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Short Communication

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Short Communication

Relationship between smoking and multiple colorectal cancers in patients with Japanese Lynch syndrome: a cross-sectional study conducted by the Japanese Society for Cancer of the Colon and Rectum

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Abstract

The positive correlation between smoking and cancer risk is well estimated in sporadic colorectal cancer, whereas little is known with regard to Lynch syndrome-associated colorectal cancer. A total of 118 familial colorectal cancer patients from the Hereditary Nonpolyposis Colorectal Cancer Registry and Genetic Testing Project of the Japanese Society for Cancer of the Colon and Rectum, were assessed to determine whether smoking alters the incidence of multiple colorectal cancers. In male patients with Lynch syndrome ($n = 29$), the incidence of multiple colorectal cancers in patients who had ever smoked (smoking duration: median of 19 years) was higher than that in those who never smoked (58.8% vs. 10.0%, $P = 0.02$). The cumulative risk for metachronous colorectal cancer was significantly higher in male Lynch syndrome patients who had previously smoked than in those who had never smoked ($P = 0.03$). Our data suggest that long-term cigarette smoking might be a strong risk factor for the development of multiple colorectal cancers in male Lynch syndrome patients.

Key words: Lynch syndrome, smoking, colorectal cancer, multiple cancer, male

Introduction

Lynch syndrome is among the most common hereditary cancer syndromes, and is caused by the germline mutation of one of the mismatch repair genes, including *MLH1*, *MSH2*, *MSH6* or *PMS2* (1,2). This syndrome accounts for ~1–4% of all colorectal cancer (CRC) patients, and increases the risk of developing CRC, as well as of extra-colonic cancers, including endometrial, ovarian and stomach cancers (1,2). The tumor spectrum of Lynch syndrome has changed over time. The spectrum observed in the first Lynch syndrome family reported by Warthin (3) included mainly gastric cancers and endometrial cancer. However, follow-up reports of this family showed that in current generations, CRC is now the most common tumor. This change suggests that the cancer spectrum of Lynch syndrome is influenced by environmental factors (2). In sporadic CRC, environmental factors such as dietary and lifestyle habits, smoking, alcohol consumption and obesity interact with a host's genetic factors, and may modulate the risk of CRC (4,5). However, there have been only limited reports on the association between smoking and CRC development in patients with Lynch syndrome (6–8). Our objective was to evaluate whether cigarette smoking would alter the age of first CRC onset, incidence of multiple CRCs or occurrence of extra-colonic cancers in individuals with Lynch syndrome.

Patients and methods

This nationwide Japanese study was conducted by the Hereditary Nonpolyposis Colorectal Cancer Registry and Genetic Testing Project of the Japanese Society for Cancer of the Colon and Rectum (9) between September 2002 and July 2010. To determine the patient eligibility, a modified version of the Amsterdam II criteria was used, in which gastric cancer was included as an extra-colonic cancer. All participants provided written informed consent. Clinical information, such as the personal and family cancer history, smoking habits, alcohol use, body height and body weight, were collected either from medical records or directly from patients who were provided genetic counseling. The Brinkman index was calculated as the product of the number of cigarettes smoked per day times the number of years of smoking (9). The body mass index [BMI, (weight, kg)/(height, m^2)] was calculated from the height and weight. Participants with a BMI $\geq$ 25.0 kg/m^2 were classified as overweight.

Mutation analyses were performed by direct sequencing of the entire coding region in the *MLH1*, *MSH2* and *MSH6* genes. Large deletions/duplications in the *MLH1* and *MSH2* genes were also investigated by multiplex ligation-dependent probe amplification. Participants were stratified into four groups based on gender and the genetic testing results. For each group, we assessed whether smoking altered the age of first CRC onset, incidence of multiple CRCs or the occurrence of extra-colonic cancers. Metachronous CRC was defined as primary CRC diagnosed >12 months after the first diagnosis of primary colon cancer.

The data were calculated as totals, medians (range) or percentages. Fisher's exact test, Mann–Whitney *U*-test and the log-rank test were used for the statistical analyses, as appropriate. The statistical analyses were carried out using JMP 10.0.2 (SAS Institute, Cary, NC); a value of $P < 0.05$ was considered to be statistically significant.

