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RESEARCH

Frameshift Mutations of *MUC15* Gene in Gastric and its Regional Heterogeneity in Gastric and Colorectal Cancers

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Abstract Mucins are important in tumorigenesis and expressional alterations of mucins are common in human cancers. A membrane-bound mucin MUC15 and secreted mucins MUC4 and MUC7 are known to involve in tumorigenesis, but their mutation status in cancers remains unknown. Aim of this study was to explore whether *MUC4*, *MUC7* and *MUC15* genes are mutated and expressionally altered in gastric (GC) and colorectal cancers (CRC). In a public database, we found that *MUC15* and *MUC7* genes had mononucleotide repeats in the coding sequences that might be mutation targets in the cancers with microsatellite instability (MSI). We analyzed the mutations in 90 GC and 141 CRC (high MSI (MSI-H) or stable MSI/low MSI (MSS/MSI-L)) by single-strand conformation polymorphism analysis and DNA sequencing. In the present study, we found *MUC15* frameshift mutations (14.7% of GC and 15.2% of CRC with MSI-H), *MUC 7* frameshift mutations (2.9% of GC with MSI-H) and *MUC4* frameshift mutations (8.8% of GC and 3.8% of CRC with MSI-H). These mutations were not found in in MSS/MSI-L (0/118). Additionally, we analyzed intratumoral heterogeneity (ITH) of *MUC15* mutation in 16 CRC and found that seven CRC (43.8%) harbored regional ITH of *MUC15*. We also analyzed MUC15 expression in GC and CRC by immunohistochemistry. Negative MUC15 expression was identified in 15–41% of the GC and CRC irrespective of MSI status. Of note, the negative expression was more common in those with *MUC15* mutations. We identified alterations of MUC genes at various levels (frameshift mutations, genetic ITH and expression loss), which together might play a role in tumorigenesis of GC and CRC with MSI-H. Our data suggest that mutation analysis in multiple regions is needed for a better evaluation of mutation status in CRC with MSI-H.

Keywords MUC15 · MUC7 · Heterogeneity · Mutation · Cancer · Microsatellite instability

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Introduction

Mucin is a glycoprotein that constitutes the major components of mucus. Mucin family genes are categorized into secreted mucins (MUC2, MUC5AC, MUC5B, MUC6, MUC7, MUC8 and MUC19) and membrane-bound mucins (MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17 and MUC20) [1–5]. The secreted mucins are secreted to outside of the cells and) provide lubrication and protection for mucosa [1]. The membrane-bound mucins serve as sensors of external environment into cells as well as a protective barrier [1].

Expression of the MUC genes is frequently altered during cell differentiation, inflammation and tumorigenesis [1–5]. For example, MUC2, the major secreted mucin, protects mucosal cells from inflammation and tumorigenesis [6]. Also, altered expression of membrane-bound mucins accompanies cancer development and is involved cancer properties, including proliferation, adhesion, migration and invasion [4]. For example, MUC4, MUC12 and MUC13 are downregulated in colorectal cancer (CRC), whereas MUC1 is upregulated [7–10]. MUC15 is a membrane-bound mucin, expression of which has been identified in many organs, including colon, small intestine and lymph node [11, 12]. Expression of

MUC15 is upregulated in CRC compared with their normal counterparts [13]. Experimentally, MUC15 overexpression activates ERK1/2 signaling and confers enhanced proliferation and invasion with cancer cells [13], suggesting that MUC15 promotes oncogenic potentials. MUC7 is expressed in a limited tissue category, including salivary glands, bronchial glands and pancreas [14, 15]. MUC7 expression is highly upregulated in transitional cell carcinoma (TCC) and detection of MUC7 in lymph nodes of TCC patients is proven to a sensitive and specific method for TCC metastasis [16, 17].

