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Colitic Cancer Develops Through Mutational Alteration Distinct from that in Sporadic Colorectal Cancer: A Comparative Analysis of Mutational Rates at Each Step

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Abstract. *Background: Patients with ulcerative colitis (UC) are at risk of UC-associated colorectal cancer (CRC); however, little is known about genetic alterations occurring during UC carcinogenesis. We examined mutational changes in patients with colitic cancer and the features that differed between the carcinogenesis of UC and sporadic CRC. Material and Methods: Specimens were obtained from the non-neoplastic mucosa and cancer cells of 12 patients with colitic cancer. The mutational rate of oncogenes in colitic cancer was analyzed and compared to that of oncogenes in sporadic CRC. Results: We observed a lower mutation rate in adenomatous polyposis coli (APC) (16.7%(2/12) vs. 75.9%(161/212), respectively, p=0.0001) and KRAS (16.7%(2/12) vs. 42% (89/212), respectively, p=0.04) in colitic cancer than in sporadic CRC. With respect to cadherin 1 (CDH1) and fibroblast growth factor receptor 2 (FGFR2), the mutational rates for non-neoplastic colorectal mucosa were similar to those in sporadic CRC. Conclusion: We demonstrated that mutational rates for APC and KRAS differ between colitic cancer and sporadic CRC. Furthermore, we revealed that CDH1 and FGFR2 become mutated at an earlier stage in colitic carcinogenesis than in sporadic CRC.*

Colorectal carcinogenesis is considered to develop through multistep genetic or epigenetic alteration along with the pathological change called the adenoma–carcinoma sequence (1-5). Further accumulation of genetic changes confers invasiveness or metastatic potential on the tumor (6, 7), and to date, several indicators have been identified to predict outcomes (7, 8). In contrast, colitic cancer, which develops through inflammation-prone carcinogenesis in patients with ulcerative colitis (UC), has a somewhat different etiology (9). UC is characterized by chronic inflammation of the colonic mucosa, and the underlying causes of inflammation are the disturbance or disorganization of the epithelial barrier, alteration of colonic microflora, and abnormal immune response caused by the dysregulation of the mucosal immune system (10). During the progression of colitis, genetic alterations associated with mucosal permeability [*e.g.* those in extracellular matrix protein 1 (*ECM1*), cadherin 1 (*CDH1*), and hepatocyte nuclear factor 4 alpha (*HNF4A*)] have been observed and considered to confer the risk of severe UC (11, 12). The pathogenesis of colitic cancer originates from long-standing and severe bowel inflammation and differs from that of sporadic CRC in several aspects. For instance, colitic cancer often occurs multifocally and is widespread; therefore, performing total coloproctectomy is recommended if any dysplastic or cancerous lesions are identified in patients with UC. Moreover, the behavior and morphology of colitic cancer differ from those of sporadic CRC in that colitic cancer often invades to deeper layers of the bowel wall at an earlier stage of progression; this feature makes it difficult to identify dysplastic lesions when the tumor elevation and size is not large. These distinctive characteristics of colitic cancer are likely to be caused by its genetic etiology, which is different from that of sporadic cancer. However, to date, little information is available in this regard.

In this study, we compared the mutation of oncogenes in colitic cancer to those in sporadic cancer. In addition, we examined the non-neoplastic mucosa of patients with colitic cancer, so as to identify the step in UC carcinogenesis in

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which mutation occurs. To our knowledge, this is the first study examining the mutational difference between colitic cancer and sporadic CRC together with non-neoplastic mucosa in UC.

Materials and Methods

Study design and patient selection. This was a retrospective study conducted at a single institute. Patients with UC were recruited from the Department of Surgical Oncology, University of Tokyo Hospital, Tokyo, Japan. This study was approved by the Ethics Committee of University of Tokyo Hospital (approval number: G3551), and patients gave their written informed consent for the storage and the use of their specimens in advance. Twelve patients with UC whose resected specimens were stored at our institute were enrolled in this study.

