

Mutational and expressional analysis of MLL genes in gastric and colorectal cancers with microsatellite instability

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MLL genes encode histone methyltransferases that are required for proper expression of a variety of genes. The pathologic implications of *MLL* genes have been studied not only in leukemias, but also in some solid cancers. We found in a public database that *MLL*, *MLL2*, *MLL3*, *MLL4* and *MLL5* genes had mononucleotide repeats that might be mutated in cancers with microsatellite instability (MSI). Frameshift mutations in a repeat of *MLL3* have been found in colorectal cancers (CRC), but there is no frameshift mutation data of the other genes. In this study, we analyzed these repeats in 32 gastric cancers (GC) with high MSI (MSI-H), 59 GC with low MSI (MSI-L)/stable MSI (MSS), 40 CRC with MSI-H and 59 CRC with MSI-L/MSS by single-strand conformation polymorphism and DNA sequencing. We also analyzed *MLL3* expression in GC and CRC tissues using immunohistochemistry. We found *MLL*, *MLL2*, *MLL3* and *MLL5* frameshift mutations in two (one GC and one CRC), three (one GC and two CRC), 17 (14 GC and three CRC) and six (four GC and two CRC) cancers, respectively. They were detected exclusively in MSI-H cancers, but not in MSI-L/MSS cancers. All of the cancers with *MLL3* mutations showed loss of *MLL3* expression, and their values were significantly lower than in those without *MLL3* mutation (50.9%). Of note, the GC with MSI-H had significantly higher incidences in both *MLL* mutations and *MLL3* expression loss than the CRC with MSI-H. Our data indicate that frameshift mutations of *MLL* genes and loss of expression of *MLL3* protein are common in GC and CRC with MSI-H.

Key words: MLL, frameshift mutation, MLL protein expression, cancer, microsatellite instability

Mixed lineage leukemia (MLL) genes include *MLL*, *MLL2*, *MLL3*, *MLL4* and *MLL5*, and encode proteins that act as histone methyltransferases [1-3]. They contain multiple domains in common, including Su(var)3-9, Enhancer-of-zeste, Trithorax (SET), plant homeodomain (PHD) zinc finger and DNA-binding domains (AT hook, Bromo and CXXC domains) [3]. The *MLL* proteins regulate histone 3- lysine 4 (H3K4) methylation, a modification associated with transcriptionally active genes [1]. *MLL* is a prototype of the *MLL* genes and is the best studied among them [2]. *MLL* is essential for proper maintenance of *HOX* gene expression during hematopoiesis and embryonic development [4-6]. The *MLL* gene is frequently involved in chromosomal translocations associated with acute leukemias [2, 7-9]. Although nomenclature of the *MLL* gene came from its function in hematopoiesis

and its alterations in leukemias, it is ubiquitously expressed in most tissues [10]. Also, expressions of other *MLL* genes are identified not only in hematopoietic cells, but also in non-hematopoietic tissues [11-14]. Germline mutations of *MLL2* gene are associated with Kabuki syndrome, a multiple malformation disorder [15]. Somatic mutations of the *MLL2* gene have also been identified in medulloblastomas and lymphomas [16, 17]. Somatic mutations of *MLL3* gene have been found in colorectal cancers (CRC) [18, 19]. An earlier study found missense mutations of *MLL3*, while another study reported absence of *MLL3* missense mutation in CRC [19, 20]. *MLL4* gene is amplified in glioblastomas and pancreatic cancers [21]. These data suggest that mutational and expressional alterations of *MLL* genes are implicated in diverse human malignancies.

In a public genome database (<http://genome.cse.ucsc.edu/>), we found that human *MLL* genes (*MLL*, *MLL2*, *MLL3*, *MLL4* and *MLL5*) have mononucleotide repeats in the coding sequences that could be targets for frameshift mutation in cancers with microsatellite instability (MSI). Frameshift mutations of genes containing mononucleotide repeats are features of gastric (GC) and CRC with microsatellite instability (MSI) [22]. It is possible that frameshift mutations of the *MLL*-encoding genes could cause alterations of *MLL* functions and contribute to cancer pathogenesis in the cancers with MSI. However, frameshift mutations among these genes are known only in the *MLL3* in CRC [18]. In this study, we used tumors that had already been evaluated their MSI status by a well developed criteria. With these tumor tissues, we explored whether the *MLL* genes are somatically mutated in GC and CRC with MSI.

