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hMLH1 promoter methylation and *BRAF* mutations in high-frequency microsatellite instability colorectal cancers not fulfilling the revised Bethesda guidelines

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Purpose: Sporadic colorectal cancers with high-frequency microsatellite instability (MSI-H) are related to hypermethylation of mismatch repair (*MMR*) genes and a higher frequency of *BRAF* mutations than Lynch syndrome. We estimated the feasibility of hereditary colorectal cancer based on *hMLH1* methylation and *BRAF* mutations.
Methods: Between May 2005 and June 2011, we enrolled all 33 analyzed patients with MSI-H cancer (male:female, 23:10; mean age, 65.5 ± 9.4 years) from a prospectively maintained database that didn't match Bethesda guidelines and who had results of *hMLH1* methylation and *BRAF* mutations.
Results: Among the 33 patients, *hMLH1* promoter methylation was observed in 36.4% (n = 12), and was not significantly related with clinicopathologic variables, including MLH1 expression. *BRAF* mutations were observed in 33.3% of the patients (n = 11). Four of 11 and five of 22 patients with MSI-H colon cancers were *BRAF* mutation (+)/ *hMLH1* promoter methylation (–) or *BRAF* mutation (–)/*hMLH1* promoter methylation (+). Of the 33 patients, 21.2% were *BRAF* mutation (+)/ *hMLH1* promoter methylation (+), indicating sporadic cancers. Seventeen patients (51.5%) were *BRAF* mutation (–)/ *hMLH1* promoter methylation (–), and suggested Lynch syndrome.
Conclusion: Patients with MSI-H colorectal cancers not fulfilling the Bethesda guidelines possibly have hereditary colorectal cancers. Adding tests of *hMLH1* promoter methylation and *BRAF* mutations can be useful to distinguish them from sporadic colorectal cancers.
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Key Words: Colorectal neoplasms, hMLH1, BRAF, Hereditary colorectal cancer

INTRODUCTION

Microsatellite instability (MSI) is one of the main colorectal carcinogenic mechanisms. MSI is associated with germ line mutations of mismatch repair (*MMR*) genes in patients with Lynch syndrome and promoter hypermethylation of MMR in patients with sporadic colorectal cancer [1]. These high-frequency MSI (MSI-H) tumors share the same distinctive clinicopathologic characteristics and have different molecular profiles. Therefore, treatment and surveillance approaches need to be specific. Lynch syndrome occurs in approximately 5% of patients with colorectal cancer [2]. Lynch syndrome has the following characteristics: 80% lifetime risk of colorectal cancer; early-onset; family history of cancers; and multiple tumors and multiorgan involvement, including the endometrium, stomach, small intestine, hepatobiliary and genitourinary tracts, and ovary [3]. Mutations in the *hMLH1* or *hMSH2* genes are the most common defects in these families, and comprise

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approximately 94% of germ line mutations in equal proportions [4,5].

The revised Bethesda guidelines (BGs) [6] are based on clinicopathologic features, rather than molecular changes, as a screening tool to select patients who need to undergo MSI analysis for detecting Lynch syndrome. Current laboratory algorithms detecting Lynch syndrome include MSI testing, immunochemistry (IHC) of MMR proteins, and germ line testing for mutations in *MMR* genes [7]. The sensitivity and specificity of BGs have been reported to be 94% and 25%, respectively [8]. As a result, a number of published studies [7,9] have approached colorectal carcinomas in terms of molecular biology and an effective strategy to detect Lynch syndrome.

BRAF encodes a cytoplasmic serine/threonine kinase, which is an essential component of the mitogen-activated protein kinase-signaling pathway [10]. Mutations in *BRAF* occur in 15% of colorectal cancers, are frequently present in sporadic colorectal cancers with methylated *hMLH1* promoters, and are rare in the Lynch syndrome [10,11]. This discrepancy may be useful to distinguish MSI-H sporadic colorectal cancers from Lynch syndrome.