Sample collection and nucleic acid isolation. Specimens were obtained from carcinoma and non-neoplastic colorectal mucosa from all the patients with colitic cancer for DNA analysis. Samples were obtained either from surgically resected specimens or during surveillance colonoscopy. The samples were snap-frozen in liquid nitrogen immediately after resection and were stored at −80˚C until DNA extraction. Nuclear DNA was extracted from epithelial cells and carcinoma cells using a DNAeasy kit (Qiagen, Tokyo, Japan). The normal mucosa samples were microscopically verified by experienced pathologists as not containing neoplastic lesions. For the samples of carcinoma, the pathologists verified that the samples contained viable carcinoma cells without necrotic tissue. The concentrations of extracted double-stranded DNA were measured using a Qubit 2.0 fluorometer and Qubit proprietary reagents and dyes (Life Technologies, Grand Island, NY, USA).

Mutational analysis by next generation sequencing (NGS). Targeted resequencing was performed using the TruSeq Amplicon Cancer Panel with the MiSeq® sequencing platform according to the manufacturer's instructions (Illumina, San Diego, CA, USA). The panel contains primer sets to generate 212 amplicons from 48 cancer-related genes, which include ABL proto-oncogene 1 (*ABL1*), AKT serine/threonine kinase 1 (*AKT1*), ALK receptor tyrosine kinase (*ALK*), adenomatous polyposis coli (*APC*), ATM serine/threonine kinase (*ATM*), B-Raf proto-oncogene, serine/threonine kinase (*BRAF*), cadherin 1 (*CDH1*), cyclin-dependent kinase inhibitor 2A (*CDKN2A*), colony stimulating factor 1 receptor (*CSF1R*), beta-catenin 1 (*CTNNB1*), epidermal growth factor receptor (*EGFR*), Erb-B2 receptor tyrosine kinase 2 (*ERBB2*), Erb-B2 receptor tyrosine kinase 4 (*ERBB4*), F-Box and WD repeat domain containing 7 (*FBXW7*), fibroblast growth factor receptor 1 (*FGFR1*), *FGFR2*, *FGFR3*, Fms-related tyrosine kinase 3 (*FLT3*), G protein subunit alpha 11 (*GNA11*), *GNAQ, GNAS,* hepatocyte nuclear factor 1-alpha (*HNF1A*), HRas proto-oncogene, GTPase (*HRAS*), cytosolic isocitrate dehydrogenase 1 (*IDH1*), Janus kinase 2 (*JAK2*), *JAK3*, kinase insert domain receptor (*KDR*), KIT proto-oncogene receptor tyrosine kinase (*KIT*), Kirsten RAS proto-oncogene (*KRAS*), MET proto-oncogene (*MET*), MutL homolog 1 (*MLH1*), MPL proto-oncogene (*MPL*), *NOTCH1*, nucleophosmin (*NPM1*), NRAS proto-oncogene (*NRAS*), platelet-derived growth factor receptor alpha (*PDGFRA*), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), phosphatase and tensin homolog (*PTEN*), protein tyrosine phosphatase, non-receptor type 11 (*PTPN11*), RB transcriptional corepressor 1 (*RB1*), Ret proto-oncogene (*RET*), SMAD family member 4 (*SMAD4*), SWI/SNF-related matrix-associated actin-dependent regulator of chromatin, subfamily B, member 1 (*SMARCB1*), Smoothened, frizzled class receptor (*SMO*), SRC proto-oncogene (*SRC*), serine/threonine kinase 11 (*STK11*), tumor protein P53 (*TP53*), and Von Hippel-Lindau tumor suppressor (*VHL*). Multiplexed libraries were subjected to cluster generation using a MiSeq Reagent Kit v2 (300 cycles) in an MiSeq desktop sequencing systems (Illumina). MiSeq Reporter software performs secondary analysis on the base calls and Phred-like quality score (Qscore) generated by Real Time Analysis software during the sequencing run. Alignment of paired-end raw reads to the amplicon sequences specified in the manifest file (*Homo sapiens*, hg19, build 37.2) was performed with a banded Smith–Waterman alignment algorithm. The Somatic Variant Caller algorithm v3.5.2.1 was used for identification of variants from aligned reads. Variants were annotated by Variant Studio ver.1.0.0 on BaseSpace Cloud (Illumina). Each single variant reported in the VCF output file was evaluated for coverage and the Qscore and visualized *via* AVADIS NGS software ver.2.7 (Strand Genomics Inc., San Francisco, CA, USA). We applied a minimum threshold in Qscore of 30 (base call accuracy of 99.9%) and a sequencing depth >100 on reads, which was considered suitable for further analysis. Variants with a global minor allelic frequency of greater than 1.0% were removed because they were considered common single nucleotide polymorphisms. Copy number variations were also estimated in samples using normal reference coverage profiles *via* AVADIS NGS.