Results

A total of 125 CRC patients were eligible to enter the study, and clinical information was available for review in 118 of these cases. Of the 118 CRC patients, 29 (24.6%) were male Lynch syndrome patients, 34 (28.8%) were female Lynch syndrome patients, 27 (22.9%) were male familial CRC cases [negative for mismatch repair (MMR) genetic testing] and 28 (23.7%) were female familial CRC cases. The male to female ratio of these 118 patients was about three times higher in those who had ever smoked than in those who had never smoked. When the male and female LS patients were analyzed together, there was no significant difference in the incidence of multiple CRCs between patients who had a history of smoking ($n = 25$) and those who had never smoked (44% vs. 29%, $P = 0.22$). When the male and female LS were analyzed separately, the incidence of multiple CRCs in patients who had a history of smoking tended to be lower (17% vs. 57%, $P = 0.15$), and the rate of smoking was significantly lower (18% vs. 66%, $P < 0.01$) in female LS patients than in male LS patients. In addition, the median Brinkman Index of female LS patients who had ever smoked was approximately one-third of that seen in the male LS patients who had ever smoked (170 vs. 550, $P < 0.01$). Thus, we proceeded with a further analysis, focusing on the relationship between multiple CRCs and smoking in the male LS patients.

In the male Lynch syndrome patients, no significant differences were observed in the mismatch repair mutation (*MLH1* in 11 patients and *MSH2* in 18 patients) ($P = 0.33$), age at last follow-up ($P > 0.99$), follow-up period from the first CRC ($P > 0.99$), alcohol consumption ($P = 0.47$) and BMI ($P > 0.99$) between those who had ever smoked ($n = 18$) and those who had never smoked ($n = 9$). The age at which smokers started smoking was 20.0 year (18–30), the years of smoking was 19 (1–44) and the Brinkman index was 550 (75–2300). Among the 19 who had smoked, 57.9% were current smokers and 42.1% were former smokers. The incidence of multiple CRCs in male Lynch syndrome patients who had smoked was 58.8%, compared with 10% in those who had never smoked. Smoking significantly increased the risk ($P = 0.02$) of male Lynch syndrome patients developing multiple CRCs (Table 1). The Brinkman index was applied with a

Table 1. The background characteristics and the cancer risk of the male patients with Lynch syndrome according to the smoking status

	Ever smoked ($n = 19$)	Never smoked ($n = 10$)	*P* value
Mismatch repair gene mutation (*MLH1/MSH2/MSH6*)	6/13/0	5/5/0	0.43
Age at the last follow-up (years)	56 (27–78)	47 (28–74)	>0.99
Follow-up period from first CRC (years)	13.3 (0.2–28)	6.5 (0.3–22.4)	>0.99
Consumption of alcohol ≧3 days/week	10/18 (55.6%)	4/10 (40.0%)	0.47
BMI (kg/m^2) ≧ 25.0	5/17 (29.4%)	2/9 (22.2%)	>0.99
Age at first CRC (years)	42 (24–70)	34.5 (25–66)	0.69
Multiple CRC	10/17 (58.8%)	1/10 (10.0%)	0.02
Metac/Sync/Metac + Sync	8/1/1	0/1/0	
Extra-colonic cancer	5/18 (27.7%)	4/10 (40.0%)	>0.99

CRC, colorectal cancer; BMI, body mass index; Sync, synchronous; Metac, metachronous.

cut-off of ≥400, which resulted in an area under the receiver operating characteristic *curve* of 0.84, and identified multiple CRCs with 81.8% sensitivity and 76.5% specificity. A Kaplan–Meier plot of the metachronous CRC-free rates showed that the cumulative risk for metachronous CRC was significantly higher in male Lynch syndrome patients who had previously smoked than in those who had never smoked ($P = 0.03$, log rank) (Fig. 1).

We observed no significant positive trend in multiple CRC risk with an increasing duration of smoking or an increasing amount smoked. Smoking was not associated with either the age at first CRC onset ($P = 0.69$) or the occurrence of extra-colonic cancers ($P > 0.99$) in male Lynch syndrome patients. The observed extra-colonic cancers in male Lynch syndrome patients included three gastric cancers, one renal pelvis cancer, one urinary bladder cancer and one prostate cancer in five patients with a history of smoking, whereas two gastric cancers, one duodenal cancer, one urinary bladder cancer and one thigh liposarcoma were observed in four patients who had no history of smoking. In the other groups, smoking was not associated with the age at the first CRC onset, the incidence of multiple CRCs, or the occurrence of extra-colonic cancers.

Discussion

Colorectal cancer is one of the most common cancers in Western countries, and its incidence has increased recently in Eastern Asia, especially in Japan, as it has become westernized over the past few decades (10). In fact, the high incidence in Japanese immigrants to Hawaii suggests that the changes in environmental factors, including the westernization of dietary habits and lifestyle, may contribute to this increase (4). Moreover, according to the Asia Pacific Cohort Studies Collaboration, changes in lifestyle, such as smoking, the BMI and a lack of physical activity, increase the risk of CRC (5). Many studies have reported a 20–60% increase in the risk of CRC associated with smoking (11). Cigarette smoke contains numerous carcinogens and toxicants. A strong relationship between tobacco smoking, carcinogen–DNA adduct formation, smoke exposure and cancer risk has been demonstrated.