In the current study, we estimated the feasibility of hereditary colorectal cancer based on *hMLH1* promoter methylation and *BRAF* mutations in MSI-H colorectal cancers not fulfilling revised BGs.

METHODS

Patients

Between May 2005 and June 2011, 1,867 patients who had available clinicopathologic data, as well as MSI status, were selected from a prospectively maintained database, analyzed, and categorized based on the revised BGs [6]. We asked about their family and operation histories through individual interviews. BGs (–) means patients do not have any BGs components (n = 958, 51.3%), and 47 patients (4.9%) had MSI-H. Among the 47 patients with BGs (–) and MSI-H colorectal cancers, 33 who had available tissue blocks were analyzed with respect to *hMLH1* promoter methylation and *BRAF* mutations. We excluded families with polyposis syndrome (familial adenomatous polyposis, Peutz-Jeghers syndrome, juvenile polyposis syndrome, and Cowden disease), hereditary nonpolyposis colorectal syndrome fulfilling the Amsterdam criteria, and inflammatory bowel disease-related cancer. The mean age was 65.5 ± 9.4 years (range, 51–84 years).

MSI-H tumors are defined as >2 mutations of the 5 microsatellite sequences in the tumor DNA [12,13]. Tumors are classified as right-sided (proximal of the splenic flexure) and left-sided (distal of the splenic flexure and rectum). This study was approved by the Institutional Review Board of Samsung Medical Center.

MSI analysis

DNA was extracted from formalin-fixed, paraffin-embedded tissues of tumor mucosa and corresponding normal mucosa by a standard procedure [14]. Areas containing >50% tumor cells were selected by microscopic evaluation on a reference H&E-stained slide. Slides (50 μm thick) were made, and if necessary, tumor cells were prepared using a scalpel. MSI status was determined by polymerase chain reaction (PCR) analysis using a DNA autosequencer (Applied Biosystems 373A sequencer, Applied Biosystems, Foster City, CA, USA). We used five microsatellite markers (BAT 25, BAT 26, D5S346, D17S250, D2S123), as recommended by the National Cancer Institute (NCI; Bethesda, MD, USA) [12].

hMLH1 promoter methylation using methylation-specific PCR

By comparing the signals from tumor-derived tissues with signals from normal tissues, we determined *hMLH1* promoter methylation. Methylationspecific PCR (MS-PCR) was used to distinguish unmethylated from methylated DNA based on sequence alterations produced by bisulfate treatment of DNA, which converted unmethylated cytosine to uracil (EZ DNA methylation kit, Zymo Research, Burlington, ON, Canada). These changes were identified by subsequent PCR using primers specific to the methylated (unchanged) or unmethylated (changed) DNA. The forward primer for the methylated *hMLH1* promoter was 5'-GATAGCGATTTTTAACGC. The reverse primer was 5'- TCTATAAATTACTAAATCTCTTCG. In unmethylated *hMLH1* promoter, the forward primer was 5'-AGAGTGGATAGTGATTTTTAATGT, and the reverse primer was 5'-ACTCTATAA ATTACTAAATCTCTTCA.

PCR reactions were performed using the primer pairs described below in the following reaction mix: 10× PCR buffer; deoxynucleotide triphosphates (each at 2.5 mM/L); primers (10 μM/L each per reaction); 0.5 unit of AmpliTaq Gold DNA polymerase (Applied Systems); and 50 ng of bisulfate-modified DNA (from paraffin-embedded tissue) in a final volume of 10 μL. PCR cycling conditions were as follows: 30 seconds at 94°C; 30 seconds at 94°C; 30 seconds at 54°C; 30 seconds at 72°C; and 10 minutes at 72°C for 40 cycles. The presence of a band in the unmethylated tumor and matched normal tissue with the absence of a methylation band in the tumor was defined as unmethylated. However, when a methylated band was present for tumor and absent for the normal tissue, we defined the sample as methylated.

We used control methylation DNA (Millpore CpGenome universal methylated DNA, Millipore Co., Billerica, MA, USA) and control unmethylated DNA (Millipore CpGenome universal unmethylated DNA set, Millipore Co.). All resections were in duplicate.

