Identification of a Japanese Lynch syndrome patient with large deletion in the 3′ region of the EPCAM gene

Hidetaka Eguchi1,†,*, Kensuke Kumamoto2,3,†, Okihide Suzuki2, Masakazu Kohda1, Yuhki Tada1, Yasushi Okazaki1, and Hideyuki Ishida2

1Division of Translation Research, Research Center for Genomic Medicine, Saitama Medical University, Hidaka, 2Department of Digestive Tract and General Surgery, Saitama Medical Center, Saitama Medical University, Kawagoe, and 3Department of Organ Regulatory Surgery, Fukushima Medical University, Fukushima, Japan

*For reprints and all correspondence: Hidetaka Eguchi, Division of Translation Research, Research Center for Genomic Medicine, Saitama Medical University, Hidaka, Japan. E-mail: eguchi@saitama-med.ac.jp

†These authors contributed equally to this work.

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Abstract

Germline deletion of the 3′ portion of the Epithelial Cell Adhesion Molecule (EPCAM) gene located 5′ upstream of MutS Homolog 2 (MSH2) is a novel mechanism for its inactivation in Lynch syndrome. However, its contribution in Japanese Lynch syndrome patients is poorly understood. Moreover, somatic events inactivating the remaining allele of MSH2 in cancer tissue have not been elucidated in Lynch syndrome patients with such EPCAM deletions. We identified a Japanese Lynch syndrome patient with colon cancer who evidenced germline deletion of a 4130 bp fragment of EPCAM encompassing exons 8 and 9 (c.859-672_*2170del). In normal colonic mucosa, two known fusion-transcripts of EPCAM/MSH2 generated from the rearranged gene were observed and heterozygous methylation of the MSH2 gene promoter was detected. In cancer tissue, dense methylation of MSH2 was observed and MLPA analysis demonstrated somatic deletion of the remaining EPCAM allele including exon 9, indicating that somatic deletion of EPCAM is responsible for complete inactivation of MSH2.

Key words: genetics-cancer genetics, GI-colorectum-basic, Lynch syndrome, EPCAM

Introduction

Lynch syndrome (LS) (previously called hereditary non-polyposis colorectal cancer, HNPCC) is an autosomal-dominant inherited cancer syndrome caused by inactivation of four mismatch repair (MMR) enzymes: MLH1, MutS Homolog 2 (MSH2), MSH6 and PMS2 (1). Accumulated reports of germline mutations of these MMR genes have recently been reviewed according to the five-tiered scheme for standardized classification by the International Society for Gastrointestinal Hereditary Tumors (InSiGHT) (2). Although mutations in these four genes account for the majority of all LS patients (1), approximately 3% of colorectal cancer is thought to develop among LS patients (1). Accumulated reports of germline mutations of these MMR genes have recently been reviewed according to the five-tiered scheme for standardized classification by the International Society for Gastrointestinal Hereditary Tumors (InSiGHT) (2). Although mutations in these four genes account for the majority of all LS cases (3), a small fraction of suspected LS cases did not reveal any genetic alterations of these genes.

A novel mechanism inactivating the MSH2 gene in LS, germline deletion of the 3′ portion of the Epithelial Cell Adhesion Molecule (EPCAM) gene (formerly called TACSTD1) located 5′ upstream of the MSH2 gene, was discovered in 2009 (4). The deletion was associated with methylation of the promoter region of the MSH2 gene. Since then, dozens of different types of EPCAM gene deletions have been reported in several populations (3). Nonetheless, the contribution of EPCAM deletion in Japanese LS patients is poorly known thus far.

During the course of cancer development in LS patients with germline deletion of the EPCAM gene, inactivation of the remaining allele of MSH2 has to occur, and this can take place through various mechanisms. Despite dozens of reports identifying LS patients harboring
EPCAM gene deletions among Caucasian and Chinese populations, only a limited number of studies have been reported describing such mechanisms. A large somatic deletion of the 3’ region of the EPCAM gene associated with methylation of the promoter region of the downstream MSH2 gene is one mechanism for inactivation of the gene (4–7).

We report here on a Japanese LS patient with germline deletion of the 3’ region of the EPCAM gene, which resulted in aberrant transcripts generated from an EPCAM/MSH2 fusion gene. Furthermore, as a mechanism of inactivation of the remaining allele of MSH2 gene, we observed somatic deletion of the remaining EPCAM gene that was associated with dense methylation of the promoter region of the MSH2 gene promoter region. This correlated well with an absence of MSH2 protein in the patient’s colorectal cancer.