Materials and methods

Tissue samples. For the mutation analysis, methacarn-fixed tissues of sporadic 91 GC and 99 CRC were used in this study. All of the cancer patients were Koreans. The GC consisted of 32 with high MSI (MSI-H), 14 with low MSI (MSI-L) and 45 with stable MSI (MSS). The CRC consisted of 40 with MSI-H, 14 with MSI-L and 45 with MSS. According to the NCI criteria tumors may be characterized as: MSI-H, if two or more of the five markers show instability, and MSI-L, if only one of the five markers shows instability by evaluating a panel of five validated microsatellites [23]. Previously we classified the GC and CRC specimens with these criteria. The pathologic features of the GC and CRC are summarized in Table 1. Approval for this study was obtained from the Institutional Review Board of Catholic University of Korea. Tumor cells and corresponding normal cells from the same patients were selectively procured from hematoxylin and eosin-stained slides using a 30G1/2 hypodermic needle affixed to a micromanipulator, as described previously [24, 25]. DNA extraction was performed by a modified single-step DNA extraction method [24, 25].

Single strand conformation polymorphism (SSCP) analysis and genomic sequencing. *MLL* exon 3 (A7 and C7 repeats), *MLL2* exon 10 (C7), *MLL3* exon 38 (A9 and A7), *MLL4* exon 3 (C7 and C7), *MLL5* exon 8 (A7), *MLL5* exon 14 (A7) and *MLL5* exon 20 (A7) have mononucleotide repeats in their coding sequences. Genomic DNA from the microdissected cells was isolated, and was amplified by polymerase chain reaction (PCR) with 10 specific primer pairs (Table 2). Radioisotope ($[^{32}P]$ dCTP) was incorporated into PCR products for detection by autoradiography. Procedures of PCR and SSCP analysis were performed as described previously [24, 25]. After SSCP, mobility shifts on the SSCP gels (FMC Mutation Detection Enhancement system; Intermountain Scientific, Kaysville, UT) were determined by visual inspection. Direct DNA sequencing reactions were performed in the cancers with the mobility shifts in the SSCP. Sequencing of the PCR products was carried out using a capillary automatic sequencer (3730 DNA Analyzer, Applied Biosystem, Carlsbad, CA, USA). When mutations in the *MLL* genes were suspected by SSCP, analysis of an independently isolated DNA from another tissue block of the same patients was performed to exclude potential artifacts originated from PCR.

Immunohistochemistry. Using the sections from GC and CRC tissues, immunohistochemistry for *MLL3*, which showed most frequent mutations, was performed in this study. The tissues consisted of 32 GC and 40 CRC with MSI-H, and 59 GC and 59 CRC with MSI-L/MSS. We used DAKO REAL EnVision System (DAKO, Glostrup, Denmark) for human *MLL3* (Abcam, Cambridge, UK; dilution 1/50). After deparaffinization, heat-induced epitope retrieval was conducted by immersing the slides in Coplin jars filled with 10 mmol/L citrate buffer (pH 6.0) and boiling the buffer for 30 min in a pressure cooker (Nordic Ware, Minneapolis, MN, USA) inside a microwave oven at 700 W. The immunohistochemical procedure was performed as described previously [26]. The reaction products were developed with diaminobenzidine and counterstained with hematoxylin. By visual inspection under light microscope, tumors were interpreted as positive when 20-100% of the cells

Table 1. Summary of pathologic features of the gastric and colorectal cancers.