BRAF mutation analysis

The fragment encompassing exon 15 was amplified by PCR in 33 paraffin-embedded carcinoma samples. Primer sequences and PCR conditions were based on those reported previously [11]. The *BRAF* p.Val600Glu primer of exon 15 was 10 and 1 pmole (forward and reverse, respectively). PCR was performed with 10 pmole of Mut R. Genomic DNA (100 ng) was amplified by PCR using the following cycling conditions: 30 seconds at 94°C; 30 seconds at 54°C; and 30 seconds at 72°C for 40 cycles. Genomic DNA was placed in a 100v PAGE-gel running for 40–60 minutes. We used DLD-1, which is a colon cancer cell line, as a negative control. Colorectal cancer cell line (RKO) was a positive control in *BRAF*-controlled DNA. All the resections were in duplicate.

Immunohistochemistry

DNA MMR protein expression (MLH1, MSH2, and MSH6) was evaluated by IHC. IHC was performed on paraffin sections of normal and tumor tissues (4 μm thick) using mouse monoclonal antibodies specific for each MMR protein as follows: MLH1 (clone G168-15, 1:200; BD Pharmingen, San Diego, CA, USA), MSH2 (clone FE11, 1:400; Calbiochem, La Jolla, CA, USA), and MSH6 (clone 44, 1:400; BD Transduction Laboratories, San Diego, CA, USA). MMR protein expression was described as negative for absent or <10% nuclear staining, and positive for ≥10% nuclear staining. Normal colonic epithelium adjacent to the tumor and lymphocytes served as positive controls.

Statistical analysis

We used Fisher exact test to compare MSI status and BGs with clinicopathologic factors. All P-values were two-tailed and P-values <0.05 were considered statistically significant. All statistical analyses were carried out using SPSS ver. 17.0 (SPSS

Table 1. Clinicopathologic characteristics according to *hMLH1* methylations

Characteristic	Total (n = 33)	Methylations (+) (n = 12, 36.4%)	Methylations (–) (n = 21, 63.6%)	P-value
Gender				0.114
Male	23 (69.7)	6 (50.0)	17 (81.0)	
Female	10 (30.3)	6 (50.0)	4 (19.0)	
Location				0.067
Right	20 (60.6)	10 (83.3)	10 (47.6)	
Left	13 (39.4)	2 (16.7)	11 (52.4)	
Differentiation				0.065
Well	6 (18.2)	0 (0)	6 (28.6)	
Moderate	27 (81.8)	12 (100)	15 (71.4)	
Lymphatic invasion				0.686
(+)	7 (21.2)	3 (0.25)	4 (19.0)	
(–)	26 (78.8)	9 (0.75)	17 (81.0)	
Vascular invasion				0.538
(+)	3 (9.1)	2 (16.7)	1 (4.8)	
(–)	30 (90.9)	10 (83.3)	20 (95.2)	
Neural invasion				1.000
(+)	1 (3.0)	0 (0)	1 (4.8)	
(–)	32 (97.0)	12 (100)	20 (95.2)	
Tumor size(cm)				0.259
<5	10 (30.3)	2 (16.7)	8 (38.1)	
≥5	23 (69.7)	10 (83.3)	13 (61.9)	
Tumor stage				0.206
T1/T2	8 (24.2)	1 (8.3)	7 (33.3)	
T3/T4	25 (75.8)	11 (91.7)	14 (66.7)	
Node stage				0.206
N0	25 (75.8)	11 (91.7)	14 (66.7)	
N1/N2	8 (24.2)	1 (8.3)	7 (33.3)	
MLH1 expression				0.145
(+)	15 (45.5)	3 (0.3)	12 (57.1)	
(–)	18 (54.5)	9 (0.8)	9 (42.9)	
MSH2 expression				1.000
(+)	29 (87.9)	11 (91.7)	18 (85.7)	
(–)	4 (12.1)	1 (8.3)	3 (14.3)	
MSH6 expression				1.000
(+)	24 (72.7)	9 (0.8)	15 (71.4)	
(–)	9 (27.3)	3 (0.3)	6 (28.6)	

Values are presented as number (%).

