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SHORT COMMUNICATION

Tight Junction-Related CLDN5 and CLDN6 Genes, and Gap Junction-Related GJB6 and GJB7 Genes Are Somatically Mutated in Gastric and Colorectal Cancers

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Abstract

Tight junction and gap junction are major cell junctions that play important roles in cellular communication and structural integrity. Alterations of these junctions are known to be involved in cancer pathogenesis. Claudins and connexins are major tight and gap junction proteins, but genetic alterations of these genes have not been reported in gastric (GC) and colorectal cancers (CRC) with microsatellite instability (MSI). Claudin genes *CLDN5* and *CKDN6*, and connexin genes *GJB6* and *GJB7* have mononucleotide repeats in the coding sequences that might be mutation targets in the cancers with MSI. We analyzed 79 GCs and 145 CRCs, and found *CLDN5* frameshift mutations in 3 (3%) CRCs and 1 (2.9%) GC, *CLDN6* frameshift mutations in 6 (6%) CRCs, *GJB6* frameshift mutations in 5 (5%) CRCs and *GJB7* frameshift mutation in one CRC (1%) with high MSI (MSI-H). We also analyzed intratumoral heterogeneity (ITH) of the frameshift mutations in 16 CRCs and found that *CLDN6* and *GJB6* frameshift mutations showed regional ITH in 2 (12.5%) and 2 (12.5%) cases, respectively. Our results show that *CLDN5, CLDN6, GJB6* and *GJB7* genes harbor not only frameshift mutations but also mutational ITH, which together may be features of GC and CRC with MSI-H. Based on the roles of cellular junctions in cancers, frameshift mutations of tight junction and gap junction genes might contribute to tumorigenesis by altering their functions in GC and CRC.

Keywords *CLDN5* · *CLDN6* · *GJB6* · *GJB7* · Mutation · Cancer

Introduction

Cell junctions are specialized intercellular connections between cells that consist of tight junction, gap junction and anchoring junction [1]. Gap junctions allow for physical and chemical communication between neighboring cells [1]. Persistent gap junction perturbation can cause cancer, and many tumor promoters are known to inhibit gap junction communication [2]. Tight junctions are junctional complexes that function not only to prevent paracellular leakage but also to maintain cell to cell integrity [1]. Of note, loss of cohesion of tight junction structures can lead to invasion and metastasis of cancer cells [3]. Claudin proteins are the most important components of tight junctions and interact with each other. Connexin proteins are a family of transmembrane proteins that consist of gap junctions [1]. Many of the gap junction and tight junction genes have features of tumor suppressor genes (TSGs) [2, 3].

Both gastric (GC) and colorectal (CRC) cancers are classified high microsatellite instability (MSI-H) cancers or microsatellite stable (MSS) cancers. About 10–20% of GC and CRC are MSI-H cancers that exhibit genetic hypermutability caused by impaired DNA mismatch repair ability [4]. Many TSGs show frameshift mutations at monocleotide repeats in MSI-H cancers, which would result in suppression of TSG and promote cancer development [4]. In a genome database, we observed that claudin-encoding genes *CLDN5* and *CLDN6*, and connexin-encoding genes *GJB6* and *GJB7*

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possess nucleotide repeats in the coding exons that might be altered in MSI-H cancers. However, frameshift mutations of these in GC and CRC have not been reported. In this study, we analyzed these repeats for the detection of frameshift mutations in gastrointestinal cancers with MSI-H (GCs and CRCs).

Materials and Methods

Tissue Samples and Microdissection

For our study, we used human tissues of 34 GCs with MSI-H, 45 MSS GCs, 100 CRCs with MSI-H and 45 MSS CRCs. These cases overrepresented MSI-H cases (Table 1) compared to the ordinary incidence of MSI-H [4]. Since our study focused on mutation in MSI-H cancers, we used more MSI-H cancers during different periods. We used an MSI evaluation system with five markers (BAT25, BAT26, NR-21, NR-24 and MONO-27) [5]. The histologic features of cancers 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. In cancer tissues, malignant cells and normal cells were selectively procured by Microdissection [6].

