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

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 (HNPPC) and familial adenomatous polyposis (FAP), whereas the vast majority show non-syndromic 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 HNPPC, have a more than 2-fold increased risk of developing tumors of the large intestine (12).

HNPPC 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                        | 18           | 15           | 0.17    |
| 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 ddH2O. PCR was performed using a mixture containing 10 × PCR buffer (16.6 mM ammonium sulphate, 67 mM Tris (pH 8.8), 6.7 mM MgCl2, 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

|                | Case 2 | Case 6 | Case 9 | Case 15 | Case 17 |
|----------------|--------|--------|--------|---------|---------|
| COX2           | M      | T      | M      | M       | M       |
| hMLH1          | M      | T      | M      | T       | T       |
| TIMP3          | M      | T      | M      | T       | T       |
| MGMT           | M      | T      | M      | T       | T       |
| p16            | M      | T      | M      | T       | T       |
| MINT2          | M      | T      | M      | T       | T       |

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.

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-

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.).
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 COX2, 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, COX2, 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.

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).
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 \(hMLH1\) 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 \(MINT\) 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: \(MINT2\), \(p16\), \(TIMP3\), \(hMLH1\), \(hMLH1\), 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-
CIMP in Familial Colorectal Cancer

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 carcinogenic 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 carcinogenic pathway. Although hypermethylation is not a rare event, either in sporadic colorectal cancers or in colorectal cancers with familial 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 exist 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.
REFERENCES

1. Baylin SB. Tying it all together: epigenetics, genetics, cell cycle, and cancer. Science 1997; 277: 1948-9.

2. Tomlinson I, Bodmer W. Selection, the mutation rate and cancer: ensuring that the tail does not wag the dog. Nat Med 1999; 5: 11-2.

3. Jackson AL, Loeb LA. The mutation rate and cancer. Genetics 1998; 148: 1483-90.

4. Feinberg AP, Tycko B. The history of cancer epigenetics. Nat Rev Cancer 2004; 4: 143-53.

5. Jones PA. DNA methylation and cancer. Oncogene 2002; 21: 5358-60.

6. Issa JP, Baylin SB. Epigenetics and human disease. Nat Med 1996; 2: 281-2.

7. Issa JP. CpG island methylator phenotype in cancer. Nat Rev Cancer 2004; 4: 988-93.

8. Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. Int J Cancer 2001; 94: 153-6.

9. McGrath DR, Spiegelman AD. Hereditary colorectal cancer: keeping it in the family—the bowel cancer story. Intern Med J 2002; 32: 325-30.

10. Salovaara R, Loukola A, Kristo P, Kaaraiainen H, Ahtola H, Eskelinen M, Harkonen N, Julkunen R, Tulikoura J, Valkamo E, Jarvinen H, Mecklin J, Aaltonen LA, de la Chapelle A. Population-based molecular detection of hereditary nonpolyposis colorectal cancer. J Clin Oncol 2000; 18: 2193-200.

11. Olsson L, Lindblom A. Family history of colorectal cancer in a Swedish county. Fam Cancer 2003; 2: 87-93.

12. Johns LE, Houlston RS. A systematic review and meta-analysis of familial colorectal cancer risk. Am J Gastroenterol 2001; 96: 2992-3003.

13. Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, Meltzer SJ, Rodriguez-Bigas MA, Fodde R, Ranzani GN, Srivastava A. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res 1998; 58: 5248-57.

14. Umar A, Risinger JJ, Hawt ET, Barrett JC. Testing guidelines for hereditary non-polyposis colorectal cancer. Nat Rev Cancer 2004; 4: 153-8.

15. Frazier ML, Xi L, Zong J, Viscofsky N, Rashid A, Wu EF, Lynch PM, Amos CI, Issa JP. Association of the CpG island methylator phenotype with family history of cancer in patients with colorectal cancer. Cancer Res 2003; 63: 4905-8.

16. Kim JC, Lee KH, Koo KH, Roh SA, Kim HC, Yu CS, Kim TW, Chang HM, Gong GY, Kim JS. Characterization of mutator phenotype in familial colorectal cancer patients not fulfilling Amsterdam criteria. Clin Cancer Res 2004; 10: 6159-68.

17. Herman JG, Graff JR, Myohuanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 1996; 93: 9821-6.

18. Kang GH, Lee S, Kim WH, Lee HW, Kim JC, Ryu MG, Ro JY. Epstein-barr virus-positive gastric carcinoma demonstrates frequent aberrant methylation of multiple genes and constitutes CpG island methylator phenotype-positive gastric carcinoma. Am J Pathol 2002; 160: 787-94.