		No. of Gastric carcinomas					No. of Colorectal carcinomas		
		MSI-H (n=32)	MSI-L (n=14)	MSS (n=45)			MSI-H (n=40)	MSI-L (n=14)	MSS (n=45)
TNM	I	12	5	13	TNM	I	6	2	7
	II	13	5	20		II	14	4	16
	III	6	3	10		III	17	7	18
	IV	1	1	2		IV	3	1	4
Lauren's subtype	Diffuse	16	8	22	Location (colon)	Cecum	6	0	0
	Intestinal	13	5	18		Ascending	22	2	2
	Mixed	3	1	5		Transverse	9	2	2
					Descending & sigmoid	3	4	17	

Table 2. Primer sequences of the MLL genes used in this study

Gene	Sequences	Size (bp)	Annealing temperature (°C)
<i>MLL</i> Exon 3-1	F: 5'-CCTTCCCTGGAGTAAAAATC-3' R: 5'-TAATCTTGTGGCTTGCTG-3'	132	49
<i>MLL</i> Exon 3-2	F: 5'-ACAAGAAGTGAAGGCTTAG-3' R: 5'-AGACTGAGTCAGGGAATGAG-3'	150	52
<i>MLL2</i> Exon 10	F: 5'-TCTGTCCCACCATTTGAAGAG-3' R: 5'-GGATAGGCGCGATACCTCA-3'	169	58
<i>MLL3</i> Exon 38-1	F: 5'-GCATGTTTAATGAGGAACTA-3' R: 5'-GTGGTGAATGTTTATCAGAG-3'	126	47
<i>MLL3</i> Exon 38-2	F: 5'-AATGAGGTAAAAACGGAAGT-3' R: 5'-TCATTTAGGTCTGAGTGAGC-3'	134	49
<i>MLL4</i> Exon 3-1	F: 5'-TTGATCCATCTCCCCACAAC-3' R: 5'-CTTCTCACCCCGTTTCC-3'	151	58
<i>MLL4</i> Exon 3-2	F: 5'-CCGCTCCTCCCGTGCATC-3' R: 5'-TGGAGAGGGGACTGGTGCTG-3'	148	60
<i>MLL5</i> Exon 8	F: 5'-TGCTTCAAGAGTTTCCAAAG-3' R: 5'-AGTTCCCTATCCGCTATCC-3'	134	49
<i>MLL5</i> Exon 14	F: 5'-AGAGTGCCTGTTCTAAAAC-3' R: 5'-GGTCGTTCCCTTACAATC-3'	154	49
<i>MLL5</i> Exon 20	F: 5'-AAAGTCATAACTGCCATTGT-3' R: 5'-ACCGAATCTAGCAACTGAT-3'	162	47

showed moderate to intense staining in cytoplasm or nucleus, and as negative when 0-19% of the cells showed no or weak or moderate or intense staining by immunohistochemistry. Intensity of MLL3 immunostaining was interpreted as positive or negative independently by two pathologists. The immunostaining was judged to be specific by absence of consistent immunostaining of cells with replacement of primary antibody with the blocking reagent and reduction of immunostaining intensity as dilution of the antibody was increased. For the statistical analysis of the immunohistochemical data, we used Fisher's exact test and χ^2 test.

Results

MLL gene mutations. Out of 10 evaluated mononucleotide repeats in 91 GC and 99 CRC, we found aberrant bands in two cases of *MLL* exon 3 (C7), three cases of *MLL2* exon 10, 16 cases of *MLL3* exon 38 (A9), one case of *MLL3* exon 38 (A7), one case of *MLL5* exon 8, one case of *MLL5* exon 14 and four cases of *MLL5* exon 20 (Figure 1; Table 3). DNA from normal tissues from the same patients showed no evidence of the mutation in SSCP, indicating the mutations had arisen somatically (Figure 1A). Direct DNA sequencing analysis of

Table 3. Summary of the frameshift mutations in the gastric and colorectal cancers with MSI