Table 1 Summary of pathologic features of gastric and colorectal cancers

Feature	MSI-H	MSS
Gastric carcinomas		
Total cases	34	45
TNM stage		
I	13	15
II	13	18
III	7	11
IV	1	1
Lauren's subtype		
Diffuse	4	21
Intestinal	20	15
Mixed	3	6
Indeterminate	7	3
EGC vs. AGC		
EGC	3	4
AGC	31	41
Colorectal carcinomas		
Total cases	100	45
TNM stage		
I	17	6
II	41	20
III	39	16
IV	3	3
Location		
Cecum	19	0
Ascending colon	56	3
Transverse colon	18	2
Descending & sigmoid colon	6	17
Rectum	1	23

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

Single Strand Conformation Polymorphism (SSCP) Analysis

In the present study, we analyzed a G7 repeat in *CLDN5*, a G7 and a G6 in *CLDN6*, an A7 and a T4 in *GJB6* and an A7 in *GJB7* by polymerase chain reaction (PCR)-based SSCP analysis. PCR was performed with following primer sets (*CLDN5*: 5′-CGCAGCGTTGGAGATCCT-3′ and 5′-ACCA CGCACGACATCCAC-3′, *CLDN6*: 5'-CTTTTGTT GCTGGGTGGGG-3′ and 5′-TTGGTAGGGTACTC AGAGGGC-3′, *GJB6*: 5'-GTGGCAGAGTTGTGCTACCT-3′ and 5′-GCTTGGGAAACCTGTGATTGC-3′ and *GJB7*: 5'-TCTTGGTCAT CACCTCATGC-3′ and 5′-ACAT ATCTGAGGCTGTGGCA-3′). [^{32}P]dCTP was incorporated to the PCR products for visualization in autoradiogram. We determined aberrant gel motility in the SSCP (FMC Mutation Detection Enhancement system; Intermountain Scientific, Kaysville, UT, USA) using visual inspection, which subsequently sequenced by Sanger DNA sequencing (3730 DNA Analyzer, Applied Biosystem, Carlsbad, ca., USA). Other procedures in detail were described in our earlier studies [6]. In addition, to detect intratumoral heterogeneity (ITH) of the mutations, we analyzed 16 cases of MSI-H CRCs with 4 to 7 regional fragments per CRC [6]. After the surgery, a surgeon picked four to seven different tumor areas and one normal mucosal area from each fresh colectomy specimen. The tumor areas were 0.027-1 cm^3 and at least 1.0 cm apart from each other. Normal mucosae were collected at least 5 cm apart from tumor margins. All of the picked fragments from tumor and normal areas were frozen, cut, and stained with hematoxylin & eosin (H&E). Two pathologists selected areas with rich tumor cell population (at least 80%), which were subsequently used as tumor areas 1–7 in this study (Fig. 1). Also, the pathologists confirmed that none of the colectomy samples used in this study was multifocal tumors. In each patient's sample, histologic grades of the selected areas were found not to be discernable from each other by the pathologists, indicating that the selected areas represented the most common histologic patterns with minimal histologic differences in each sample. Each tumor area was further sliced into a fragment, which was subsequently used for genomic DNA extraction.