Individuals with Lynch syndrome have a very high risk of developing CRC (25–70%) and endometrial cancer (30–70%), and an increased risk of developing other tumors (12). The main clinical features are an early age of onset and the occurrence of multiple tumors. For Lynch syndrome patients who undergo segmental resection of their initial cancer, the cumulative risk of metachronous CRC is 16% at 10 years, 41% at 20 years and up to 62% after 30 years (13). Thus, in patients with Lynch syndrome, managing the risk of developing CRC is very important.

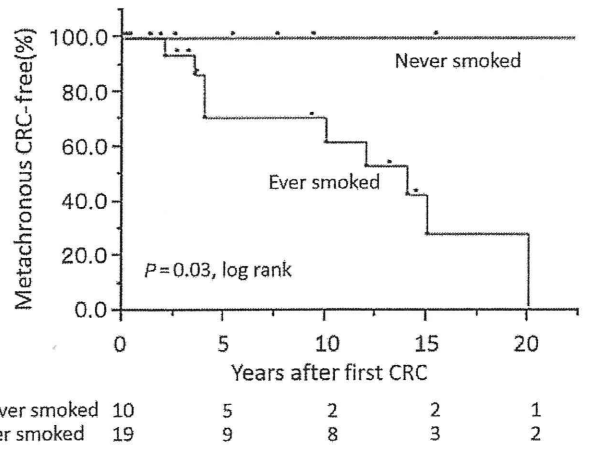

Figure 1. The Kaplan–Meier plots of the metachronous colorectal cancer-free rates by smoking status in male patients with Lynch syndrome. CRC, colorectal cancer.

There have been many studies performed to determine which environmental and lifestyle factors influence the development of CRC in patients with Lynch syndrome. Two studies demonstrated that a higher BMI was associated with an increased risk of colorectal neoplasia (12). Alcohol was not found to be associated with the risk, while fruit and fiber intake were found to be possibly related to decreased risk (12). Concerning smoking, two studies (one retrospective cohort (6) and another case–control (8) study) showed a significant association between smoking and an increased risk of developing CRC in patients with Lynch syndrome. The retrospective study (6) showed that, for former smokers, the CRC risk decreased as the number of years since quitting increased ($P < 0.01$).

Our data suggest that long-term smoking (median of 19 years) significantly increased the risk of developing multiple CRCs in male patients with Lynch syndrome. We believe that intensive colorectal surveillance and smoking cessation are very important in patients with Lynch syndrome, especially in males. One limitation is the small sample size and lack of standardization for the surveillance program. Besides smoking, we tried to identify any other risk factors for developing CRC. However, we could not identify any significant association between alcohol consumption, BMI, MMR gene mutation and multiple CRC risk. The other limitation was a lack of sufficient data regarding the effect of smoking on the development of multiple CRCs in female LS syndrome patients, because the smoking habits of females are generally different from those of males in Japan, which also seems to be applicable to female LS patients. The relationship between smoking and multiple CRCs in female LS patients is considered to be important, but further investigations with larger series are needed.

The present results obtained from male LS syndrome patients also need further confirmation, preferably in prospective studies. However, smoking is also a major cause of many other deadly health problems, such as heart disease, aneurysms, bronchitis, emphysema and stroke. Although inconclusive data are available about reducing the risk of developing multiple CRCs at this time, the recommendation of smoking cessation is a reasonable option for all patients, including males with Lynch syndrome, as it can help prevent the development of various conditions.

Conclusions

Our data suggest that cigarette smoking might be a strong risk factor for the development of multiple CRCs after long-term exposure in male patients with Lynch syndrome.

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Conflict of interest statement

None declared.

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Appendix

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BMC Research Notes

RESEARCH ARTICLE Open Access

Haplotype defined by the *MLH1*-93G/A polymorphism is associated with *MLH1* promoter hypermethylation in sporadic colorectal cancers

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Abstract

Background: Methylation of the *MLH1* promoter region has been suggested to be a major mechanism of gene inactivation in sporadic microsatellite instability-positive (MSI-H) colorectal cancers (CRCs). Recently, single-nucleotide polymorphism (SNP) in the *MLH1* promoter region (*MLH1*-93G/A; rs1800734) has been proposed to be associated with *MLH1* promoter methylation, loss of MLH1 protein expression and MSI-H tumors. We examined the association of *MLH1*-93G/A and six other SNPs surrounding *MLH1*-93G/A with the methylation status in 210 consecutive sporadic CRCs in Japanese patients.