Inc., Chicago, IL, USA).

RESULTS

Among 1,867 patients, MSI-H existed in 141 (7.5%). Nine hundred and nine patients (48.7%) were BGs (+). Of the 909 patients, 94 (10.3%) had MSI-H tumors. Nine hundred and fifty-eight patients (51.3%) were BGs (–), 47 (4.9%) of whom had MSI-H.

hMLH1 promoter methylation and protein expression of MLH1, MSH2, and MSH6

Among 33 patients, *hMLH1* promoter methylation existed in 36.4% (n = 12). Of the 12 patients, 3 had MLH1 expression and 9 did not express MLH1. Eleven patients expressed MSH2 and 9 patients expressed MSH6. Among 21 patients (63.7%) without *hMLH1* promoter methylation, 12 (36.4%) had expression of MLH1 and the remaining patients did not express MLH1. Eighteen patients expressed MSH2 and 15 patients expressed MSH6 (Table 1).

There was no statistical significance between *hMLH1* pro-

Table 2. Clinicopathologic characteristics according to *BRAF* mutations

Characteristic	Total (n = 33)	*BRAF* (+) (n = 11, 33.3%)	*BRAF* (–) (n = 22, 66.7%)	P-value
Gender				1.000
Male	22 (66.7)	8 (72.7)	15 (68.2)	
Female	11 (33.3)	3 (27.3)	7 (31.8)	
Location				0.714
Right	20 (60.6)	6 (54.5)	14 (63.6)	
Left	13 (39.4)	5 (45.5)	8 (36.4)	
Differentiation				0.077
Well	6 (18.2)	0 (0)	6 (27.3)	
Moderate	27 (81.8)	11 (100)	16 (72.7)	
Lymphatic invasion				0.661
(+)	7 (21.2)	3 (27.3)	4 (18.2)	
(–)	26 (78.8)	8 (72.7)	18 (81.8)	
Vascular invasion				1.000
(+)	3 (9.1)	1 (9.1)	2 (18.2)	
(–)	30 (90.9)	10 (90.9)	20 (81.8)	
Neural invasion				1.000
(+)	1 (3.0)	0 (0)	1 (4.8)	
(–)	32 (97.0)	11 (100)	21 (95.2)	
Tumor size (cm)				0.109
<5	10 (30.3)	1 (9.1)	9 (40.9)	
≥5	23 (69.7)	10 (90.9)	13 (59.1)	
Tumor stage				**0.031**
T1/T2	8 (24.2)	0 (0)	8 (36.4)	
T3/T4	25 (75.8)	11 (100)	14 (63.6)	
Node stage				0.218
N0	25 (75.8)	10 (90.9)	15 (68.2)	
N1/N2	8 (24.2)	1 (9.1)	7 (31.2)	
Family history				1.000
(+)	1 (3.0)	1 (9.1)	0 (0)	
(–)	32 (97.0)	10 (90.9)	22 (100)	
MLH1 expression				0.712
(+)	15 (45.5)	4 (36.4)	11 (50)	
(–)	18 (54.5)	7 (63.6)	11 (50)	
MSH2 expression				0.276
(+)	29 (87.9)	11 (100)	18 (81.8)	
(–)	4 (12.1)	0 (0)	4 (18.2)	
MSH6 expression				0.681
(+)	24 (72.3)	9 (81.8)	15 (68.2)	
(–)	9 (27.7)	2 (18.2)	7 (31.8)	
hMLH1 methylation				0.052
(+)	12 (48.5)	7 (63.6)	5 (22.7)	
(–)	21 (51.5)	4 (36.4)	17 (77.3)	

Values are presented as number (%).

moter methylation and clinicopathologic variables (Table 1). Fifteen patients (45.5%) had MLH1 expression, but did not demonstrate a relationship between clinicopathologic features and MLH1 protein expression. Even if there was no significant difference, *hMLH1* promoter methylation was more frequent with respect to loss of MLH1 (Table 1).