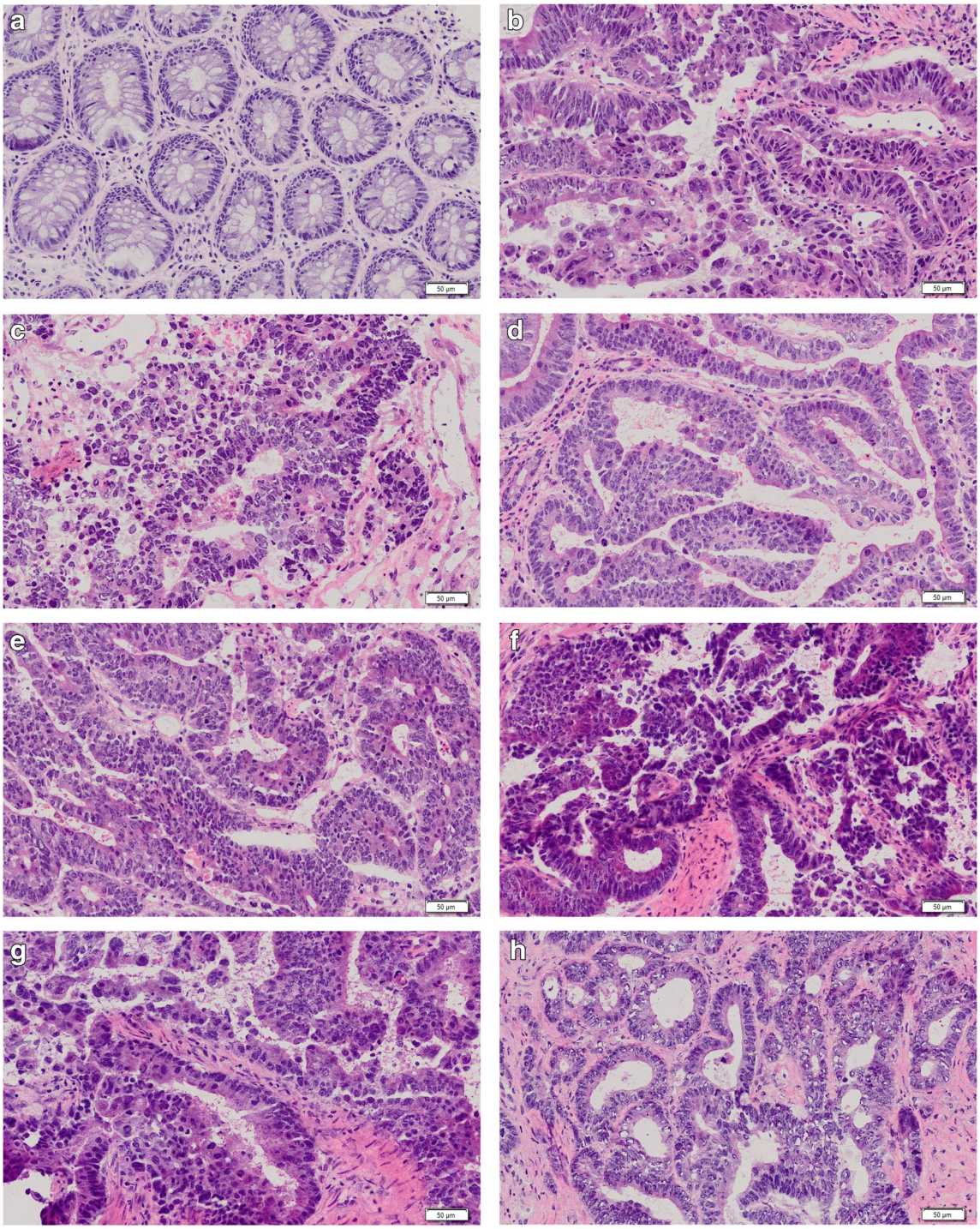

Fig. 1 Representative histologic images of the regional tissues in a colon cancer for the ITH analysis. Normal mucosa (**a**) and seven different areas of a colon cancer tissue (**b**-**h**) are shown (hematozylin and eosin stain)

Results and Discussion

In the present study, we found *CLDN5* frameshift mutations in 3 (3%) CRCs and 1 (2.9%) GC, *CLDN6* frameshift mutations in 6 (6%) CRCs, *GJB6* frameshift mutations in 5 (5%) CRCs and *GJB7* frameshift mutation in one CRC (1%) with MSH-H (Table 2). DNA from the patients' normal tissues showed no evidence of mutation in Sanger sequencing, indicating the mutations had risen somatically. These mutations were deletion or duplication of one or two base (s) in the repeats that would result in frameshift of amino acid translation (Table 2). These mutations were detected in 9 cancers (16/134) with MSI-H, but not in those with MSS (0/90) ($P < 0.01$). In addition, we analyzed these repeats in 16 cases of CRCs with 4 to