Methods: Methylation of the *MLH1* promoter region was evaluated by Na-bisulfite polymerase chain reaction (PCR)/single-strand conformation polymorphism (SSCP) analysis. The genotype frequencies of SNPs located in the 54-kb region surrounding the *MLH1*-93G/A SNP were examined by SSCP analysis.

Results: Methylation of the *MLH1* promoter region was observed in 28.6% (60/210) of sporadic CRCs. The proportions of *MLH1*-93G/A genotypes A/A, A/G and G/G were 26% (n = 54), 51% (n = 108) and 23% (n = 48), respectively, and they were significantly associated with the methylation status (p = 0.01). There were no significant associations between genotype frequency of the six other SNPs and methylation status. The A-allele of *MLH1*-93G/A was more common in cases with methylation than the G-allele (p = 0.0094), especially in females (p = 0.0067). In logistic regression, the A/A genotype of the *MLH1*-93G/A SNP was shown to be the most significant risk factor for methylation of the *MLH1* promoter region (odds ratio 2.82, p = 0.003). Furthermore, a haplotype of the A-allele of rs2276807 located −47 kb upstream from the *MLH1*-93G/A SNP and the A-allele of *MLH1*-93G/A SNP was significantly associated with *MLH1* promoter methylation.

Conclusions: These results indicate that individuals, and particularly females, carrying the A-allele at the *MLH1*-93G/A SNP, especially in association with the A-allele of rs2276807, may harbor an increased risk of methylation of the *MLH1* promoter region.

Keywords: MSI, SNP, *MLH1*, Methylation, Colorectal cancer, Haplotype

Background

Microsatellite instability (MSI) is a form of genomic instability that can be detected as changes in the length of repetitive microsatellite sequences. MSI occurs in the majority of tumors from patients with Lynch syndrome carrying germline mutations in the DNA mismatch repair (*MMR*) genes [1]. MSI also occurs in approximately 15-20% of sporadic colorectal cancers (CRCs) [1,2]. Methylation of the *MLH1* promoter region has been suggested to be a major mechanism of gene inactivation in sporadic MSI-positive CRCs [3]. Bi-allelic methylation of the *MLH1* promoter region induced transcriptional silencing in cell lines showing MSI and this epigenetic mechanism of gene inactivation is analogous to Knudson's two-hit hypothesis [4,5].

We previously reported that all CpG sites in a 1-kb region encompassing the *MLH1* promoter were methylated (designated as full methylation) in CRCs showing

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high-frequency MSI (MSI-H) [6]. Full methylation was particularly observed in proximal colon cancers in females older than 70 years old and led to the transcriptional silencing of the *MLH1* gene and the MSI-H phenotype [7]. Methylation of the *MLH1* promoter region was also observed in 20% of MSI-negative CRCs, although all of these cases displayed partial methylation (limited to the most upstream region of the *MLH1* promoter); MLH1 protein expression was maintained in such cases showing partial methylation. Thus, there is an important correlation between the methylation status of the *MLH1* promoter and the development of sporadic MSI-positive CRCs.

Single-nucleotide polymorphisms (SNPs) are the most common type of human genetic variation and occur at about every 1000 bases in the human genome. Numerous studies are now ongoing to assess their possible associations with human diseases or phenotypes [8-10]. SNPs in the promoter region have been shown to influence gene transcription [11]. G/A SNP −93 bp upstream of the translation start site was identified in the *MLH1* gene promoter region [*MLH1*-93G/A SNP (rs1800734)] [12]. The *MLH1*-93G/A SNP has recently been proposed to have an association with CRCs, ovarian and endometrial cancer [13-16]. In particular, it was shown to be associated with *MLH1* promoter methylation, loss of MLH1 protein expression and MSI-H tumors [14,16-21]. However, these results are based largely on analyses of cancers in Caucasian populations. Allelic frequencies of the *MLH1*-93G/A SNP differ between Caucasians and Japanese, and the frequency of the A-allele in Caucasians (20-23%) is lower than that in Japanese (46%) [12,16,20].

The purpose of this study was to address the association between the *MLH1*-93G/A SNP and methylation status in the *MLH1* promoter region in a series of sporadic CRCs in a Japanese population. Furthermore, we also examined the association between six other SNPs located in the 54-kb region surrounding the *MLH1*-93G/A SNP and methylation status in the *MLH1* promoter region. We report here that the *MLH1*-93G/A SNP was significantly associated with *MLH1* promoter methylation in a Japanese population. Furthermore, a haplotype comprising the A-allele of rs2276807 and the A-allele of *MLH1*-93G/A SNP showed a significant association with *MLH1* promoter methylation. This haplotype may be associated with an increased risk for methylation-positive CRCs, especially in females.