In a public genome database (http://genome.cse.ucsc.edu/), we found that *MUC4*, *MUC7* and *MUC15* genes have mononucleotide repeats in their coding sequences that could be targets for frameshift mutation in cancers with microsatellite instability (MSI). Frameshift mutations of genes with mononucleotide repeats are features of gastric (GC) and CRC with microsatellite instability (MSI) [18]. Frameshift mutations in *MUC4*, *MUC7* and *MUC15* might cause alterations of their functions and contribute to cancer pathogenesis. However, the data on mutations in *MUC4*, *MUC7* and *MUC15* genes are known neither in GC nor CRC. In this study, we analyzed the mononucleotide repeats in *MUC4*, *MUC7* and *MUC15*.

A cancer is established by clonal expansion of a single cell, but becomes heterogenous. Such an intratumoral heterogeneity (ITH) contributes to acquire aggressiveness in cancers and may impede accurate diagnosis and proper selection of cancer therapies [19]. In this study, we analyzed ITH of *SMUC15* mutation in GC and CRC with MSI.

Materials and Methods

Tissue Samples and Microdissection

For the mutation analysis, methacarn-fixed tissues of 90 sporadic GC and 141 sporadic CRC were used in this study. All of the patients with the cancers were Koreans. The GC consisted of 34 GC with high MSI (MSI-H), 56 GC with stable MSI/low MSI (MSS/MSI-L), 79 CRC with MSI-H and 62 CRC with 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 [20]. For 54 CRC of the 141 CRC described above, we collected four to seven different tumor areas and one normal mucosal area from each fresh CRC specimen. The tumor areas were 0.027–1 cm^3 and at least 1.0 cm apart from each other. Sixteen of the 54 CRC were identified as MSI-H. These four to seven different tumor areas in the 16 CRC were used for detecting regional heterogeneity of *MUC15* gene.

The 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 [21, 22]. 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.

Single Strand Conformation Polymorphism (SSCP) Analysis

MUC15 exon 3 (A9 repeat), *MUC7* exon 4 (C7) and *MUC7* exon 16 (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 specific primer pairs. Radioisotope ([^{32}P]dCTP) was incorporated into the PCR products for detection by

Table 1 Summary of pathologic features of the cancers

No. of gastric carcinomas				No. of colorectal carcinomas			
		MSI-H (n=34)	MSS/MSI-L (n=56)			MSI-H (n=79)	MSS/MSI-L (n=62)
TNM	I	13	19	TNM	I	15	9
	II	13	22		II	29	21
	III	7	12		III	32	29
	IV	1	3		IV	3	3
Lauren's subtype	Diffuse	20	31	Location (colon)	Cecum	16	0
	Intestinal	14	25		Ascending	46	3
					Transverse	14	3
EGC Vs. AGC	EGC	3	4		Descending & sigmoid	3	23
	AGC	31	52		Rectum	0	33

EGC early gastric cancer, *AGC* advanced gastric cancer, *TNM* tumor, lymph node, metastasis

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 *MUC15*, *MUC7* and *MUC4* 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.

Immunohistochemistry

Using the sections from GC and CRC tissues, immunohistochemistry for MUC15 was performed. The tissues consisted of 32 GC and 47 CRC with MSI-H, and 59 GC and 53 CRC with MSS/MSI-L. We used ImmPRESS System (Vector Laboratories, Burlingame, CA, USA) and antibody for human MUC15 (Abnova, Taipei, Taiwan; dilution 1/50). The immunohistochemical procedure was performed as described previously [23, 24]. The staining intensity was graded as follows: 0, negative; 1+ when the cells showed weak staining in cytoplasm; 2+, moderate; and 3+, intense. The extent was graded according to the percentage of positive cells as follows: 0, 0–5%; 1, 6–19%; 2, 20–49%; 3, > 50%. The percentage of positive cells and the staining intensity were then multiplied to generate the immunohistochemistry score (IS). We categorized the IS 0–3 as negative, 4–5 as+and 6–9 as ++. Both+and ++ were considered positive. For the statistical analysis of the immunohistochemical data, we used Fisher's exact test and χ^2 test.