Gene	Location	Repeats (wild type)	Repeats (Mutation)	MSI status of the mutation cases (n)	Incidence in cancers with MSI-H (%)	Nucleotide change (predicted amino acid change)
<i>MLL</i>	Exon 3	C7	C6	MSI-H (2)	Gastric: 1/32 (3.1) Colorectal: 1/40 (2.5)	c.2318delC (p.Ala773ArgfsX8)
<i>MLL2</i>	Exon 10	C7	C6	MSI-H (2)	Gastric: 1/32 (3.1) Colorectal: 1/40 (2.5)	c.1940delC (p.Pro647HisfsX283)
		C7	C8	MSI-H (1)	Colorectal: 1/40 (2.5)	c.1940insC (p.Pro648ThrfsX2)
		A9	A8	MSI-H (12)	Gastric: 9/32 (28.1) Colorectal: 3/40 (7.5)	c.8390delA (p.Lys2797ArgfsX26)
<i>MLL3</i>	Exon 38	A9	A7	MSI-H (2)	Gastric: 2/32 (6.3)	c.8389_8390delAA (p.Lys2797GlyfsX11)
		A9	A10	MSI-H (2)	Gastric: 2/32 (6.3)	c.8390insA (p.Glu2798GlyfsX11)
		A7	A6	MSI-H (1)	Gastric: 1/32 (3.1)	c.8525insA (p.Asn2842LysfsX2)
<i>MLL5</i>	Exon 8	A7	A6	MSI-H (1)	Gastric: 1/32 (3.1)	c.691delA (p.Ser231AlafsX73)
	Exon 14	A7	A6	MSI-H (1)	Colorectal: 1/40 (2.5)	c.1482delA (p.Asp495ThrfsX26)
	Exon 20	A7	A6	MSI-H (4)	Gastric: 3/32 (9.4) Colorectal: 1/40 (2.5)	c.2619delA (p.Lys873AsnfsX8)

