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ORIGINAL ARTICLE

Intratumoral Heterogeneity of Frameshift Mutations in *MECOM* Gene is Frequent in Colorectal Cancers with High Microsatellite Instability

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Abstract *MECOM* gene, also known as *EVI,* encodes a transcriptional regulator involved in hematopoiesis, apoptosis, development and proliferation. In blood system, *MECOM* is considered an oncogene, but in solid tumors it has both oncogenic and tumor suppressor activities. Low frequent somatic mutations of *MECOM* have been detected in many cancers including colorectal cancers (CRC), but the mutation status with respect to the microsatellite instability (MSI) has not been studied. There is an A7 mononucleotide repeat in *MECOM* coding sequences that could be a mutation target in the cancers with MSI. We analyzed the A7 of *MECOM* in 79 CRCs with high MSI (MSI-H) and 65 microsatellite stable/low MSI (MSS/MSI-L) CRCs by single-strand conformation polymorphism analysis and DNA sequencing. Overall, we found *MECOM* frameshift mutations in 6 (7.6 %) CRCs with MSI-H, but not in MSS/MSI-L cancers (0/65) ($p < 0.025$). We also analyzed intratumoral heterogeneity (ITH) of the *MECOM* frameshift mutation in 16 CRCs and found that four CRCs (25.0 %) harbored regional ITH of the frameshift mutations. Our data indicate that *MECOM* gene harbors both somatic frameshift mutations and mutational ITH, which together may be features of CRC with MSI-H.

Keywords MECOM · Frameshift mutation · Colon cancer · Microsatellite instability · Intratumoral heterogeneity

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Introduction

MECOM gene, also known as *EVI,* encodes a transcriptional regulator that may be involved in hematopoiesis, apoptosis, development, differentiation and proliferation [1–4]. The encoded protein of *MECOM* interacts with TGF-β, PI3K/AKT and JNK signaling components [5–7]. *MECOM* frequently translocates with *AML1* gene, resulting in overexpression of this gene and promotes leukemogenesis [8–10]. From these observations, MECOM is considered an oncoprotein in hematopoietic system. In solid tumors, however, MECOM has shown both oncogene and tumor suppressor gene (TSG) functions. Overexpression of MECOM protein is observed in many types of cancers, including ovary and colorectal cancers (CRCs) compared to the corresponding normal tissues [11]. By contrast, amplification of *MECOM* gene is associated with favorable prognosis of ovarian cancer patients [12]. Malignant melanomas harbor *MECOM* somatic mutations (16 % of the cases) that include truncating (1/4 of the mutations) as well as missense mutations (3/4) [13]. Together, these data suggest that alterations of *MECOM* are possibly involved in tumorigenesis by either upregulation or downregulation. However, inactivating mutation status of *MECOM* remains unknown in most carcinomas along with their pathologic features.

In the genome database, we observed that *MECOM* gene had a mononucleotide repeat (A7) in its coding sequences that could be a target for frameshift mutation (loss-of-function mutation) in cancers with microsatellite instability (MSI). Frameshift mutation of genes containing mononucleotide repeats is a feature of CRC with MSI [14], but *MECOM* frameshift mutations in CRC with MSI has not been explored. In this study, we analyzed the repeat of *MECOM* gene in CRC. Intratumoral heterogeneity (ITH) is a cancer hallmarks and results from cancer evolution [15–17]. Understanding ITH

status is important in determining diagnostic and therapeutic strategies that may influence on clinical outcome of the cancer patients. In this study, we also studied the genetic ITH of *MECOM* mutation in CRC.