### Patients and methods

#### Patient and blood sample collection

The Japanese patient (III-2 in Fig. 1) was a 47-year-old woman who visited a clinic suffering from epigastric pain. Lower gastrointestinal endoscopic examination revealed a type II tumor (ulcerated type with clear margin) (8) in her cecum and pathological examination of the biopsy sample provided a diagnosis of well-differentiated adenocarcinoma. She then underwent laparoscopic ileocecal resection and D3 lymphadenectomy for Stage IIA colon cancer (T3N0M0) (AJCC 7th Ed.) at Saitama Medical Center, Saitama Medical University. The patient had a family history of cancers related to LS; her grandmother and two sisters (subjects I-2, I-6 and I-8, respectively; Fig. 1) had colorectal or gastric cancers. The MSI test of the patient’s cancer specimen showed instability in all five out of five microsatellite markers, indicating high MSI (MSI-H). Considering that the subject fulfilled the revised Bethesda guidelines (9), we suspected LS. Genetic testing was initiated at 3 months after surgery. We also contacted her mother, who had no evidence of colorectal cancer, and obtained a sample of the mother’s peripheral blood. DNA was extracted from whole blood using a QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany).

#### Ethical considerations

Informed consents were obtained from the subjects. This research was approved by the Institutional Review Board at Saitama Medical Center, Saitama Medical University (926) and the Ethics Committee at Saitama Medical University (592-II), and was conducted according to the guidelines put forth in the Declaration of Helsinki.

#### Immunohistochemistry

Formalin-fixed and paraffin-embedded (FFPE) 5 μm tissue sections were used to examine expression of MMR enzymes. Primary antibodies used were as follows: Purified Mouse Anti-MLH1 Monoclonal Antibody, clone G168-15 (BD Pharmingen, San Diego, CA) for MLH1; Anti-MSH2 Antibody, clone FE11 (Calbiochem, La Jolla, CA) for MSH2; Purified Mouse Anti-MSH6, clone 44/MSH6 (BD Pharmingen) for MSH6; and Purified Mouse Anti-PMS2, clone A16-4 (BD Pharmingen) for PMS2. Dilution rates of ×50, ×50, ×100 and ×50, respectively, were used. Staining was conducted using the DAKO EnVision™ system (Agilent Technologies Dako, Glostrup, Denmark) and diaminobenzidine (SIGMA, St. Louis, MO) was used as the substrate chromogen. Slides were counterstained with hematoxylin. Normal colonic mucosa was used as a positive control.

#### Microsatellite instability test

The microsatellite instability test using a panel of five markers—BAT25, BAT26, D2S123, D5S346 and D17S250—recommended by the revised Bethesda guideline (9), was conducted at a clinical testing laboratory, FALCO Biosystems Co. Ltd. (Kyoto, Japan). When two or more markers evidenced altered lengths in electrophoresis, the case was recognized as high-MSI (MSI-H).

![Figure 1. Pedigree of the patient who carried a 3′ deletion of the EPCAM gene. Filled symbols indicate individuals affected with LS associated cancers. Plus signs denote individuals who are carriers of the deletion.](https://academic.oup.com/jjco/article-abstract/46/2/178/2384934)
Detection of large deletion of the 3′ region of EPCAM gene

Deletion in exon 9 of the EPCAM gene was detected using a SALSA MLPA kit P003 C1-0512 MLH1/MSH2 (MRC-Holland, Amsterdam, the Netherlands) and a 3500 Genetic Analyzer with POP-7 polymer (Life Technologies, Carlsbad, CA), according to the manufacturer’s instructions. The resulting data were analyzed using the Coffalyser.Net software (MRC-Holland).