Results

Mutational Analysis

Genomic DNAs isolated from normal and tumor tissues of the 90 GC and 141 CRC were analyzed for detection of mutation in *MUC15* (exon 3 (A9), *MUC7* (exon 4 (C7) and *MUC4* (exon 16 (A7) by PCR-SSCP analysis. On the SSCP, we observed aberrant bands in 17 cases of *MUC15*, one case of *MUC7* and six cases of *MUC4* (Table 2). DNA from normal tissues from the same patients showed no evidence of aberrant migration in SSCP, indicating the mutations had risen somatically. All of the mutations were interpreted as heterozygous according to the SSCP and direct sequencing analyses. Direct DNA sequencing analyses of the cancer tissues with aberrantly migrating bands confirmed that the aberrant bands represented somatic mutations of *MUC15*, *MUC7* and *MUC4* genes. All of the mutations were deletion or duplication mutations of bases in the repeats that would cause premature stop codons, which lead to the termination of translation (Table 2). Five of 34 GC (14.7%) and 12 of 79 CRC (15.2%) with MSI-H harbored *MUC15* frameshift mutations, while three of 34 GC (8.8%) and three of 79 CRC (3.8%) with MSI-H harbored *MUC4* frameshift mutations. *MUC7* frameshift mutation was detected in one of 34 GC (2.9%) with MSI-H.

The mutations were detected in the cancers with MSI-H, but not in those with MSS/MSI-L (Table 2). There was a statistical difference in the *MUC15* frameshift mutation frequencies between the cancers with MSI-H (17/113) and MSS/MSI-L (0/118) (Fisher's exact test, $p<0.001$). Also, there was a statistical difference in the *MUC4* frameshift mutation frequencies between the cancers with MSI-H (6/113) and MSS/MSI-L (0/118) (Fisher's exact test, $p=0.013$). There was no significant association of the mutations with the clinicopathologic data of the patient (age, sex, histologic grade and stage) (χ^2 test, $p>0.05$).

Regional Heterogeneity of MUC15 Mutation

Seven of the 16 CRC (43.8%) harbored *MUC15* frameshift mutations in two or more fragments. Of note, all of the seven CRC with the mutations showed regional ITH of the *MUC15* frameshift mutation, meaning that none of them showed uniform patterns of the mutations across the fragments (Table 3). Six CRC harbored a mutation type (c.60delA) while one CRC harbored two mutation types (c.60delA and c.59delAA).

Table 2 Summary of *MUC15*, *MUC7* and *MUC4* mutations in gastric and colorectal cancers

Gene	Location	Wild type	Mutation	MSI status of the mutation cases (n)	Incidence in MSI-H cancers (%)	Nucleotide change (predicted amino acid change)
MUC15	Exon 3	A9	A7	MSI-H (1)	Colorectal: 1/79 (1.3)	c.59_60delAA (p.Lys20Thrfsx47)
MUC15	Exon 3	A9	A8	MSI-H (14)	Gastric: 4/34 (11.8) Colorectal: 10/79 (12.7)	c.60delA (p.Lys20Asnfsx12)
MUC15	Exon 3	A9	A10	MSI-H (2)	Gastric: 1/34 (2.9) Colorectal: 1/79 (1.3)	c.60dupA (p.Pro21Thrfsx47)
MUC4	Exon 16	A7	A6	MSI-H (6)	Gastric: 3/34 (8.8) Colorectal: 3/79 (3.8)	c.14705delA (p.Asn4902Thrfsx8)
MUC7	Exon 4	C7	C6	MSI-H (1)	Gastric: 1/79 (2.9)	c.246delC (p.Lys83Asnfsx23)

Table 3 Intratumoral heterogeneity of MUC15 mutations in colorectal cancers

Case	Regional biopsy sites						
	#1	#2	#3	#4	#5	#6	#7
CRC3	c.60delA	c.60delA	c.60delA	c.60delA	c.60delA	Wild type	n.d.
CRC15	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
CRC27	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type
CRC34	Wild type	Wild type	Wild type	c.60delA	c.60delA	Wild type	Wild type
CRC35	Wild type	Wild type	n.d.	n.d.	n.d.	Wild type	Wild type
CRC39	Wild type	Wild type	Wild type	Wild type	n.d.	Wild type	Wild type
CRC41	Wild type	n.d.	Wild type	Wild type	n.d.	Wild type	Wild type
CRC43	c.60delA	Wild type	Wild type	n.d.	n.d.	Wild type	n.d.
CRC45	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type
CRC47	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type
CRC48	c.60delA	n.d.	n.d.	Wild type	c.60delA	c.60delA	c.60delA
CRC49	n.d.	Wild type	c.60delA	Wild type	Wild type	c.60delA	c.60delA
CRC51	Wild type	Wild type	c.60delA	Wild type	Wild type	Wild type	Wild type
CRC53	c.60delA	c.60delA	Wild type	Wild type	Wild type	c.59delAA	c.60delA
CRC55	Wild type	Wild type	n.d.	n.d.	Wild type	Wild type	Wild type

