed in 20.0%, 13.3%, and 13.3%, respectively, of these tumors. In contrast, only 6.7% of tumors were methylated at the *TIMP3* promoter (Fig. 3). Methylation of the *p16*, *C0X2*, and *MGMT* loci were observed in 13.3%, 6.7%, and 3.4%, respectively, of the normal mucosa samples, whereas we did not observe methylation of the *hMLH1*, *TIMP3*, and *MINT2* loci in any of these tissues.

Table 2. Clinicopathologic features of patients according to methyation status

Variables	MR	MP	*p* value
Sex			
Male	21	8	0.58
Female	16	10	
Age (yr)			
≤60	21	6	0.15
>60	16	12	
Location			
Colon	17	7	0.77
Rectum	20	11	
Size (cm)			
≤5	21	10	0.58
>5	10	8	
T			
1	1	1	0.29
2	11	2	
3	25	15	
N			
0	25	8	0.14
1-2	12	10	
Serum CEA (ng/mL)			
≤6	27	11	0.54
>6	10	7	
Differentiation			
WD, MD	34	16	1.00
PD, MUC	3	2	
Group			
Familial	16	9	0.74
Sporadic	21	9	
MSI			
MSI-H	4	2	1.00
MSI-L, MSS	33	16	
Total	37	18	

MR, methylation-resistant; MP, methylation-prone colorectal cancer; CEA, carcinoembryonic antigen; WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated; MUC, mucinous; MSI-H, microsatellite instability high frequency; MSI-L, microsatellite instability low frequency; MSS, microsatellite stable.

Correlation of clinicopathologic characteristics and MSP results

To clarify the clinical significance of the methylation status of individual genes or the extent of methylation of multiple CpG islands, we compared molecular and clinicopathologic features of colorectal cancer patients (Table 2). We did not detect any differences in clinicopathologic features between the MP and MR groups or between groups of patients with or without a family history of colorectal cancer. In the group of sporadic colorectal cancers, however, we found that the methylation index was higher in older patients (p<0.001) and in those with right colon tumors (p=0.041).

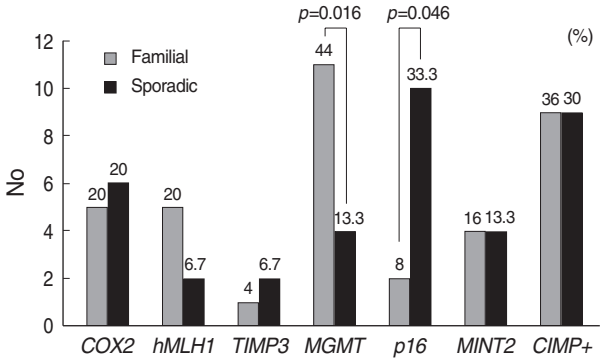

Fig. 4. Differences in methylation status of *MGMT* and *p16* in tumors of patients with a family history of colorectal cancer and in those with sporadic colorectal cancer. Compared with sporadic colorectal cancers, *MGMT* was more frequently (p=0.016) and *p16* was less frequently (p=0.046) methylated in colorectal cancers from patients with a family history.

Comparisons of methylation in patients with and without a family history of colorectal cancer

The overall frequency of methylation in tumors did not differ between patients with and without a family history of colorectal cancer (p=0.524 for methylation index; p=0.774 for MP vs. MR). When we analysed methylation of individual genes, however, we found that *MGMT* was more frequently methylated in colorectal cancers of patients with a family history (p=0.016), whereas *p16* was more frequently methylated in sporadic colorectal cancers (p=0.046) (Fig. 4). We found that the normal mucosa in patients with family history showed more frequent methylation than did normal mucosa of sporadic cancer patients (p=0.016 for methylation index; p=0.037 for MP vs. MR), but there was no difference in methylation of any of the individual genes.