Methods

Tissue samples

Tumor tissues and corresponding normal mucosa were obtained from 210 consecutive CRC patients who underwent surgery from 1996 through 1998 at Jichi Medical University Hospital. Genetic analyses were carried out in the Oncogene Research Unit/Cancer Prevention Unit, Tochigi Cancer Center Research Institute. As all samples were derived from archived tissue samples, they were coded anonymously, prior to the analysis of methylation status and genotyping of the vicinity of the *MLH1* promoter regions, according to the Ethical Guidelines for Human Genome/Gene Analysis Research (Ministry of Education, Culture, Sports, Science and Technology, Ministry of Health, Labour and Welfare and Ministry of Economy, Trade and Industry, March 29, 2001) and the study protocol was approved by the institutional review board of each institution. Normal mucosa was obtained from the surgical margin of the resected specimen before sampling the tumor tissue to avoid contamination by tumor cells. Kindred in whom the Lynch syndrome was suspected were excluded from this study, since patients with at least one CRC among first-degree relatives were not included. Tumors were staged according to a modified version of Dukes classification [22]. DNA was extracted from fresh tumor material by a standard procedure using proteinase K digestion and subsequent phenol-chloroform extraction. Methylation status and the genotype of the *MLH1* promoter region were also examined in peripheral blood lymphocytes (PBLs) from 100 anonymous samples obtained from normal healthy donors over 50 years of age undergoing routine health checkups.

Analysis of MSI

Genomic DNA was subjected to PCR amplification at two mononucleotide microsatellite repeat loci, BAT26 and BAT25. The BAT26 locus contains a 26-repeat adenine tract and is located in intron 5 of the *MSH2* gene, whereas the BAT25 locus contains a 25-repeat thymine tract located in intron 16 of the *c-kit* oncogene. Both loci have been shown to be sensitive markers of MSI, which manifests as alteration in the size of the respective mononucleotide repeats in tumor DNA. PCR reactions were performed as described previously [6]. When either BAT26 or BAT25 showed MSI, we examined 7 microsatellite repeat loci, D2S123, D5S346, D17S250, MSH3, MSH6, TGFBR2 and BAX, according to the recommendations of the Bethesda guidelines [23]. Tumors were classified as MSI-H when ≥30% of the 9 markers showed MSI. Low-frequency MSI (MSI-L) is classified as MSI-negative.

Methylation analysis of the *MLH1* promoter region

Na-bisulfite PCR/single-strand conformation polymorphism (SSCP) (BiPS) analysis was performed as described previously [6,24]. With the adenine residue at the initiation codon numbered as +1 nt, the *MLH1* promoter (−755 to +86) was divided into five regions [region A (from −755

to −574, containing 23 CpG sites), B (from −597 to −393, 12 CpG sites), C (from −420 to −188, 16 CpG sites), D (from −286 to −53, 13 CpG sites) and E (from −73 to +86, 13 CpG sites)] and amplified with 5 sets of PCR primers. Sequences of PCR primers were as reported previously [6]. Each primer set was designed to anneal to both methylated and unmethylated DNA sequences and the amplicons could be separated by SSCP analysis depending on their methylation status (Figure 1). Amplified DNA fragments were visualized using SYBR® Gold nucleic acid gel stain (Cosmo Bio Co., Tokyo, Japan) and scanned with a Fluorescent Image Analyzer Model FLA-3000G (Fuji Photo Film Co., Tokyo, Japan). When the bands showed mobility shifts, they were cut from the gel, reamplified and directly sequenced using an ABI 310 PRISM™ sequencer (Perkin-Elmer Co., Branchburg, NJ) with a Big-Dye Terminator Cycle Sequencing Ready Reaction Kit™ (Perkin-Elmer Co., Branchburg, NJ). Methylation profiles were classified as follows: full methylation if all CpG sites in regions A to E showed methylation, partial methylation if some CpG sites, in any region, showed methylation, and no methylation if all CpG sites in any region did not show methylation. Methylation status of the *MLH1* promoter region D was also analyzed by methylation-specific PCR (MSP) and the allelic status of methylation was examined by direct sequencing of the *MLH1*-93G/A SNP in region D (Figure 1) [7].

Analysis of the genotype frequencies of the *MLH1* promoter region

The genotype frequencies of the *MLH1*-93G/A SNP were examined by SSCP analysis using an ALFexpress DNA sequencer (Pharmacia, Tokyo, Japan). Briefly, primer sequences were 5′Cy5-gacgaagagacccagcaaccc3′ (forward) and 5′tagatgctcaacggaagtgc 3′ (reverse). The 5′-terminus of the forward primer was labeled with indodicarbocyanine (Cy5) fluorescent dye. PCR reactions were performed as described previously [7]. The data were analyzed using the software package Fragment Manager™ (Pharmacia, Tokyo, Japan).