Materials and Methods

Tissue Samples and Microdissection

For mutation analysis, 144 sporadic CRCs were used in this study. The CRCs consisted of 79 CRCs with high MSI (MSI-H) and 65 CRCs with microsatellite stable/low MSI (MSS/MSI-L). The MSI evaluation system used five mononucleotide repeats (BAT25, BAT26, NR-21, NR-24 and MONO-27), tumoral MSI status of which was characterized as: MSI-H, if two or more of these markers show instability, MSI-L, if one of the markers shows instability and MSS, if none of the markers shows instability [18]. For 16 out of the 79 CRCs with MSI-H, we collected three to seven different tumor areas and one normal mucosal area from each fresh CRC specimen to analyze the mutational ITH. The tumor areas were 0.027–1 cm^3 and at least 1.0 cm apart from each other. To confirm that these multi-regional biopsies were all areas of carcinoma (as opposed to areas of normal or dysplasia), they were frozen, stained with hematoxylin and eosin and examined under light microscope. The tumor cell purities of the ITH tissues were at least 70 %. These three to seven different tumor areas in the 16 CRCs were used for detecting regional ITH of *MECOM* gene. Pathologic features of the cancers are summarized in Table 1. The histologic features of CRC with MSI-H, including mucinous histology, tumor infiltrating lymphocytes, medullary pattern, and Crohn's like inflammation, were evaluated in all blocks of all cases by a pathologist. Malignant cells and normal cells were selectively procured from hematoxylin and eosin-stained slides using a 30G1/2 hypodermic needle by microdissection as described previously [19, 20]. DNA extraction was performed by a modified single-step DNA extraction method by proteinase K treatment. Approval of this study was obtained from the Catholic University of Korea, College of Medicine's institutional review board for this study.

Table 1 Summary of pathologic features of the colorectal cancers

Feature	MSI-H	MSS/MSI-L
Colorectal carcinomas		
Total cases	79	65
TNM stage		
I	15	16
II	29	28
III	32	18
IV	3	3
Location		
Cecum	16	0
Ascending colon	46	3
Transverse colon	14	2
Descending & sigmoid colon	3	27
Rectum	0	33

TNM tumor, lymph node, metastasis, *MSI-H* high microsatellite instability, *MSI-L* low microsatellite instability, *MSS* stable microsatellite instability

Single Strand Conformation Polymorphism (SSCP) Analysis

We analyzed mononucleotide repeats of *MECOM* in their coding sequences (an A7 in exon 8). Genomic DNA from the microdissected cells was isolated, and was amplified by polymerase chain reaction (PCR) with specific primer pairs. Radioisotope ([^{32}P]dCTP) was incorporated into the PCR products for detection by autoradiogram. After SSCP, mobility shifts on the SSCP gels (FMC Mutation Detection Enhancement system; Intermountain Scientific, Kaysville, UT, USA) were determined by visual inspection. Direct DNA sequencing reactions in both forward and reverse sequences were performed in the cancers with the mobility shifts in the SSCP using a capillary automatic sequencer (3730 DNA Analyzer, Applied Biosystem, Carlsbad, CA, USA). When mutations in the genes were suspected by SSCP, analysis of an independently isolated DNA from another tissue section of the same patients was performed to exclude potential artifacts originated from PCR. Other procedures for PCR-SSCP were described in our previous reports [19, 20].

Results and Discussion

Genomic DNAs isolated from tumor and matched normal tissues of 144 CRCs were studied for detection of mutations in the nucleotide repeats (an A7 in exon 8) of *MECOM* gene by PCR-SSCP analysis. In the SSCP, we found aberrantly migrating bands in six cases (Fig. 1 and Table 2). DNA from matched normal tissues showed no evidence of aberrant migration in SSCP, indicating the mutations had risen somatically (Fig. 1a). Direct DNA sequencing of the cancer tissues with the aberrant bands on SSCP confirmed that they represented somatic mutations of *MECOM* gene (Fig. 1b). All of the mutations detected were interpreted as heterozygous according to the SSCP and direct sequencing analyses (Fig. 1a and b). The mutations were deletion mutations in the A7 repeat that would cause a premature stop, which would lead to termination of amino acid translation (Table 2).