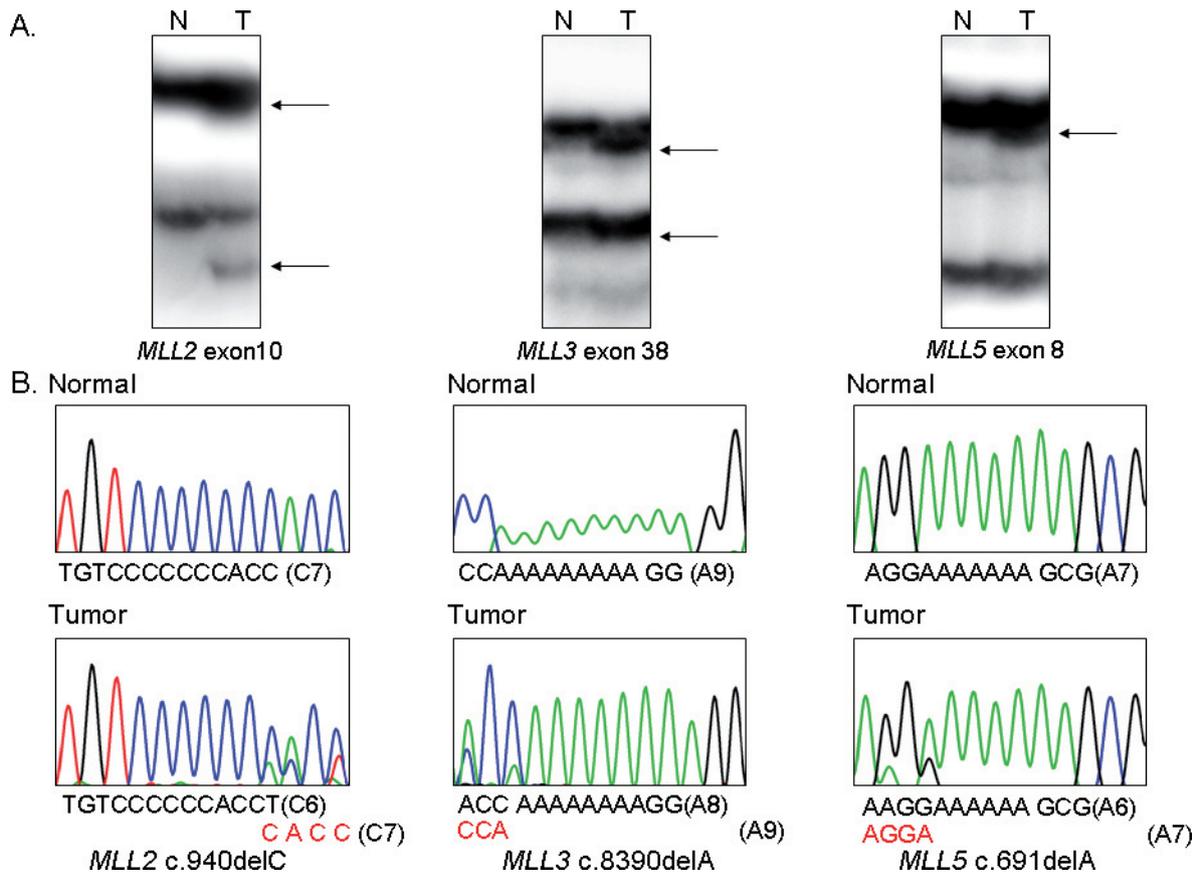


Figure 1. Representative SSCP and DNA sequencing of *MLL2*, *MLL3* and *MLL5* genes in the cancers. A: In the SSCP, the arrows (Lane T) indicate aberrant bands compared to the SSCP from normal tissues (N). B: Direct DNA sequencing analyses of *MLL2* (left), *MLL3* (middle) and *MLL5* (right) show heterozygous deletions of a nucleotide in tumor tissue as compare to normal tissues.

the cancer tissues with the aberrantly migrating bands led to identification of *MLL*, *MLL2*, *MLL3* and *MLL5* gene mutations in two, three, 17 and six cancers, respectively (Table 3). All of the mutations were interpreted as heterozygous according to the SSCP and direct sequencing analyses (Figure 1). All of the 28 mutations were deletion or insertion mutations of the bases in the repeats that could cause the premature stop codon, which leads to the termination of translation (Table 3). There was no aberrant band in the SSCP of *MLL4* gene.

All of the mutations were detected in the cancers with MSI-H (Table 3), but not in those with MSI-L or MSS. There were statistical differences between the frequencies of the mutations together in the cancers with MSI-H (28/72) and MSI-L (0/28) (Fisher's exact test, $p < 0.001$), and those with MSI-H (28/72) and MSS (0/90) (Fisher's exact test, $p < 0.001$). Of the 32 GC with MSI-H, 16 cancers harbored one mutation each and two cancers harbored two mutations (18/32; 56.3%). Of the 40 CRC with MSI-H, six cancers harbored one mutation each and one cancer harbored two mutations (7/40; 17.5%). The GC with MSI-H had a significantly higher incidence in *MLL* mutations than the CRC with MSI-H (Fisher's exact test,

$p = 0.001$). At individual gene level, there were significant differences of mutation frequencies of *MLL3* (Fisher's exact test, $p < 0.001$) and *MLL5* (Fisher's exact test, $p = 0.003$) genes between cancers with MSI-H and non-MSI-H. There was no significant association of the mutations with the clinicopathologic data of the patient (age, sex, stage, and metastasis). There was no correlation between histological features of the tumors (histologic grade, subtypes, mucinous histology, medullary pattern and tumor-infiltrating lymphocytes) and the presence of the mutations, either.