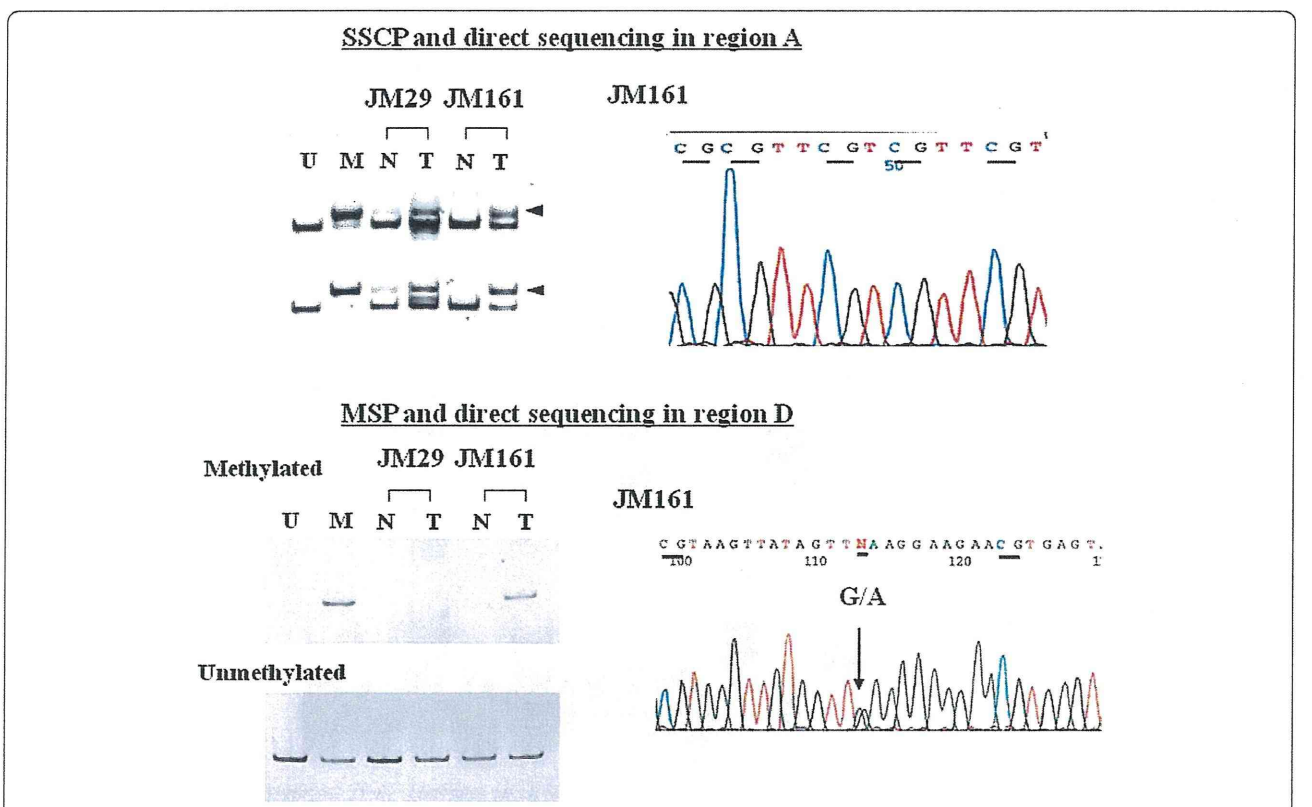

Figure 1 BiPS and MSP analysis of *MLH1* promoter region. Upper panel: BiPS analysis of *MLH1* promoter region A is shown. U indicates control unmethylated normal DNA; M indicates control methylated DNA. Cancer tissue (T) from cases JM29 and JM161 displayed a methylated band in region A. After SSCP analysis, the extra bands were cut from the electrophoresed gels, reamplified by PCR and sequenced by the dideoxy sequencing procedure. Results of the direct sequencing are shown in the right panel. Sequences (JM161) with methylated cytosines of CpG dinucleotides in region A are shown. Lower panel: MSP analysis of *MLH1* promoter region D and direct sequencing of the amplified DNA fragments. U, control unmethylated normal DNA; M, control methylated DNA. Tumor tissue (T) from case JM161 (full methylation case) also showed a methylated band in promoter region D. In contrast, tumor tissue (T) from case JM29 (partial methylation case) did not show a methylated band in the same region.