Statistical analysis and software. The mutational rates of genes in colitic cancer observed in our specimens were compared with the existing data of The Cancer Genome Atlas (TCGA) obtained from Bio-portal (13). The difference between these two categorized values was assessed by Fisher's exact or chi-square test. *p*-Values of less than 0.05 were considered to indicate statistical significance. All statistical analyses were carried out using JMP v9.02 (SAS Institute, Cary, NC, USA).

Results

Table I shows the gene mutations that were found in the 12 patients with UC. *APC*, known to occur at an early stage of sporadic colorectal carcinogenesis, was mutated in only two (16.7%) out of 12 patients with UC. Other key molecules of carcinogenesis showed moderate-to-high mutational rates in colitic cancer (*KRAS*: 16.7%, *TP53*: 58.3%, and *FBXW7*: 25.3%). These mutational changes had already occurred in non-neoplastic mucosa – albeit at lower mutational rates. *CHD1*, known to modulate cell adhesion, mutated in one (8.3%) case of non-neoplastic mucosa. *FGFR2*, a molecule involved in the development of fibroblasts, healing and degeneration, was mutated in three (25.0%)case of non-dysplastic UC mucosa. Neither *CHD1* nor *FGFR2* accumulated further mutations in colitic cancer cells. No mutation was observed in other molecules in non-neoplastic mucosa.

Next, we compared our results to the mutational rate of sporadic CRC obtained from Bioportal (TCGA Nature, 2012). Table II shows the categorized comparison and the result of

Table I. *Genes found to be mutated (X) in non-neoplastic mucosa (M) and carcinoma epithelium (T) of patients with colitic cancer.*

	Patient																							
	P01		P02		P03		P04		P05		P06		P07		P08		P09		P10		P11		P12		Frequency (%)	
Gene	M	T	M	T	M	T	M	T	M	T	M	T	M	T	M	T	M	T	M	T	M	T	M	T	M	T
APC														X							X	X			8.3	16.7
CDH1																			X	X					8.3	8.3
ERBB4																						X			0.0	8.3
FBXW7												X					X	X						X	8.3	25.0
FGFR2											X	X				X	X	X			X				25.0	25.0
KRAS		X										X					X								8.3	16.7
PIK3CA																				X					0.0	8.3
SMAD4																X						X			0.0	16.7
TP53				X				X				X		X	X	X		X		X				X	16.7	58.3

Fisher's test of oncogenes. The mutational rates for *CDKN2A* and *NOTCH1* were not registered in Bioportal (TCGA Nature, 2012) and were, therefore, excluded from further analysis.

The mutational rate of *APC* was lower in colitic than sporadic CRC (16.7% *vs*. 75.9%, respectively, p=0.0001). We also observed a lower mutational rate of *KRAS* in colitic than in sporadic CRC (67% *vs*. 42%, respectively, p=0.04). Regarding *FGFR2*, the mutational rate was higher in colitic than in sporadic CRC (25% *vs*. 1.5%, p=0.0025). No statistical difference was found in mutational rates for *CDH1* or other genes.