BRAF mutations and *hMLH1* promoter methylation

We identified 11 patients who had *BRAF* mutations (33.3%) and 22 patients (66.7%) who had no *BRAF* mutations. *BRAF* mutations were more frequent in T3/T4 cancers than T1/T2 (P = 0.031) (Table 2).

Of the 11 patients with *BRAF* mutations, 7 (21.2%) had *hMLH1* promoter methylation. In the 22 patients without *BRAF* mutation, 5 (15.2%) had *hMLH1* promoter methylation and 17 (51.5%) had no *hMLH1* promoter methylation. The incidence of *BRAF* mutations was higher in patients with methylated *hMLH1* promoters, but there was not a significance difference between *BRAF* mutations and *hMLH1* promoter methylation (P = 0.052) (Table 2).

Seven patients (21.2%) had *hMLH1* promoter methylation (+)/*BRAF* mutation (+), which was indicative of sporadic colorectal cancer. Five patients (15.2%) had MSI colon cancers with *hMLH1* promoter methylation (+)/*BRAF* mutation (–) or *hMLH1* promoter methylation (–)/*BRAF* mutation (+). The remaining 16 patients (48.4%) had *hMLH1* promoter methylation (–)/*BRAF* mutation (–), which was suggestive of Lynch syndrome (Fig. 1).

Table 3 shows the profile of *BRAF* mutations, *hMLH1* promoter methylation, and MMR expression.

DISCUSSION

Detection of patients or families with hereditary colorectal cancer is requisite of treatment, surveillance provision, and adequate counseling of family members. The Amsterdam criteria are widely used to identify putative patients with Lynch syndrome, but these criteria are strictly defined and do not take into account most suspected hereditary colorectal cancers without a strong family history [15]. The 2002 revision of the BGs was developed to select patients for molecular analysis of MSI [15]. If the results of MSI suggest Lynch syndrome, it is recommended to carry out germ line testing of *MMR* genes [7]. The majority of the patients who do not fulfill the BGs cannot be considered to have Lynch syndrome, but rather sporadic colon cancer. However, there are several reports showing that the Lynch syndrome is related to colorectal cancer and can be diagnosed at 60 years of age, suggesting the BGs are not entirety adequate [16,17].

In the present study, about one-half of patients did not have any components of the BGs; approximately 5% of the patients had MSI colorectal cancers. One-third of MSI colorectal cancer patients did not have any components of the BGs. With respect to Korean patients with colon cancer, if only patients who satisfied the BGs were genetically tested, one-third of MSI colon cancers would be missed, which had the possibility of hereditary colon cancers. MSI colorectal cancer has been reported in approximately 95% of patients with Lynch syndrome-related colon cancers and 10%–20% of patients with sporadic colon cancer [17,18]. The proportion of MSI tumors was less compared to other series [19-21]. This discrepancy may be attributed, in part, to ethnic differences [22].

The detection of germ line mutations in MMR is an important supplement to clinical criteria and crucial for the definitive diagnosis of Lynch syndrome, especially for patients with an uncertain family history and small family numbers [17,23]. However, germ line tests have several obstacles to overcome, including high cost and time-consumption in performing the tests [10].

Many researchers [10,11,24] have reported a high frequency of *BRAF* mutations in patients with sporadic colorectal cancer with MSI and a methylated *hMLH1* promoter, and the lack of *BRAF* mutations in patients with Lynch syndrome is useful in detecting families with Lynch syndrome. *BRAF* mutations have rarely been found in colorectal cancers and cell lines of Lynch