MSI

Of the 25 colorectal cancers with family history, four (16.0 %) were MSI-H and five (20.0%) were MSI-L. In contrast, of the 30 colorectal cancer without family history, two (6.7 %) were MSI-H and 28 (93.3%) were MSS (MSI-H vs. MSI-L vs. MSS, p=0.014). When we divided the microsatellite status into two groups (MSI-H vs. MSI-L plus MSS), we observed no difference between colorectal cancers with and without family history. We found that the *hMLH1* gene was methylated in three of the MSI-H tumors (50.0%), three of the MSI-L tumors (60.0%), and one of the MSS tumors (2.3%). When we analysed the correlations between MSI and clinicopathologic characteristics, we found that the tumors with MSI showed right side predominance (MSI-H vs. MSI-L plus MSS; p=0.022) and were more frequently methylated at *hMLH1* (p=0.022) and *TIMP3* (p=0.029).

DISCUSSION

Aberrant methylation in promoter CpG islands of tumor suppressor genes is associated with transcriptional silencing of these genes and is thought to be an alternative mechanism in carcinogenesis (5, 6). Colorectal cancers with CpG island methylation are thought to have a distinct clinicopathologic phenotype and genetic profiles, including MSI and frequent mutations of the K-ras gene, but they lack *p53* mutations (19, 20). There is as yet no consensus about the definition of CIMP and the panel of CpG sites that should be analysed to classify tumors as CIMP. This is not trivial, given that each study uses different methods to assay DNA methylation, assays different genes, has different definitions of CIMP, and examines a different minimal number of genes. Genes related to carcinogenesis may be tissue-dependent, and the definition of CIMP for colorectal cancer might not be applicable to other cancers. CIMP has been generally defined as methylation of at least two *MINTs* or target genes such as p16, p14, or hMLH1, when a small panel of such markers is examined (7, 21, 22).

In the present study, we have assayed six markers: *MINT2*, *p16*, *TIMP3*, *hMLH1*, *MGMT*, and *COX2*. All of these have been found to be methylated mainly in cancers and are frequently methylated in gastrointestinal tumors (23, 24). Moreover, these genes have been reported to be closely related to colorectal carcinogenesis; specifically, cell-cycle regulation (*COX2* and *p16*), DNA repair or protection (*MGMT*, and *hMLH1*), and metastasis and invasion (*TIMP3*). In our opinion, this selection satisfies the minimum conditions for studying methylation in colorectal cancer (19). We categorized colorectal tumors as MP or MR to avoid confusion with other definitions of CIMP (7, 20, 25), and calculated and compared methylation indices to reduce the bias from our selection of genes.

In this study, we have focused on a subgroup of familial colorectal cancers; i.e., tumors from patients with first-degree relatives with colorectal cancers and no germline mutations in *hMLH1* and *hMSH2* (16). This group of cancers may be a mixture of heterogeneous tumors with different molecular profiles. Several mechanisms of genetic predisposition to colorectal cancer with a familial tendency have been suggested. These include defects in MMR genes other than *hMLH1* and *hMSH2*, low penetrance of polymorphisms, simple phenocopy, or technically undetectable alterations in *hMLH1* and *hMSH2* (16, 26, 27). Therefore, in interpreting the results of this study, our definition of familial cancers should be considered. That is, we enrolled and classified patients as having familial colorectal cancer, who have been known not to have a mutation in *hMLH1* and *hMSH2*, and have at least one first-degree relative without considering familial history of cancers in other organs and in second-degree relatives. Therefore, our results may not be representative of the methylation status in hereditary cancers not fulfilling the Amster-

dam criteria of HNPCC. The present study was also limited by the small number of samples studied.

Several reports have described an association between the methylation status of multiple genes and a familial tendency to colorectal cancer (15, 28). A recent large study, however, found no evidence that patients with heavily methylated colorectal cancers were more likely to develop a second malignancy or have a positive family history of cancer (29). Aberrant methylation may result from an inherited defect in the methylation apparatus. In this study, considerable proportions of cancer in both groups with or without familial history of colorectal cancer presented the methylated genes that may involved in carcinogenetic pathways. It still remains to be elucidated whether promoter methylation in multiple genes is one of main mechanism to evoke cancer or simple bystander. However, our results confirm that CpG island promoter methylation may be a universal event in sporadic or familial colorectal cancer, and is suggested as one of the mechanisms for 'second hits' by which tumor suppressor genes are inactivated. Evidence for this mechanism in familial colorectal cancers was previously reported in studies showing aberrant methylation of individual genes, including *CDH1*, *VHL,* and *hMLH1*, in hereditary cancer syndromes (30-32).