Analysis of six SNPs surrounding the *MLH1*-93G/A SNP

The genotype frequencies of the following six SNPs, rs2276807 (C/A), rs4678922 (A/T), rs6789043 (T/C), rs1046512 (C/A), rs3774343 (G/A) and rs4647215 (C/A) (Figure 2a), located in the 54-kb region surrounding the *MLH1*-93G/A SNP, were examined by SSCP analysis. The locations of the SNPs and the PCR primer sequences are described in Additional file 1: Table S1. Each PCR was carried out using 0.1 μg of genomic DNA as a template, 10×PCR buffer, 1.25 mM dNTP, 10 μM each primer and 0.625 units of *Taq* DNA polymerase in a total volume of 25 μL. The PCR conditions were as follows: heat denaturation at 95°C for 12 min, 30 cycles of PCR comprising 95°C for 30 sec, an annealing step with different temperatures, namely, 55°C (rs4678922, rs6789043, rs1046512, rs3774343), 63°C (rs2276807) or 68°C (rs4647215), for 30 sec, and an extension step at 72°C for 30 sec, followed by a final extension at 72°C for 10 min. Conditions of the SSCP analysis are described in Additional file 1: Table S1. The data were analyzed using the software package Fragment Manager™ (Pharmacia, Tokyo, Japan).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed using the Gelshift™ Kit (Active Motif, Carlsbad, CA) (Figure 2b). The sequences of ^{32}P-labeled complementary oligonucleotide pairs and complementary competitor oligonucleotide pairs corresponding to the *MLH1* promoter sequence were as follows: MLH-184 to −132: 5′-ACCCACAGAGTTGA GAAATTTGACTGGCATTCAAGCTGTCCAATCAATA GCTG-3′ [12], *MLH1*-93G: 5′-AGCTACAGCTGAAGG AAGAA-3′, and *MLH1*-93A: 5′-AGCTACAGCTAAAGG AAGAA-3′. After the reaction mixtures with and without