Determination of break point of rearranged genome

Long-range PCR was conducted to amplify fragments spanning the putative break points using KOD FX neo (TOYOBO Co. Ltd., Osaka, Japan) and the following primer sets: EPCAM_I7_F01, 5′-GGA AAA GGA GGA TGT GGA AGA ATG C-3′ and EPCAM_5Kdel_R01, 5′-CAG TGT TGA CCT TAG CGA AAG TAG C-3′ (4) (Supplementary Fig. S1). Thermal cycle conditions were 94°C, 2 min for activation, followed by 45 cycles of 98°C, 10 s for denaturation, 65°C, 30 s for annealing and 68°C, 5 min for extension. The amplified DNA fragments were electrophoresed on 0.7% agarose gel, excised from the gel, then purified using NucleoSpin Gel and a PCR Clean-up kit (Macherey-Nagel). The purified fragments were subjected to sequencing reaction using a BigDye 3.1 cycle sequencing kit (Life Technologies) with primer EPCAM_5Kdel_R02: 5′-TAG CCA AGT ATA GTG GTG GGT G-3′ (4) (Supplementary Fig. S1), on an ABI 3130 DNA sequencer (Life Technologies). The CAlu program (http://clustbu.cc.emory.edu/calu/index.cgi) was used for identification of Alu sequences (10).

Methylation analysis of the promoter region of MSH2 gene

DNA was extracted from 10 μm sections of FFPE normal colonic mucosa or cancer tissue specimens using a QIAamp DNA FFPE Tissue Kit (QIAGEN). For analysis of the cancer tissue, the FFPE tissue sections were mounted on glass slides with a PEN membrane film coated with 0.1% poly-L-Lysine solution (Nacalai tesque Inc., Kyoto Japan). After deparaffinization using Lemosol A (Wako Pure Chemicals, Col Ltd.), the tissue was stained with 0.05% toluidine blue solution (Wako Pure Chemicals, Col Ltd.), and cancerous cells were collected using an LMD 7000 laser microdissection system (Leica Microsystems GmbH, Wetzlar, Germany). Bisulfite conversion of the extracted DNA was conducted using an EZ DNA Methylation-Gold™ Kit (Zymo Research Corporation, Irvine, CA). The converted DNA was used as a template for PCR amplification using AmpliTag Gold 360 Master mix (Life Technologies) and primer sets NP2-F: 5′-GGT GTA GTA AGG GTA GGT TGT TAT T-3′ and NP2-R: 5′-AAA CAC ACR TTT TTA CAA AAT ACT AA-3′ (11). After 2% agarose gel electrophoresis of the products, the bands were excised and the fragments were purified using NucleoSpin Gel and a PCR Clean-up kit (Macherey-Nagel). The purified fragments were subjected to Sanger sequencing reaction using NP2-F as a sequencing primer as described above.

Analysis of aberrant transcripts in normal mucosa

Total RNA was extracted from a snap frozen tissue specimen of the normal colonic mucosa using a NucleoSpin RNA Plus kit (Macherey-Nagel). A reverse-transcription reaction using random primers was carried out with ReverTra Ace qPCR RT Master Mix (TOYOBO). The cDNA obtained was used as a template for PCR using AmpliTag Gold 360 (Applied Biosystems) and primer sets EPCAM_ex7_F01: 5′-GGT GTA GTA AGG GTA GGT TGT TAT T-3′ and MSH2_ex2_R01: 5′-ATG CCA AAT ACC AAT CAT TC-3′ or MSH2_ex2_R02: 5′-CTT CAA CTC TAT ACT GAC GAA CC-3′ (Supplementary Fig. S2). As controls, SW480 and Colo210 colorectal cancer cells were also analyzed.

Results

Immunohistochemical staining

Immunohistochemical staining of four MMR enzymes evidenced an absence of both MSH2 and MSH6 proteins in the cancer tissue specimens of the patient (Fig. 2A–D), strongly indicating that inactivation of the MSH2 gene might be responsible for the deficiency of these MMR enzymes.
Germline alteration analysis

Sanger sequencing analyses of exons and intron boundary regions of MSH2 together with MSH6 genes revealed no causative germline mutation in the patient (data not shown). Examination of copy number alterations in MSH2 and EPCAM genes using the MLPA test revealed that two probes (9-1 and 9-2) of the MLPA set within exon 9 of the EPCAM gene indicated a heterozygous deletion of the 3′ region (Fig. 3, upper and Supplementary Fig. S3). To determine the precise breakpoint of the EPCAM gene, we performed long-range PCR amplification of the 3′ region using germline DNA extracted from lymphocytes of the patient as template (Fig. 4A, Supplementary Fig. S1). Agarose gel electrophoresis of PCR products (lane 1) showed a major band of 1.9 kb in length with a minor band of 6 kb, while the control (lane 3) showed only a 6 kb band (Fig. 3A). The patient’s mother (subject II-2) also showed the shortened fragment together with the longer one (lane 2). Direct sequencing of the amplified fragment revealed that both subjects had a 4130 bp deletion including exons 8 and 9 of the EPCAM gene (c.859-672_*2170del) (Fig. 4B and C). The breakpoints, located within two interspersed elements AluSz in intron 7 and AluSq in the 3′ downstream region of the EPCAM gene, were novel. Information regarding the EPCAM gene breakpoints in the patient is summarized in Supplementary Table S1.