Cancer is a genetic disease. Most cancer-causing mutations are somatic, occurring in the affected tissue during the course of carcinogenesis. However, most cancers also have a hereditary component, caused by predisposing mutations that affect the germline, are heritable, contribute to the initiation of carcinogenesis, and influence the carcinogenesis pathway. Although hypermethylation is not a rare event, either in sporadic colorectal cancers or in colorectal cancers with family history, predisposing germline alterations can affect detailed aspects of methylation. Our data showed two of the genes methylated at their promoters, *p16* and *MGMT*, differ according to the tumor type. These findings suggest that the methylation of *p16*, leading to its loss of function, may be a dominant and necessary event for sporadic colorectal carcinogenesis, whereas methylation of *MGMT* may be dominant and necessary in colorectal carcinogenesis in individuals with a family history. It was reported that the frequency and pattern of gene methylation varied between HNPCC syndrome and sporadic adenomas, implying differences in the molecular pathogenesis of tumors (33). MGMT has been considered as a critical step of genetic instability and methylation of MGMT are proposed to show a distinctive phenotype of MSI-L or mild family history of colorectal cancer (34, 35). The present study also showed a possibility that different pathways to cancer may exit according to the molecular background and some subgroup of familial colorectal cancer are related to the loss of function of MGMT. Thus, genes selected for methylation-induced functional loss may differ according to the genetic background. While methylation seems to be a universal mechanism by which gene function is inactivated, the germline mutations in familial tumors confer a selective advantage for their tumorigenic growth, but other genetic and epigenetic lesions are also necessary. Genetic predisposition to CpG island methylation may be a modifying factor that contributes to the penetrance of HNPCC. Our findings thus expand these early observations on methylation in familial colorectal cancer and highlight the selective advantage of epigenetic gene silencing.

A 'field defect' is an area of abnormal tissue that predisposes to the development of cancer. The molecular basis is relatively simple to understand when it occurs in patients who have a genetic predisposition for cancer development or massive exposure to a carcinogen. Within this defective field, a second change may confer a growth advantage on a given cell relative to other cells. In colorectal cancer patients with germline genetic defects, all cells in the colonic mucosa have the same genetic alteration, leading to the frequent development of tumors in these individuals. In some of these patients, however, the selective advantage of genetic alterations is not great (36). Methylation has been proposed as a candidate mediator of this field defect and methylation status of normal mucosa was explored to verify the role of methylation as an earliest event in carcinogenetic pathway (37, 38). A recent study reported that some colorectal cancers arise from a field defect defined by epigenetic inactivation of some genes such as MGMT (38). Detection of this abnormality may useful in predict the colorectal cancer risk. Germline defects that alter methylation machinery may increase methylation frequency in normal colonic mucosa of individuals with a family history of colorectal cancer, and this may be associated with the frequent and multiple incidences of colorectal tumors in these patients. We found that methylation status of mucosa differed between in the patients of sporadic and familial colorectal cancers, although we did not detect differences in individual genes. This finding might be a small clue that field defect related to methylation has variable effects regarding selective power and involved genes for field defect is also diverse according to the specific carcinogenetic pathway. A methylation field effect in the entire whole colonic mucosa may be associated with the process of carcinogenesis in familial colorectal cancer, at least in those tumors with high methylation rates in normal mucosa.

Conclusively, alterations in the methylation machinery may also be associated with both sporadic and familial colorectal carcinogenesis, although there are qualitative and quantitative differences in methylation between sporadic and familial tumors. Our findings, however, do not support the concept that a germline defect in the methylation machinery is responsible for the development of most tumors with multiple epimutation. This epigenetic mechanism can silence different genes affected by other genetic backgrounds, leading to divergent pathways of development in hereditary and sporadic colorectal cancers.

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