Table 2 Summary of *CLDN5, CLDN6, GJB6* and *GJB7* mutations in gastric and colorectal cancers

Gene	Wild type	Mutation	MSI status of the mutation cases (n)	Incidence in MSI-H cancers (%)	Nucleotide change (predicted amino acid change)
CLDN5	G7	G8	MSI-H (3)	Colorectal: 3/100 (3.0)	c.314dupG (p.Leu106SerfsX232)
		G6	MSI-H (1)	Gastric: 1/34 (2.9)	c.314delG (p.Gly105ValfsX2)
CLDN6	G6	G6	MSI-H (1)	Colorectal: 1/100 (1.0)	c.537delG (p.Leu180CysfsX80)
	G7	G8	MSI-H (2)	Colorectal: 2/100 (2.0)	c.567dupG (p.Ser190ValfsX24)
		G6	MSI-H (3)	Colorectal: 3/100 (3.0	c.567delG (p.Ser190ProfsX70)
GJB6	T4	T3	MSI-H (1)	Colorectal: 1/100 (1.0)	c.657delT (p.Phe219LeufsX18)
	A7	A8	MSI-H (1)	Colorectal: 1/100 (1.0)	c.689dupA (p.Asn230LysfsX11)
		A6	MSI-H (2)	Colorectal: 2/100 (2.0)	c.689delA (p.Asn230IlefsX7)
		A5	MSI-H (1)	Colorectal: 1/100 (1.0)	c.688_689delAA (p.Asn230SerfsX10)
GJB7	A7	A8	MSI-H (1)	Colorectal: 1/100 (1.0)	c.651dupA (p.Pro218ThrfsX40)

7 regional fragments per CRC to detect ITH of these mutations. Two of 16 CRCs (12.5%) revealed different mutation status of the *CLDN6* c.567dupG mutation in the regional areas (4 wild and 2 mutant areas in one CRC, 5 wild and 1 mutant area (s) in the other CRC). Another two CRCs (12.5%) exhibited ITH of the *GJB6* mutations in the regional areas (6 wild and 1 mutant area (s) in one CRC, 2 wild and 4 mutant areas in the other CRC), indicating there was ITH of the frameshift mutations (Fig. 2). We, however, could not find any significant histological difference among the ITH regions in these cases.

Downregulation of *CLDN5* in CRC and *CLDN6* in GC have been reported [7, 8]. Hypermethylation of *GJB6* is noted in 25% of CRCs [9] and *GJB6* loss is associated with advanced GC stages [10]. These earlier data suggest that the tight junction and gap junction genes might be altered by various mechanisms, which could inactivate TSG functions of these genes. The frameshift mutations (premature amino acid stops) detected in the present study resemble a typical inactivating mutation and would inactivate *CLDN5, CLDN6, GJB6* and *GJB7*, further suggesting that tight junction and gap junction genes might be inactivated by frameshift mutations.