Expression of *MLL3* protein. We used immunohistochemistry to examine expression of *MLL3* that had been found to be mutated most among the *MLL* genes in the cancers analyzed. We analyzed *MLL3* protein expression in 32 GC and 40 CRC with MSI-H, and 59 GC and 59 CRC with MSI-L/MSS. Immunopositivity for *MLL3* was observed in 10 (31.3%) of the GC and 18 (45.0%) of the CRC with MSI-H (Figure 2; Table 4). Of the 17 cancers with *MLL3* frameshift mutations (Table 4), none of them showed positive *MLL3* immunostaining, and there was a significant difference of *MLL3* immunostaining between MSI-H cancers harboring

mutations in *MLL3* mutations and the MSI-H cancers that have not acquired mutations in *MLL3* (Fisher's exact test, $p < 0.001$). There was a significant difference of *MLL3* immunopositivity between the GC with MSI-H and the GC with MSI-L/MSS (Fisher's exact test, $p < 0.05$), but not between the CRC with MSI-H and the CRC with MSI-L/MSS (Fisher's exact test, $p > 0.05$) (Table 4).

Discussion

Based on the earlier reports that MLL proteins are critically involved in chromatin modification and are implicated in the pathogenesis of human diseases [1-9], we attempted to determine whether somatic frameshift mutation of *MLL* genes is present in GC and CRC tissues that are the most common