Discussion

Patients with longstanding UC are at an increased risk of developing colitic cancer. Several reports, including meta-analyses, described the estimated risk of colitic cancer at 10, 20, and 30 years as 0.5-2%, 1.1-8%, and 18%, respectively, and the risk for dysplasia at these time points was estimated as 3.1%, 10%, and 15.6%, respectively (14-16). Colitic cancer develops in a widespread distribution among sites of inflammation, and this feature makes early detection difficult. In order to detect dysplastic lesions, we performed non-target biopsy during annual colonoscopy, taking a biopsied specimen every 10 cm all around the colon, and an additional specimen was taken if it presented as an elevation or had an abnormal color (16). We also introduced a dye-spraying method along with magnifying colonoscopy for the purpose of improving the detection rate of dysplasia (17, 18). Together with these efforts, the introduction of biomarkers for dysplasia or colitic cancer is expected to facilitate earlier detection. For this purpose, we examined the genetic alterations specific to colitic cancer.

In this study, we found a much lower mutational rate of *KRAS* than that of sporadic cancer, compatible to a previous report (19). *KRAS* plays a key role in intracellular signal transduction pathways, and its mutation is observed in approximately 40% of CRC. The rate of mutation in sporadic CRC increases with tumor progression, and mutations affect survivals (20). The reason for the lower mutational rate in colitic cancer is unclear; however, we consider one reason to be that most of the colitic cancer cases in our study were detected through annual colonoscopy, which led to early-stage detection. Future studies conducting further analysis to determine whether the mutational rate changes as clinical stage progresses in colitic cancer are warranted.

Another representative genetic alteration observed in sporadic CRC is of *APC*, which we found to have a lower mutational rate in colitic cancer, in line with a previous report (13). Rapozo *et al*. reported that *APC* mutation did not occur in the mucosa of patients with UC (21), and a study by Odze *et al*. showed the rates of allelic loss of *APC* were similar among precancerous lesions in both normal adenoma (33%) and UC-associated dysplasia-associated lesions or masses (29-43%) (22). In our study, the rate of *APC* mutation was much higher in sporadic CRC than in colitic cancer (73% *vs*. 17%, respectively). These results suggest that *APC* mutation is involved while adenoma progresses to sporadic cancer, whereas this alteration does not exist in UC carcinogenesis.

We also found a higher mutational rate of *FGFR2* in colitic cancer than in sporadic CRC. This alteration had already been observed in the non-neoplastic mucosa of colitis. Basic fibroblast growth factor (bFGF) is reported to be a key molecule in healing and degeneration (23). Higher serum levels of bFGF have been observed in various collagen-related diseases (24), and this is considered to be induced by immune dysfunction.

Table II. *Comparison of mutational rates in colitic cancer epithelium (UC-Ca) determined in this study by next-generation sequencing and in sporadic CRC data obtained from Bioportal (TCGA Nature 2012).*