Methylation analysis of the MSH2 promoter region in normal colonic mucosa of the patient

To examine the association between the EPCAM gene deletion and methylation of the MSH2 gene promoter, we examined methylation status of the MSH2 gene in normal colonic mucosa. Bisulfite sequencing analysis of the MSH2 gene promoter region revealed heterozygous methylation of cytosine residues at two CpG sites in the DNA extracted from normal mucosa (Fig. 5, upper), confirming an association between the rearranged gene and methylation of the MSH2 promoter.

Analysis of the EPCAM/MSH2 gene transcripts in normal colonic mucosa of the patient

To confirm the consequences of the rearranged gene, we analyzed EPCAM gene transcripts in normal colonic mucosa of the patient. When DNA fragments were amplified with primer sets EPCAM_ex7_F01 and MSH2_ex2_R01 or EPCAM_ex7_F01 and MSH2_ex2_R02 using cDNA generated by reverse-transcription of mRNA from normal colonic mucosa as a template (Supplementary Fig. S2), we observed two major fragments (Fig. 6, lanes 1-2) in agarose gel electrophoresis, while such fragments were absent in control samples SW480 and Colo210 (lanes 6–7 and 11–12, respectively). Sanger sequencing of the amplified fragments demonstrated that one aberrant transcript (aberrant transcript 1) was generated by fusion of exon 7 of EPCAM and exon 2 of MSH2 (Supplementary Fig. S4A). On the other hand, another (aberrant transcript 2) contained an insertion of cryptic exon of a 111 bp AluJo repetitive element located ∼4 kb upstream of the MSH2 gene (MSH2 c.–4237_-4127) between exon 7 of EPCAM and exon2 of MSH2 (Supplementary Fig. S4B).

Somatic events responsible for inactivation of the remaining MSH2 allele in cancer tissue of the patient

Because MSH2 protein level was diminished in the cancer tissue of the patient as assessed with immunohistochemistry, we examined
Figure 4. Rearrangement of EPCAM gene. (A) Agarose gel electrophoresis of long-range PCR products. (M) 1 kb Ladder. (1) Patient. (2) Patient’s mother. (3) Control. See Supplementary Fig. S1 for organization of the gene exons and location of the primers. (B) Electropherograms of cycle sequencing products. (Upper) The patient. (Lower) A control. The red arrow indicates the breakpoint. (C) Alignment of the sequences around the breakpoint of the EPCAM gene of the patient with those of normal, wild-type intron 7 of the EPCAM gene and 5′ upstream region of the MSH2 gene. Vertical bars indicate nucleotides that are identical with either intron 7 of the EPCAM gene or the 5′ upstream region of the MSH2 gene. The red rectangle indicates an array of identical nucleotides among the three sequences.
mechanisms responsible for inactivation of the remaining allele of MSH2 gene. Sanger sequencing demonstrated no somatic alteration with known pathogenicity in any of the exons or exon/intron boundaries examined (data not shown). On the other hand, we observed dense methylation of the promoter region of the MSH2 gene in the patient’s cancer tissue (Fig. 5, lower). In addition, MLPA analysis using the DNA sample extracted from FFPE cancer tissue indicated a drastic reduction of signals of such LS patients. According to the classification by Kempers et al. (15), our case (c.859-672_*2170del) belongs to Subgroup I; this subgroup showed a similar risk for colorectal cancer as compared with LS associated with MSH2 mutations, whereas no increased risk for endometrial cancer was observed (13). Thus far, only a single type of LS case with 3′ deletion of EPCAM has been identified among Japanese [the present study and (13) according to the reported deletion size]. Further examination of the EPCAM deletion in a suspected LS case lacking expression of MSH2 protein with no known pathogenic mutation should be performed to discover EPCAM deletions in the future to better understand their prognosis according to genotype among the Japanese population.