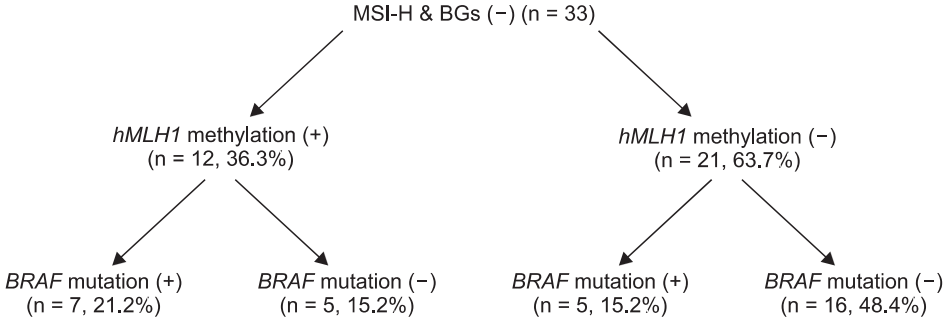

Fig. 1. Patient distribution according to *hMLH1* promoter methylation and *BRAF* mutations. MSI-H, high frequency microsatellite instability; BGs, Bethesda guidelines.

Table 3. *BRAF* mutation, *hMLH1* promoter methylation, and MMR expression profile

Case	*BRAF* mutation	*hMLH1* promoter methylation	MLH1 expression	MSH2 expression	MSH6 expression
4	√	√		√	
7	√	√	√	√	√
14	√		√	√	√
18	√			√	√
19	√		√	√	
26	√	√		√	√
27	√	√		√	√
28	√			√	√
29	√	√	√	√	√
30	√	√		√	√
33	√	√		√	√
1				√	√
2		√		√	
3		√	√	√	√
5			√		
6		√		√	√
8			√	√	√
9		√		√	√
10				√	√
11			√	√	√
12			√	√	√
13			√	√	√
15			√	√	√
16				√	√
17			√		
20				√	√
21			√		
22				√	
23				√	√
24		√			
25			√	√	
31				√	√
32			√	√	√

Check mark (√) indicate presence of *BRAF* mutation, *hMLH1* promoter methylation, and MMR expression.

syndrome related with mutations of *hMLH1* [7,10]. However, *BRAF* mutations are closely correlated to *hMLH1* promoter methylation in patients with sporadic colon cancer [10,25,26]. MSI colon cancers with *hMLH1* promoter methylation, as well as *BRAF* mutations, can be regarded as sporadic colon cancers and MSI tumors without *hMLH1* promoter methylation or *BRAF* mutations can be regarded as hereditary colon cancer. MSI colon cancers with *hMLH1* promoter methylation (+)/*BRAF* mutation (–) or *hMLH1* promoter methylation (–)/*BRAF* mutation (+) are difficult to distinguish from hereditary colon cancer and sporadic MSI tumors. However, because *hMLH1* promoter methylation is frequently reported in Lynch syndrome-related colorectal cancer, the subset of tumors with *hMLH1* promoter methylation (+)/*BRAF* mutation (–) might be Lynch syndrome-related colorectal cancer. All these things require the analysis of germ line mutations to confirm Lynch syndrome. In the present study, *BRAF* mutations were found in 33.3% of MSI colon cancers that did not meet the BGs. Among 33 patients, 48.5% (16/33; *hMLH1* promoter methylation (–)/*BRAF* mutation (–)) to 63.6% (21/33; *hMLH1* promoter methylation (–)/*BRAF* mutation (–) and *hMLH1* promoter methylation (+)/*BRAF* mutation (–)) might be a Lynch syndrome-related colon cancer among MSI tumors that do not satisfy the BGs criteria.

The combination of three molecular tests (MSI, *BRAF* mutations, and *hMLH1* promoter methylation) must be validated against analysis of MMR germ line mutations. It was a limitation of the current study that mutational analysis was not available.

In conclusion, even though the patients do not fulfill the BGs criteria, there may be a high likelihood that colon cancer results

from germ line mutations of MMR. Thus, MSI testing has been suggested as necessary for late-onset colon cancer patients or patients not satisfying BGs. Adding analysis of *BRAF* mutations and *hMLH1* promoter methylation to MSI could be an easy and efficient way to have the information of whether or not the patients have Lynch syndrome without full sequencing of *MMR* genes.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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