19. Toyota M, Ohe-Toyota M, Ahuja N, Issa JP. Distinct genetic profiles in colorectal tumors with or without the CpG island methylator phenotype. Proc Natl Acad Sci USA 2000; 97: 710-5.

20. Park SJ, Rashid A, Lee JH, Kim SG, Hamilton SR, Wu TT. Frequent CpG island methylation in serrated adenomas of the colorectum. Am J Pathol 2003; 162: 815-22.

21. Jass JR, Whitehall VL, Young J, Leggett BA. Emerging concepts in colorectal neoplasia. Gastroenterology 2002; 123: 862-76.

22. Rashid A, Issa JP. CpG island methylation in gastroenterologic neoplasia: a maturing field. Gastroenterology 2004; 127: 1578-88.

23. Laird PW. The power and the promise of DNA methylation markers. Nat Rev Cancer 2003; 3: 253-66.

24. Jubb AM, Bell SM, Quirke P. Methylation and colorectal cancer. J Pathol 2001; 195: 111-34.

25. Rashid A, Shen L, Morris JS, Issa JP, Hamilton SR. CpG island methylation in colorectal adenomas. Am J Pathol 2001; 159: 1129-35.

26. Fearnhead NS, Wilding JL, Winney B, Tonks S, Bartlett S, Bicknell DC, Tomlinson IP, Mortensen NJ, Bodmer WF. Multiple rare variants in different genes account for multifactorial inherited susceptibility to colorectal adenomas. Proc Natl Acad Sci USA 2004; 101: 15992-7.

27. de la Chapelle A. Genetic predisposition to colorectal cancer. Nat Rev Cancer 2004; 4: 709-80.

28. Esteller M, Fraga MF, Guo M, Garcia-Foncillas J, Hedenfalk I, Godwin AK, Trojan J, Vaurs-Barriere C, Bignon YJ, Raman S, Benitez J, Caldes T, Akiyama Y, Yuasa Y, Launonen V, Canal MJ, Rodriguez R, Capella G, Peinado MA, Borg A, Aaltonen LA, Ponder BA, Baylin SB, Herman JG. DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. Hum Mol Genet 2001; 10: 3001-7.

29. Ward RL, Williams R, Law M, Hawkins NJ. The CpG island methylator phenotype is not associated with a personal or family history of cancer. Cancer Res 2004; 64: 7618-21.

30. Wheeler JM, Loukola A, Aaltonen LA, Mortensen NJ, Bodmer WF. The role of hypermethylation of the hMLH1 promoter region in HNPCC versus MSI+ sporadic colorectal cancers. J Med Genet 2000; 37: 585-92.

31. Grady WM, Willis J, Guilford PJ, Dunbier AK, Toro TT, Lynch H, Wiesner G, Ferguson K, Eng C, Park JG, Kim SJ, Markowitz S. Methylation of the CDH1 promoter as the second genetic hit in hereditary diffuse gastric cancer. Nat Genet 2000; 26: 16-7.

32. Prowse AH, Webster AR, Richards FM, Richard S, Olschwang S, Resche F, Affara NA, Maher ER. Somatic inactivation of the VHL gene in Von Hippel-Lindau disease tumors. Am J Hum Genet 1997; 60: 765-71.

33. Kaz A, Kim Y, Dzieciatkowski S, Lynch H, Watson P, Washington MK, Lin L, Grady WM. Evidence for the role of aberrant DNA methylation in the pathogenesis of Lynch syndrome adenomas. Int J Cancer 2007; 120: 1922-9.

34. Jass JR. Serrated adenoma of the colorectum and the DNA-methylator phenotype. Nat Clin Pract Oncol 2005; 2: 398-405.

35. Jass JR, Whitehall VL, Young J, Leggett BA. Emerging concepts in colorectal neoplasia. Gastroenterology 2002; 123: 862-76.
36. Bodmer W. *The somatic evolution of cancer. The Harveian Oration of 1996.* *J R Coll Physicians Lond* 1997; 31: 82-9.

37. Ye C, Shrubsole MJ, Cai Q, Ness R, Grady WM, Smalley W, Cai H, Washington K, Zheng W. *Promoter methylation status of the MGMT, hMLH1, and CDKN2A/p16 genes in non-neoplastic mucosa of patients with and without colorectal adenomas.* *Oncol Rep* 2006; 16: 429-35.

38. Shen L, Kondo Y, Rosner GL, Xiao L, Hernandez NS, Vilaythong J, Houlihan PS, Krouse RS, Prasad AR, Einspahr JG, Buckmeier J, Alberts DS, Hamilton SR, Issa JP. *MGMT promoter methylation and field defect in sporadic colorectal cancer.* *J Natl Cancer Inst* 2005; 97: 1330-8.