Large deletions of the 3′ region of the EPCAM gene are known to be associated with methylation of the promoter region of the MSH2 gene (4–7). Because our observed deletion type was different from the reported ones (3), we also examined methylation and confirmed that the 4130 bp EPCAM deletion (c.859-672_*2170del) was indeed associated with heterozygous methylation of the MSH2 gene in normal colon mucosa (Fig. 5, upper). Moreover, we detected expression of aberrant transcripts in normal colon mucosa that are generated from the rearranged gene (Fig. 6 and Supplementary Fig. S4). These aberrant transcripts were identical to those found in LS patients of the family HFC121 harboring a 6.1 kb deletion of the 3′ region of the EPCAM gene including exons 8 and 9 (g.79459_85516del6058) as reported by Kovacs et al. (16). These aberrant transcripts were known to be inactive for MMR. The observed methylation of MSH2 gene promoter and expression of aberrant transcripts confirm that germline inactivation of MSH2 occurred in normal colonic mucosa of our patient.

LS patients with EPCAM deletions demonstrate an absence of MSH2 protein in the cancer tissue. However, somatic events involved in inactivation of the remaining allele of MSH2 have heretofore not been fully characterized. Huth et al. (17) reported that four out of six various cancers that developed in LS patients with germline EPCAM deletions revealed homozygous EPCAM deletion in cancer tissue. On the other hand, a recent study by Spaepen et al. (7) reported that one colorectal cancer developed in an LS patient with a large 4 Mb deletion including the EPCAM, MSH2 and MSH6 genes.

**Discussion**

Mutations in MLH1 and MSH2 account for ~90% of all mutations related to LS, while MSH6 and PMS2 account for 7–10% and <5% of LS, based on NCBI data (3). On the other hand, it has recently been reported that at least 1–3% of LS can be explained by EPCAM gene deletions among the Dutch and German populations (12). The exact prevalence of EPCAM deletions in LS patients of populations other than these is still under investigation. Specifically, such information in the Japanese population is lacking. Takahashi et al. (13) recently published a brief report written in Japanese about a Japanese LS patient having a 4130 bp deletion in the 3′ portion of the EPCAM gene encompassing exons 8 and 9, but they did not provide detailed information such as breakpoints. Nagasaka et al. (14) reported that three of four Japanese suspected LS patients with methylation of the MSH2 gene promoter evidenced EPCAM deletions as assessed with MLPA analysis, but they did not confirm deletion using long-range PCR, nor did they identify the exact breakpoints. In this sense, we believe that ours is the first report providing a detailed view of a Japanese LS patient with an EPCAM gene deletion.

Since risks of colorectal and endometrial cancers in LS with EPCAM deletion differ according to the extent of the deletion (15), it is important to determine the precise breakpoint for surveillance of such LS patients. According to the classification by Kempers et al. (15), our case (c.859-672_*2170del) belongs to Subgroup I; this subgroup showed a similar risk for colorectal cancer as compared with LS associated with MSH2 mutations, whereas no increased risk for endometrial cancer was observed (13). Thus far, only a single type of LS case with 3′ deletion of EPCAM has been identified among Japanese [the present study and (13) according to the reported deletion size]. Further examination of the EPCAM deletion in a suspected LS case lacking expression of MSH2 protein with no known pathogenic mutation should be performed to discover EPCAM deletions in the future to better understand their prognosis according to genotype among the Japanese population.
while the other showed homozygous deletion of only the EPCAM gene. In our study, we found a homozygous deletion in exon 9 of EPCAM as assessed with MLPA in colorectal cancer tissue (Fig. 3, lower and Supplementary Fig. S5). Weak signals at the two probes (9-1 and 9-2) set within exon 9 of the EPCAM gene in MLPA can be attributed to contamination of DNA from stromal cells. Because only FFPE sample was available for analysis of the cancer tissue and the extracted DNA sheared severely, we could not determine the extent of the somatic EPCAM deletion. Biallelic methylation of the MSH2 gene promoter (Fig. 5, lower) in cancer tissue strongly supported the conclusion of somatic deletion of the remaining allele of EPCAM.

In conclusion, we identified a LS patient harboring a germline deletion of the 3′ region of the EPCAM gene that has not been reported in populations other than Japanese. Somatic deletion of the remaining allele of EPCAM gene was indicated as a mechanism for inactivation of MSH2 in cancer tissue.

Supplementary data
Supplementary data are available at http://www.jjco.oxfordjournals.org.

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Conflict of interest statement
None declared.

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