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Original contribution

Frequent frameshift mutations in 2 mononucleotide repeats of *RNF43* gene and its regional heterogeneity in gastric and colorectal cancers☆,☆☆

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Summary RNF43, an E3 ligase, inhibits Wnt signaling by removing Wnt receptors and behaves as a candidate tumor suppressor. Recent studies identified that *RNF43* gene was frequently mutated in gastric (GC), colorectal (CRC), and endometrial cancers with high microsatellite instability (MSI-H). The aim of this study is to explore whether *RNF43* gene is mutated in GC and CRC in Korean patients and whether the mutations show regional intratumoral heterogeneity (ITH). We analyzed 2 exonic repeats (C6 and G7) of *RNF43* in 78 GCs and 130 CRCs by single-strand conformation polymorphism and DNA sequencing analyses. Also, we analyzed regional ITH of *RNF43* mutation in 16 CRCs. We found *RNF43* frameshift mutation in MSI-H (50/118), the incidence of which was significantly higher than that in microsatellite stable/low microsatellite instability (1/90). GCs showed a significantly higher incidence of the mutation than CRCs (66.7% of GC and 32.9% of CRC with MSI-H). Also, we found that all of the 7 CRCs with the mutations harbored mutational ITH. By immunohistochemistry, we observed that loss of RNF43 expression was significantly more common in those with *RNF43* frameshift mutation than those with wild-type *RNF43*. Our data indicate that *RNF43* gene harbored not only exceedingly high mutations but also mutational ITH, which together might play a role in tumorigenesis of GC and CRC. We suggest that regional analysis is required for a more comprehensive evaluation of the mutation status in these tumors.

1. Introduction

Wnt signaling is an evolutionarily conserved mechanism that regulates crucial cellular processes during embryonic development [1,2]. Alterations of Wnt signaling are often linked to human diseases, including cancers [1,2]. For example, *APC* and *β-catenin* mutations in Wnt signaling play critical

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roles in colorectal cancer (CRC) development [3,4]. Also, gene fusions of R-spondins (*RSPO-2* and *RSPO-3*) that activate Wnt signaling are frequently found in CRC [5,6]. RNF43 is a transmembrane E3 ligase that negatively regulates Wnt signaling by removing Wnt receptors from cell surface [7,8]. A recent study discovered that *RNF43* was frequently mutated in CRC and endometrial cancers, especially in microsatellite-unstable tumors (79.9% in microsatellite instability high [MSI-H] CRC and 50.7% MSI-H endometrial cancers) [9]. Also, 2 other studies demonstrated a higher percentage of *RNF43* frameshift mutations in MSI-H gastric cancer (GC) (55% and 33%) [10,11]. These data suggest that *RNF43* is a tumor suppressor that is commonly inactivated in MSI-H cancers by mutations. Despite these results, clinicopathological features of *RNF43*-mutated cancers including intratumoral heterogeneity (ITH) remains elusive.

Cancer development begins through a clonal expansion of single cell [12]. The resulting cell population becomes heterogeneous after branching subclonal expansions, which may lead to ITH. The ITH contributes to tumor aggressiveness and may impede the accurate diagnosis/prognosis and proper selection of tumor therapy [13]. In this study, we analyzed frameshift mutation of *RNF43* in GC and CRC of Korean patients and discovered that ITH of the mutations was common.

2. Materials and methods

2.1. Tissue samples and microdissection

For the mutation analysis, methacarn-fixed tissues of 78 sporadic GCs and 130 sporadic CRCs were used in this study. All of the patients in this study were Koreans. They consisted of 33 GCs with MSI-H, 45 GCs with microsatellite stable (MSS)/low microsatellite instability (MSI-L), 85 CRCs with MSI-H, and 45 CRCs with MSS/MSI-L. None of the CRC cases met the minimum requirement for a clinical diagnosis of Lynch syndrome by Amsterdam II criteria [14]. The microsatellite instability (MSI) evaluation system used 5 mononucleotide repeats (BAT25, BAT26, NR-21, NR-24, and MONO-27), tumoral MSI status that was characterized as follows: MSI-H, if 2 or more of these markers show instability; MSI-L, if 1 of the markers shows instability; and MSS, if none of the markers shows instability [15]. From 16 of 85 of the CRCs with MSI-H, we collected 4 to 7 different tumor areas and 1 normal mucosal area to analyze the mutational ITH. The tumor areas were 0.027 to 1 cm^3 and at least 1.0 cm apart from each other. To confirm that these multiregional biopsies were all areas of carcinoma (as opposed to areas of normal tissue or dysplasia), they were frozen, stained with hematoxylin and eosin, and examined under light microscope. The remaining CRC samples were collected separately and were not used for ITH analyses.

The pathologic features of the cancers are summarized in Table 1. The histologic features of CRCs with MSI-H, including mucinous histology, tumor-infiltrating lymphocytes, medullary pattern, and Crohn-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 [16]. 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.

2.2. Single-strand conformation polymorphism analysis

RNF43 has a mononucleotide repeat (C6 repeat) in exon 3 and another mononucleotide repeat (G7 repeat) in exon 9. Up to now, approximately 70% of *RNF43* somatic mutations in

Table 1 Summary of pathologic features of the cancers analyzed in this study

No. of gastric cancers			No. of colorectal cancers		
	MSI-H (n =33)	MSS/MSI-L (n = 45)		MSI-H (n = 85)	MSS/MSI-L (n = 45)
TNM			TNM		
I	12	15	I	15	6
II	13	18	II	35	20
III	7	11	III	32	16
IV	1	1	IV	3	3
Lauren's subtype			Location (colon)		
Diffuse	19	25	Cecum	17	0
Intestinal	14	20	Ascending	51	3
EGC *vs* AGC			Transverse	14	2
EGC	3	4	Descending and sigmoid	3	17
AGC	30	41	Rectum	0	23

Abbreviations: MSI-H, high microsatellite instability; MSS, microsatellite stable; MSI-L, low microsatellite instability; EGC, early gastric cancer; AGC, advanced gastric cancer; TNM, tumor, lymph node, metastasis.