Immunohistochemical Analysis

Immunopositivity for MUC15 was observed in 67 (74.4%) of the GC and 100 (70.9%) of the CRC (Table 4). The MUC15 expression was significantly different with respect to the MSI status (MSI-H Vs. MSI-L/MSS) (Fisher's exact test, $p<0.01$), but not with respect to the origin (GC Vs. CRC) (Fisher's exact test, $p>0.05$) (Table 4). Of the 17 cancers with *MUC15* frameshift mutations, all except one (16/17) showed a very weak or a negative MUC15 immunostaining, and there was a significant difference of MUC15 immunostaining between MSI-H cancers with *MUC15* frameshift mutations and those without *MUC15* frameshift mutations (Fisher's exact test, $p<0.001$) (Table 4).

Table 4 Summary of MUC15 expression in gastric and colorectal cancers

	Positive expression (%) (number of subcategory positivity)
GC with MSI-H (n=34)	20 (58.8) (+: 10, ++:10)
CRC with MSI-H (n=79)	47 (59.4) (+: 21, ++: 26)
GC with MSS/MSI-L (n=56)	47 (83.9) (+: 22, ++: 25)
CRC with MSS/MSI-L (n=62)	53 (85.4) (+: 25, ++: 28)
MSI-H GC and CRC with *MUC15* mutation (n=17)	1 (5.9) (+: 1)
MSI-H GC and CRC without *MUC15* mutation (n=96)	66 (78.8) (+: 30, ++; 36)

GC gastric cancer, *CRC* colorectal cancer, *MSI-H* high microsatellite instability, *MSI-L* low microsatellite instability, *MSS* stable microsatellite instability

Discussion

It is now well known that mucin is actively involved in many cellular processes as well as in cellular protection. In cancers, altered expression of MUC genes is known to underlie tumorigenesis [1]. However, somatic mutations of these genes remain elusive in cancers. Based on earlier reports that showed *MUC15*, *MUC7* and *MUC4* genes were expressionally altered in some cancers and promoted tumorigenesis [7–10, 13, 17], we attempted to disclose whether somatic frameshift mutations of *MUC15*, *MUC7* and *MUC4* were present in GC and CRC. Since mononucleotide repeat sequences are popular targets for somatic mutations in cancers with MSI-H, we focused our analysis within the repeats in *MUC15*, *MUC7* and *MUC4* genes. In the present study, we found *MUC15* frameshift mutations (14.7% of GC and 15.2% of CRC with MSI-H), *MUC 7* frameshift mutations (2.9% of GC with MSI-H) and *MUC4* frameshift mutations (8.8% of GC and 3.8% of CRC with MSI-H). In addition, there was a significant difference of the mutation frequencies between the cancers with MSI-H and MSS/MSI-L, indicating that associations of the MUC gene mutations with MSI-H were specific. Also, we analyzed tissue expression of MUC15 protein in GC and CRC by immunohistochemistry, and observed that there was a significant difference in them between those with *MUC15* mutation and

those without the mutation. These results indicate that *MUC15*, *MUC7* and *MUC4* genes are altered in GC and CRC with MSI-H by somatic frameshift mutation and that the *MUC15* mutation might underlie the expression loss.