The mutations were detected in cancers with MSI-H, but not in those with MSS/MSI-L (Table 2). There was a statistical difference in the frameshift mutation frequencies between the

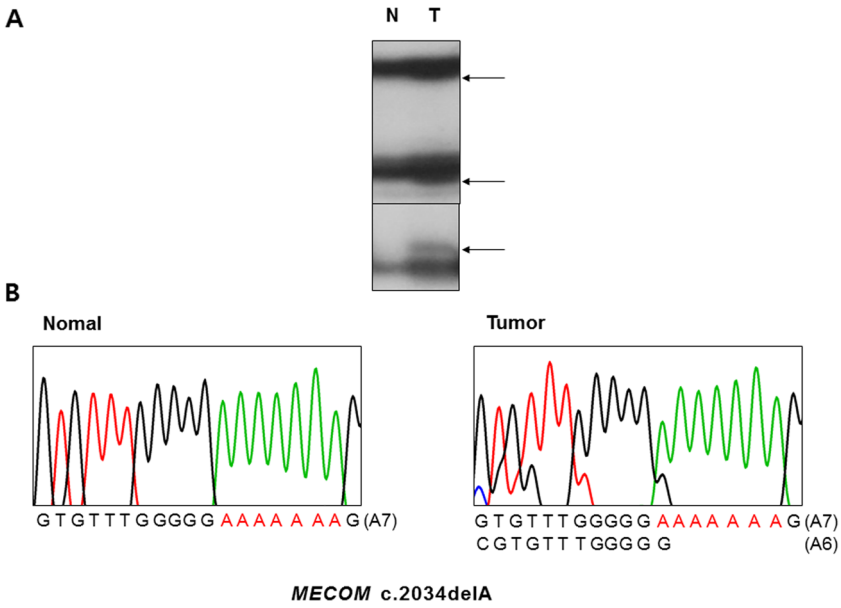

Fig. 1 Representative SSCP and DNA sequencings of the *MECOM* mutation in colon carcinomas. SSCP (A) and DNA sequencing analyses (B) of *MECOM* from tumor (Lane T) and normal tissues (Lane N). **a** SSCP of the PCR products from the A7 repeat shows aberrant bands (arrows in lane T) as compared to SSCP from matched normal tissues (N). **b** Direct DNA sequencing analyses (B) show heterozygous A deletion in the A7 in tumor tissues as compared to the normal tissues

cancers with MSI-H (6/79) and MSS/MSI-L (0/65) (Fisher's exact test, $p < 0.025$). There was no significant association of the mutations with the clinicopathologic data of the patients (age, sex, histologic grade and stage). In the cancers with MSI-H, there was no correlation between histological features of the tumors (histologic grade, subtypes, mucinous histology, medullary pattern and tumor-infiltrating lymphocytes) and presence of the mutations.

From 91 regional fragments of 16 CRCs (3–7 fragments per case) with MSI-H were collected and analyzed with respect to their regional status of the frameshift mutations of *MECOM*. The A7 repeat exhibited the deletion (A7 to A6) mutation in four of the 16 CRCs. All of the four CRCs harbored ITH of the deletion mutation (# 39, 45, 49 and 51). For example, a CRC case (#45) showed the deletion mutation in five (45–1, −2, −3, −4 and −6) of seven regional areas (Table 3 and Fig. 2).