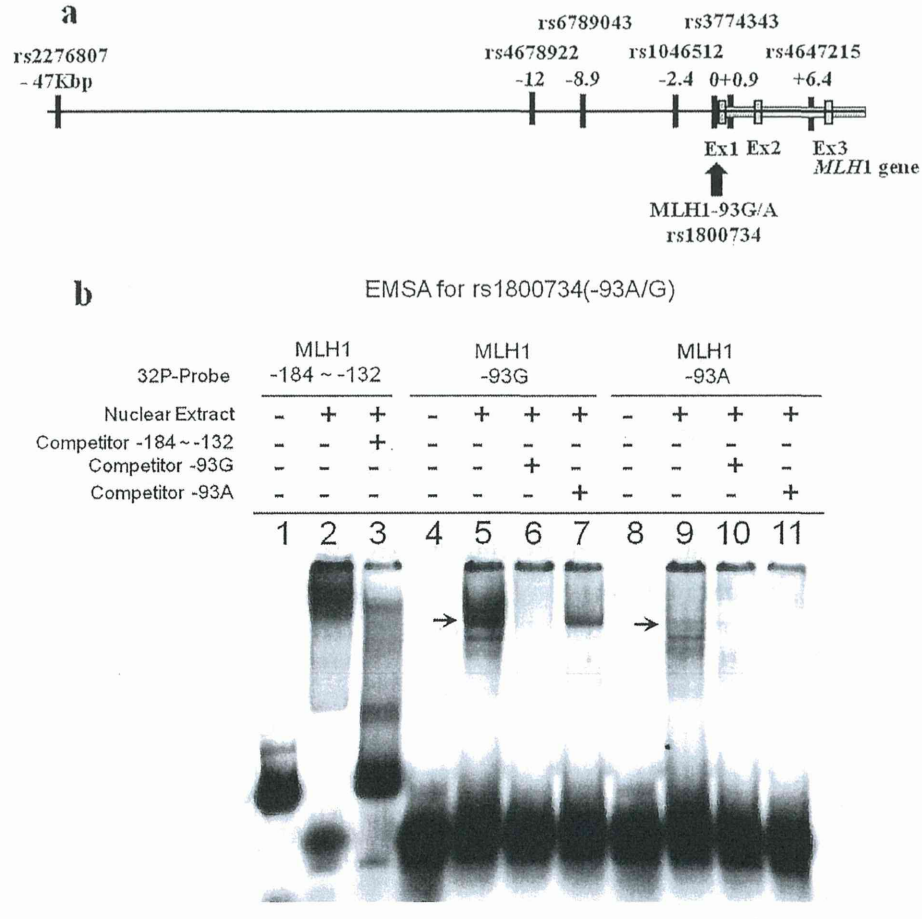

Figure 2 Map of six SNPs and EMSA for rs1800734. a. Map of six SNPs, rs2276807 (C/A), rs4678922 (A/T), rs6789043 (T/C), rs1046512 (C/A), rs3774343 (G/A) and rs4647215 (C/A), located in the 54-kb region surrounding the *MLH1*-93G/A SNP. **b**. EMSA using nuclear extracts from HeLa cells. The competition assay was performed with ^{32}P-labeled MLH1-184 to −132 and unlabeled MLH1-184 to −132 oligomer (lanes 1–3). A shifted band was observed in the presence of HeLa nuclear extract, which was blocked in the presence of excess unlabeled competitor (lanes 2 and 3). The competition assay was performed with matched sets of DNA oligomers homologous to each genotype of the *MLH1*-93G/A SNP (lanes 5–7, 9–11). When a ^{32}P-labeled MLH-93G probe was mixed with nuclear extract from HeLa cells, a shifted band was blocked by the competitor oligomer of MLH1-93G, but not MLH1-A (lanes 5–7). When ^{32}P-labeled MLH-93A probe was mixed with the nuclear extract from HeLa cells, a shifted band was not detected (lanes 9–11).

an excess of unlabeled competitor and HeLa cell nuclear extract (HeLa Nuclear Extract™; Activemotif, Carlsbad, CA) were incubated for 20 min on ice, ^{32}P-labeled oligonucleotides were added as a probe. The binding reactions were carried out according to the manufacturer's instruction manual. After the binding reaction, the DNA protein complexes were resolved by electrophoresis in a 5% non-denaturing acrylamide gel chilled at 4°C using a circulating water bath. After electrophoresis, the gel was dried and scanned with a Fluorescent Image Analyzer Model FLA-3000G (Fuji Photo Film Co., Tokyo, Japan).

Statistical analysis

Statistical analysis was carried out using Pearson's chi-square (X^2) test or Student's t-test. Multivariate logistic regression analysis was performed using Stat View 5.0 statistical package (SAS Institute Inc., Cary, NC) to identify independent variables associated with the presence of *MLH1* promoter methylation. The included variables were *MLH1*-93G/A genotype (A/A vs. A/G + G/G), tumor location (right vs. left), gender (female vs. male) and age at onset (more than 70 vs. less than 70). Absence of departure from the Hardy-Weinberg equilibrium was calculated by the asymptotic X^2 goodness of fit test. The haplotypes of the seven SNPs were analyzed using SNPAlyze software ver. 5.0 (Dynacom, Chiba, Japan) and a case–control study was performed to examine the relationship between methylation-positive and methylation-negative subjects. Permutation analysis was used to determine the empirical significance and to calculate the probability (p) values based on 10,000 replications. The global p-values represent the overall significance using the X^2-test when the observed versus expected frequencies of all haplotypes are considered together.

The individual haplotypes were tested for association by grouping all other haplotypes together and applying the X^2-test with one degree of freedom. P-values <0.05 were considered statistically significant.

Results

Comparison of clinicopathological and molecular features with methylation status of the *MLH1* promoter region

The clinicopathological background, MSI status and the association of these variables with methylation status are summarized in Table 1. The category of methylation-positive includes both full methylation (n = 13) and partial

Table 1 Association between clinicopathological features and MSI status and methylation status of the *MLH1* promoter region in sporadic colorectal cancers

	Methylation-positive				Methylation-negative	p values[4]
	Full methylation	Partial methylation	p values[3]	Total (Full + Partial methylation)	(No methylation)	
Numbers of cases	13	47		60	150	
Age at onset	72.8 ± 10.6	64.5 ± 10.1	0.012	66.3 ± 10.7	63.9 ± 11.1	NS
Gender						
Male	3	25	NS(0.054)	28	100	0.007
Female	10	22		32	50	
Tumor location[1]						
Right	13	15	<0.0001	28	39	0.004
Left	0	32		32	110	
Histological type[2]						
well	6	24	NS	30	105	0.04
mod	3	19		22	36	
muc	2	3		5	5	
por	2	1		3	4	
Dukes						
A/B	1/10	2/27	NS	3/37	8/75	NS
C/D	1/1	14/4		15/5	51/16	
Tumor size (mm)	66.4 ± 35.5	58.2 ± 26.9	NS	60.0 ± 28.9	52.2 ± 22.4	0.04
MSI-H	13	2	<0.0001	15	4	<0.0001

[1]right: cecum, ascending colon and transverse colon, left: descending colon, sigmoid colon and rectum.
[2]well: well-differentiated adenocarcinoma, mod: moderately differentiated adenocarcinoma, por: poorly differentiated adenocarcinoma, muc: mucinous adenocarcinoma.
[3]P values were analyzed using Pearson's chi-square test or Student's t-test between cases with full methylation and cases with partial methylation. NS: not significant. P values of <0.05 were statistically significant.
[4]P values were analyzed using Pearson's chi-square test or Student's t-test between methylation-positive cases (full methylation + partial methylation) and methylation-negative cases. NS: not significant. P values of <0.05 were statistically significant.