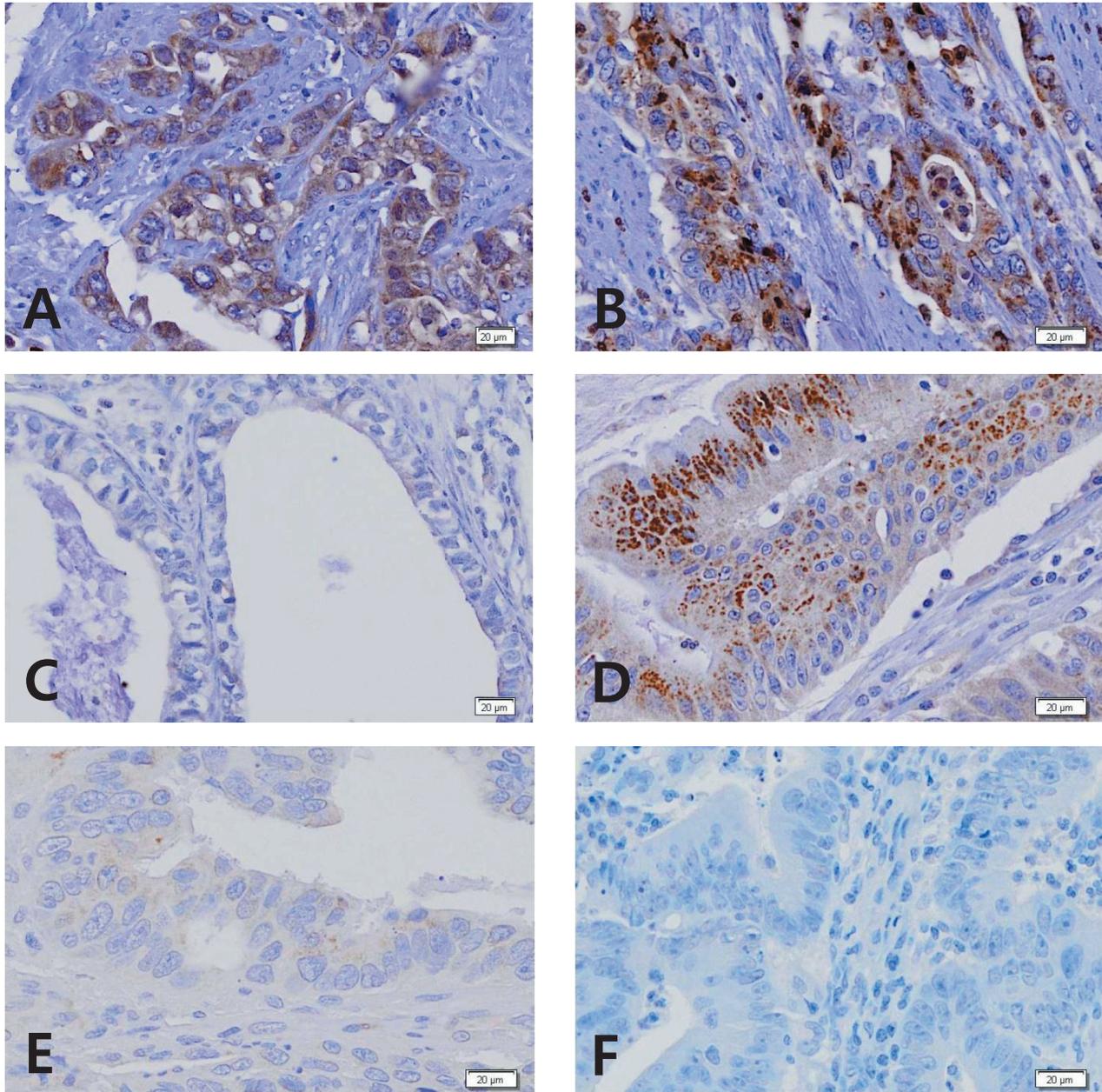


Figure 2. Visualization of *MLL3* expression in gastric and colorectal cancer tissues with MSI-H by immunohistochemistry. A: A gastric cancer without mutation in evaluated A9 repeat shows *MLL3* immunostaining in both cytoplasm and nuclei of the cancer cells. B: Another gastric cancer with a frameshift mutation in *MLL3* shows *MLL3* immunostaining mainly in cytoplasm of the cancer cells. C: In a gastric cancer with a frameshift mutation in *MLL3*, the cancer cells are negative for *MLL3* immunostaining. D: A colon cancer without mutation in evaluated A9 repeat shows *MLL3* immunostaining mainly in cytoplasm of the cancer cells. E: In another colon cancer without mutation in evaluated A9 repeat, the cancer cells are negative for *MLL3* immunostaining. F: A negative control of the immunostaining with omission of primary antibody in a colon cancer shows no *MLL3* immunostaining. (Scale bars: 20 μm)

cancers in gastrointestinal tract. We found frameshift mutations of *MLL*, *MLL2*, *MLL3* and *MLL5* genes in both GC and CRC. These mutations were found exclusively in the cancers with MSI-H, but not in those with MSI-L or MSS, indicating all but one *MLL* genes are altered in the GC and CRC with MSI-H by somatic mutations.

Among the *MLL* genes with somatic mutations in the present study, all except *MLL3* mutations were novel mutations. An earlier study documented that CRC with MSI-H harbored a type of *MLL3* frameshift mutation (c.8390delA) in 24.5% of the cases [18]. In our study, we found *MLL3* frameshift mutations in GC with MSI that had not been reported yet. In addition to the one base deletion (c.8390delA), we found one base insertion (c.8390insA) and two base deletion (c.8389_8390delAA) (Table 3). We found also frameshift mutation in the other repeat of *MLL3* (c.8525insA). We found *MLL3* frameshift mutations in 7.5% of CRC with MSI, which were in contrast with other study where the mutations had been detected in 24.5% of CRC [18] (Fisher’s exact test, $p = 0.024$). By contrast, the incidence in GC was not statistically different (Fisher’s exact test, $p > 0.05$). However, the background mechanisms of such differences between our data and the earlier data are unclear. Collectively, our data indicate that *MLL3* frameshift mutation may be common not only in CRC with MSI, but also in GC with MSI, and suggest that *MLL3* frameshift mutation might contribute to pathogenesis of cancers with MSI.

MLL gene is altered by rearrangements in leukemias and contributes to leukemogenesis [7-9]. Because such rearrangements have not been found in solid cancers and *MLL* gene expression is not limited to hematopoietic system, we hypothesized that *MLL* gene could be altered by other mechanisms. The frameshift mutations identified in this study would lead to premature stops of amino acid syntheses in *MLL* proteins and hence resembles a typical loss-of-function mutation [27-29]. Because both *MLL3* and *MLL5* are known to perform tumor suppressor functions [30, 31], the frameshift mutations may inhibit their tumor suppressor functions and contribute to tumorigenesis. By contrast, tumor-related functions of *MLL2* and *MLL4* are largely unknown. Rather, *MLL2* expression is overexpressed in colon and breast cancers [32], and *MLL4* gene is amplified in glioblastoma and pancreatic cancers [21]. These data suggest a possibility that hyperfunction of *MLL2* and *MLL4* is involved in cancer pathogenesis. It remains to be clarified, how the functions of *MLL2* and *MLL4* are different in GC and CRC with MSI, and what the consequence of the mutations in their pathogenesis is.