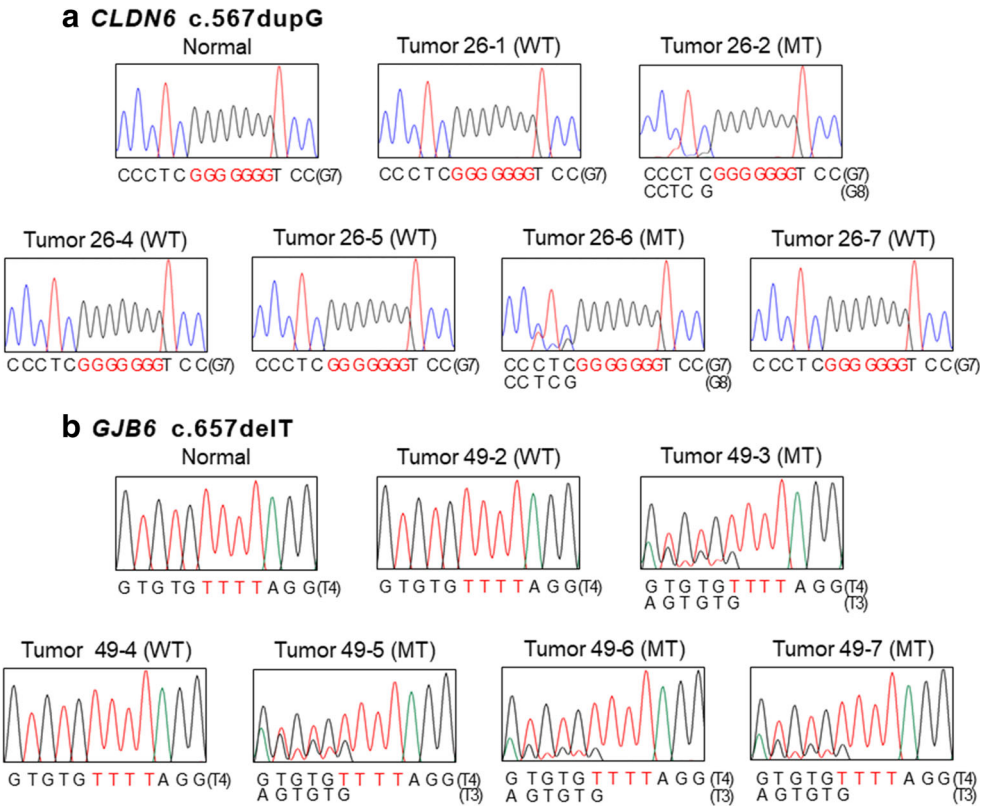

Fig. 2 Intratumoral heterogeneity of *CLDN6* and *GJB6* frameshift mutations in colon cancers. A: Direct DNA sequencings show *CLDN6* c.567dupG mutation in two regions (MT) and wild type in the other four regions (WT). B: Direct DNA sequencings show *GJB6 GJB6* c.657delT mutation in four regions (MT) and wild type in the other two regions (WT)

In the COSMIC database (https://cancer.sanger.ac.uk/cosmic) that catalogues pan-cancer genome data, somatic mutations of *CLDN5* (0-1.12%), *CLDN6* (0-2.33%), *GJB6* (0-3.96%) and *GJB7* (0-2.5%) are shown in various cancers. In the database, GCs exhibit *CLDN5* (0.47%), *CLDN6* (0.83%), *GJB6* (0.71%) and *GJB7* (0.83%) mutations while CRCs do *CLDN5* (1.12%), *CLDN6* (0.99%), *GJB6* (1.12%) and *GJB7* (1.17%) mutations. These mutation data indicate that prevalence of *CLDN5, CLDN6, GJB6* and *GJB7* mutations are different depending on the cancer types with an overall low prevalence (0-3.96%). Our prevalences in merged MSI-H and MSS cases (GC: 1.2% for *CLDN5*, 0% for *CLDN6*, 0% for *GJB6* and 0% for *GJB7*; CRC: 2.1% for *CLDN5*, 4.1% for *CLDN6*, 4.1% for *GJB6* and 0.7% for *GJB7*) revealed overall low prevalences (0-4.1%) as well, suggesting that the prevalences of tight junction and gap junction-related gene mutations in Korean patients in our study may not be different from those in worldwide data.

Also, the mutational ITH of *CLDN6* and *GJB6* in CRCs in our data may suggest that the ITH could result in a mixed effect of the inactivating mutations in the pathogenesis of MSI cancers. However, we were not able to find any discernable clinical or pathologic features ITH-positive or ITH-negative cancers, probably due to small case numbers.

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Compliance with Ethical Standards

Conflict of Interest None to declare.

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