In the present study, we found three types of *MUC15* mutations, a type of *MUC4* mutations and a type of *MUC7* mutation (Table 2), all of which were novel variants. They would delete amino acids after the frameshift mutations and hence would resemble a typical loss-of-function mutation. The *MUC15* mutations would delete amino acids after the 20th or 21st residue (Table 2). Full-length MUC15 protein is a 361 amino acid-long protein. The cytoplasmic domain of MUC15 in the C-terminus possesses many phosphorylation sites and plays an important role of MUC15 in biological functions [13]. MUC15 activates ERK signaling and changes tyrosine phosphorylation of many cellular proteins, and may possibly be involved in cell signaling [15]. All of the *MUC15* mutations in our study have 1–19 or 1–20 amino acid residues (Table 2), which were devoid of the cytoplasmic domains. It is likely that the truncating mutations detected in our study would result in loss of MUC15 functions that may be provided by the cytoplasmic domain. Currently, there are two contradicting data on MUC15 functions in tumorigenesis. In colon cancer cells, MUC15 enhanced proliferation and invasion of cancer cells and possessed oncogenic potential [13]. By contrast, in hepatocellular carcinoma MUC15 expression was reduced and overexpression of MUC15 reduced proliferation and invasion of cancer cells, suggesting its role as a tumor suppressor [25]. Provided that MUC15 behaves as an oncogene, the *MUC15* frameshit mutations seem to reduce the proliferative and invasive capabilities of cancer cells, suggesting a rationale for better prognosis of CRC with MSI-H than those with MSS [26]. On the other hand, provided that MUC15 behaves as a tumor suppressor [25], the *MUC15* frameshit mutations seem to inhibit the tumor suppressive functions of MUC15 and activate the cancer-related capabilities, and contribute to CRC development. However, molecular basis for such opposite consequences in tumorigenesis remains to be clarified.

There are reports that showed genetic ITH of repeat sequences in coding genes [27]. In the present study, we found ITH of *MUC15* mutation among the CRC with *MUC15* mutation (7/16: 43.8%). As for the clinicopathologic parameters, however, there was no definite difference between the CRCs with and without *MUC15* ITH. Roles of ITH of *MUC15* mutation remain to be clarified in conjunction with the identification of biological functions of *MUC15* in cancers. The generation of ITH may have implications for predictive and prognostic biomarker strategies. For example, low frequency mutations with a potential to metastasize define clinical outcomes since the clones with ITH easily achieve clonal dominance during the progression and affect treatment efficacy [19]. In the context of pathology practice, the data also suggest that there could be under- or over-estimation of *MUC15* mutation in cancers. The data suggest that when performing mutation analysis in cancers with MSI-H, multi-regional biopsies should be considered for a better evaluation of mutations.

We observed that MUC15 immunostaining was very weak or negative in all but one cancers with its frameshift mutations (Table 4). Because the anti-MUC15 antibody had been raised using a peptide that is located between amino acids 51 and 200 that would be removed by the *MUC15* frameshift mutations, the resulting MUC15 mutants may not be detected by the antibody. SSCP and direct sequencing of the mutated cancers showed that all of the *MUC15* frameshift mutations were heterozygous, suggesting that the second alleles were intact. Lack of immunostainings in the mutated cases might be originated by the mutated allele and by any other gene silencing in the second allele. Another possibility is that quantity of MUC15 expression from one allele might not be enough to be detected by the immunohistochemistry.

It is generally agreed that MUC genes are altered in tumorigenesis, functions and alterations in detail of *MUC15*, *MUC7* and *MUC4* remains unknown. In about 15% of GC and CRC with MSI-H, we found frameshift mutations of *MUC15* that may inactivate MUC15 functions and possibly disrupt cell proliferation and invasion. Furthermore, we observed that MUC15 expression was reduced in GC and CRC with MSI-H. Our data suggest that at least in GC and CRC with MSI-H, *MUC15* is frequently altered mutationally and expressionally. However, it remains to be elucidated that roles of MUC15 as a candidate oncogene or tumor suppressor are cancer type-specific. It is, therefore, imperative that mutations and expression status of *MUC15* should be further analyzed in other cancers. In summary, our study identified mutational, expressional and ITH alterations of MUC genes in GC and CRC with MSI-H. Our study has added concerns about ITH of the mutations, which should be considered in the clinical application to GC and CRC with MSI-H.

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Conflict of Interests The authors declare no competing interests.

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