All of the five *MLL* genes have SET domains in the C-terminals, which are predicted to be removed by the frameshift mutations in *MLL*, *MLL2* and *MLL3* genes. The SET-domain protein methyltransferase superfamily includes all but one of the proteins known to be essential for histone methylation that is important in the regulation of chromatin structure and gene expression [33]. It becomes apparent that the histone methylation can be perturbed in disease. For example,

Table 4. Summary of *MLL3* expression in gastric and colorectal cancers.

	Positive <i>MLL3</i> expression (%)
GC with MSI-H (n=32)	10 (31.3)
CRC with MSI-H (n=40)	18 (45.0)
GC with MSI-L/MSS (n=59)	30 (50.8)
CRC with MSI-L/MSS (n=59)	28 (47.5)
MSI-H GC and CRC with <i>MLL3</i> mutation (n=17)	0 (0)
MSI-H GC and CRC without <i>MLL3</i> mutation (n=55)	28 (50.9)

a SET-domain protein SMYD3 is upregulated in colorectal and liver cancers, and its histone H3 K4 methyltransferase activity activates oncogenes associated with cell cycle [34]. Another SET domain-containing protein EZH2 is involved in metastasis of prostate and breast cancers [35]. Through our study here, we could accumulate evidence that other SET-containing genes (*MLL* genes) were also altered in GC and CRC. In the affected cancers, how the loss of SET domains is involved in cancer pathogenesis (cancer types (GC/ CRC) or MSI (MSI-H)) remains to be explained in future studies.

We observed that *MLL3* immunostainings were very weakly or not detectable in all of the cancers with *MLL3* frameshift mutations (Table 4). Because the antibody against *MLL3* had been raised by a peptide immunogen within amino acid residues 3471-3501 that would be removed by the *MLL3* frameshift mutations detected in the present study, the mutated *MLL3* proteins might not be detected in the cancer cells by the antibody. Mutations involve either one of the two alleles (heterozygous mutation) or both of the two alleles (homozygous mutation). SSCP and direct sequencing of *MLL3* in the mutated cases showed that all frameshift mutations appeared heterozygous (Figure 1), suggesting that one of the alleles was intact. Although it was possible that normal cell contamination made it difficult to conclude the mutations were heterozygous, use of a microdissection method that had been proven to procure tumor cells with nearly being devoid of normal cell contamination [36] led us to conclude that the mutations were heterozygous. In these cases, loss of the *MLL3* immunostaining in the cancers might be caused by the frameshift mutation in one allele and by the other gene silencing mechanisms in the second allele. Another possibility for the absence is that quantity of *MLL3* expression from one allele might not be enough to be detected by the antibody in the immunohistochemistry. Of note, about half (27/55) of tumors with wild-type *MLL3* showed loss of *MLL3* expression by the immunohistochemistry (Table 4). These data suggest a possibility that loss of *MLL3* expression in GC and CRC may be affected not only by frameshift mutations in the repeats, but also by other mechanisms such as epigenetic mechanisms or other somatic mutations besides the frameshift mutations.

Alteration of histone modification in chromatin has long been recognized as an important step in cancer development.

However, exact mechanisms for such deregulation are partly known. In the present study, we found somatic mutations of *MLL* genes in human GC and CRC tissues. However, the consequences of such frameshift mutations in cancer development remain unknown. It is imperative that functional studies on the mutated genes and their products using in vitro models and animal models should be further performed. Also, to see whether mutations and losses of expression of *MLL* genes are common features of cancers. Mutations and expression status of the genes should be further extended even in other cancers.

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