The Pan-Cancer analyses integrated overall genome sequencing data across diverse cancer types and identified genetic commonalities [18–20]. Many mutations that occurred at low frequencies in a single cancer type were shared by sets of types and were considered significantly mutated genes [21–23]. *MECOM* mutations have been observed in many cancers with low to moderate incidences (0.87 % in CRCs) [23], but they became a driver by the Pan-Cancer analysis (search at www.intogen.org). In CRCs, all non-silent mutations were missense mutations in the database. In the present study, however, we discovered frameshift mutations of *MECOM* in 7.6 % CRCs with MSI-H (Table 2). These data show that MECOM mutation is rare in CRCs, but relatively common in CRCs with MSI-H. The frameshift mutations would alter and delete amino acids after the frameshift mutation and hence resembled a typical loss-of-function mutation. Presence of the frameshift mutation in CRCs with MSI-H suggests that loss of MECOM might be related to CRCs with MSI-H, but not those with MSS. Previous studies observed that MECOM has both oncogenic [5–7] and TSG activities [12], suggesting that it's function may be tumor type-specific. We identified the frameshift mutations in cancers with MSI-H, but not in those with MSS/MSI-L, suggesting the association of the mutations with MSI-H is specific and the mutations might possibly contribute to the MSI-H CRC development. However, there is no correlation of the mutations with clinicopathologic characterics of the CRCs with MSI-H. Studies on a large number of CRCs with MSI-H may be needed to explore the association.

In the present study we observed ITH of the *MECOM* mutations in four of 16 CRCs (25.0 %). These data are in agreement with previous reports that showed mutational ITH within coding genes in CRCs with MSI-H [24]. A cancer begins with clonal expansion of a single cell, but becomes heterogeneous after sub-clonal expansion, which results in ITH [15–17]. Presence of ITH may influence on clinical outcome of the cancer patients by impeding proper diagnosis and

Table 2 Summary of *MECOM* frameshift mutations in colorectal cancers

Location	Wild type	Mutation	MSI status of the mutation cases (n)	Incidence in MSI-H cancers (%)	Nucleotide change (predicted amino acid change)
Exon 8	A7	A6	MSI-H (6)	Colorectal: 6/79 (7.6)	c.2034delA (p. Gly679GlufsX30)

Table 3 Intratumoral heterogeneity of *MECOM* frameshift mutations in colorectal cancers

Case	Regional biopsy sites							Mutation status	ITH status
	#1	#2	#3	#4	#5	#6	#7		
CRC3	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	n.d.	Wild type	-
CRC15	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	-
CRC26	Wild type	Wild type	n.d.	Wild type	Wild type	Wild type	Wild type	Wild type	-
CRC27	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	-
CRC34	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	-
CRC35	Wild type	n.d.	n.d.	n.d.	n.d.	Wild type	Wild type	Wild type	-
CRC39	Wild type	Wild type	Wild type	Wild type	n.d.	Wild type	c.2034delA	Mutation	ITH
CRC41	n.d.	n.d.	Wild type	Wild type	n.d.	Wild type	Wild type	Wild type	-
CRC43	Wild type	Wild type	n.d	n.d.	n.d.	Wild type	n.d.	Wild type	-
CRC45	c.2034delA	c.2034delA	c.2034delA	c.2034delA	Wild type	c.2034delA	Wild type	Mutation	ITH
CRC47	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	-
CRC48	Wild type	n.d.	n.d.	Wild type	Wild type	n.d.	Wild type	Wild type	-
CRC49	n.d.	Wild type	Wild type	c.2034delA	Wild type	Wild type	Wild type	Mutation	ITH
CRC51	c.2034delA	Wild type	Wild type	n.d.	Wild type	Wild type	c.2034delA	Mutation	ITH
CRC53	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	-
CRC55	Wild type	Wild type	n.d.	n.d.	Wild type	Wild type	Wild type	Wild type	-

ITH intratumoral heterogeneity, *n.d.* not done

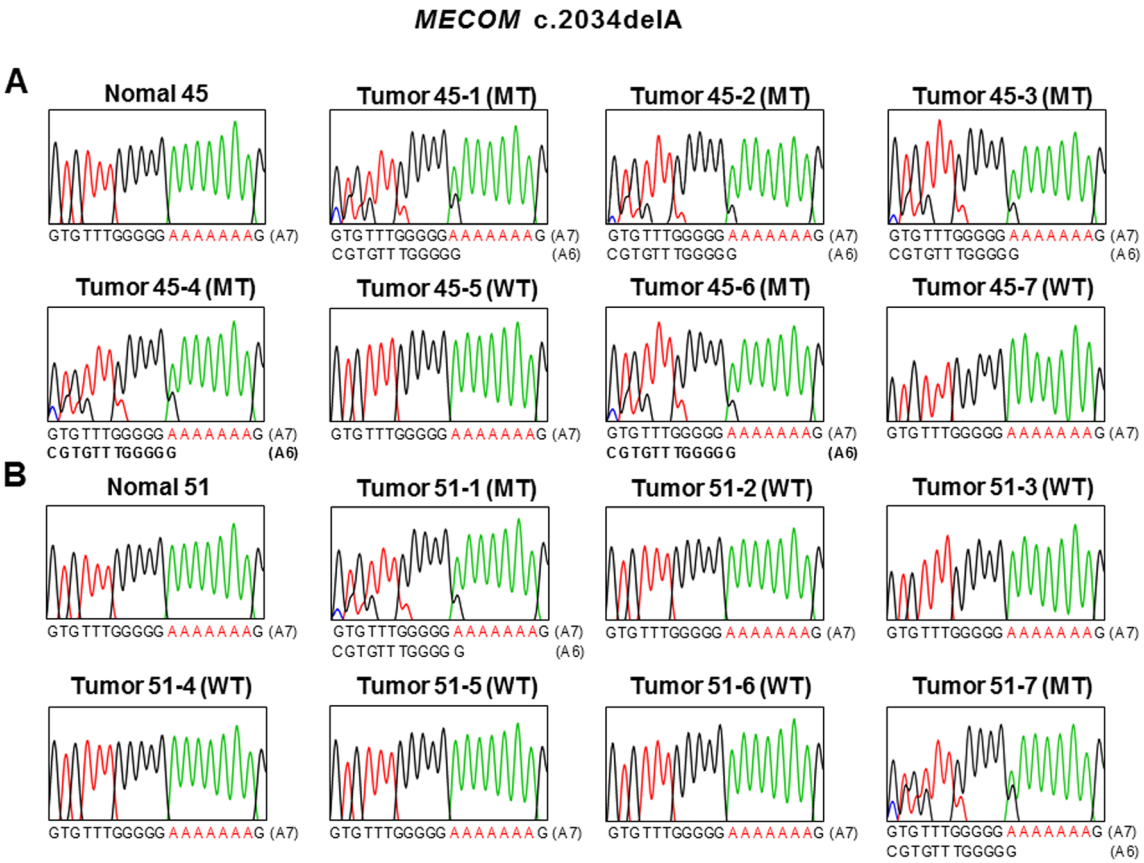

Fig. 2 Intratumoral heterogeneity of the *MECOM* frameshift mutations in colon cancers. **a** Direct DNA sequencings show *MECOM* c.2034delA mutation (MT) in five regional areas (45–1, −2, −3, −4 and −6) and wild-type (WT) in the other two areas (45–5 and −7). **b** Direct DNA sequencings show *MECOM* c.2034delA mutation (MT) in two regional areas (51–1 and −7) and wild-type (WT) in the other five areas (51–2, −3, −4, −5 and −6)

therapy. A low level mutation with a potential to metastasize may achieve clonal dominance during the progression and affect treatment efficacy [17]. One possible way to address is to analyze survival data to find out if the mutation is associated with clinical outcomes. However, survival data of the patients are not available in many patients, especially in the CRCs with ITH due to short follow-up periods after surgeries (less than 5 years). Also, due to the small number of CRCs with the *MECOM* mutational ITH ($n = 4$), we were not able to define clinical feature of the ITH in this study. For pathology practice, our data suggest that there may be under- or over-estimation of frameshift mutations in the repeat sequences in CRCs with MSI-H. Therefore, when performing mutation analysis in MSI-H cancers, multiple biopsies should be taken into account for a better evaluation of the mutation status.

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