Gene	Encoded protein	Mutation	UC-Ca, n	TCGA nature 2012, n	*p*-Value*
ABL1	ABL proto-Oncogene 1	+	0	1	n.s.
		–	12	37	
AKT1	ALT serine/threonine kinase 1	+	0	1	n.s.
		–	12	37	
ALK	ALK receptor tyrosine kinase	+	0	4	n.s.
		–	12	34	
APC	Adenomatous polyposis coli	+	2	161	0.0001
		–	10	51	
ATM	ATM serine threonine kinase	+	0	5	n.s.
		–	12	33	
BRAF	B-Raf proto-oncogene, serine/threonine kinase	+	0	21	n.s.
		–	12	191	
CDH1	Cadherin 1	+	1	5	n.s.
		–	11	207	
CSF1R	Colony-stimulating factor 1 receptor	+	0	1	n.s.
		–	12	37	
CTNNB1	Beta-Catenin 1	+	0	3	n.s.
		–	12	35	
EGFR	Epidermal growth factor receptor	+	0	3	n.s.
		–	12	35	
ERBB2	Erb-B2 receptor tyrosine kinase 2	+	0	3	n.s.
		–	12	35	
ERBB4	Erb-B2 receptor tyrosine kinase 4	+	1	17	n.s.
		–	11	195	
FBXW7	F-BOX and WD repeated domain containing 7	+	3	34	n.s.
		–	9	178	
FGFR1	Fibroblast growth factor receptor 1	+	0	1	n.s.
		–	12	56	
FGFR2	Fibroblast growth factor receptor 2	+	3	3	0.0025
		–	9	197	
FGFR3	Fibroblast growth factor receptor 3	+	0	1	n.s.
		–	12	37	
FLT3	Fms related thyrosine kinase 3	+	0	4	n.s.
		–	12	208	
GNA11	G Protein subunit alpha 11	+	0	1	n.s.
		–	12	211	
GNAQ	G Protein subunit alpha Q	+	0	1	n.s.
		–	12	56	
GNAS	G Protein subunit alpha S	+	0	6	n.s.
		–	12	122	
HNF1A	HNF1 Homobox A	+	0	1	n.s.
		–	12	53	
HRAS	Hepatocyte nuclear factor 1-alpha	+	0	1	n.s.
		–	12	37	
IDH1	Isocitrate dehydrogenase [NADP] cytoplasmic	+	0	3	n.s.
		–	12	35	
JAK2	Janus kinase 2	+	0	2	n.s.
		–	12	36	
JAK3	Janus kinase 3	+	0	2	n.s.
		–	12	36	
KDR	Kinase Insert Domain Receptor	+	0	3	n.s.
		–	12	35	
KIT	Mast/stem cell growth factor receptor Kit	+	0	5	n.s.
		–	12	36	
KRAS	GTPase Kras	+	2	89	0.04
		–	10	123	
MET	Hepatocyte growth factor receptor	+	0	1	n.s.
		–	12	37	

Table II. *Continued*

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Gene	Encoded protein	Mutation	UC-Ca, n	TCGA nature 2012, n	*p*-Value*
MLH1	DNA mismatch repair protein Mlh1	+	0	4	n.s.
		–	12	116	
MPL	MPL proto-oncogene, thrombopoietin receptor	+	0	1	n.s.
		–	12	53	
NMP1	Nucleophosmin	+	0	1	n.s.
		–	12	53	
NRAS	Transforming protein N-Ras	+	0	12	n.s.
		–	12	108	
PDGFRA	Platelet-derived growth factor receptor alpha	+	0	11	n.s.
		–	12	201	
PIK3CA	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform	+	1	43	n.s.
		–	11	169	
PTEN	Phosphatase and tensin homolog	+	0	3	n.s.
		–	12	35	
PTPN11	Protein tyrosine phosphatase, non-receptor type 11	+	0	2	n.s.
		–	12	52	
RB1	RB transcriptional corepressor 1	+	0	1	n.s.
		–	12	37	
RET	Ret proto-oncogene	+	0	2	n.s.
		–	12	36	
SMAD4	Mothers against decapentaplegic homolog 4	+	2	24	n.s.
		–	10	188	
SMARCB1	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1	+	0	3	n.s.
		–	12	117	
SMO	Smoothened homolog	+	0	1	n.s.
		–	12	53	
SRC	Proto-oncogene tyrosine-protein kinase Src	+	0	0	n.s.
		–	12	38	
STK11	Serine/threonine-protein kinase STK11	+	0	0	n.s.
		–	12	120	
TP53	Tumor protein P53	+	7	111	n.s.
		–	5	101	
VHL	Von Hippel-Lindau disease tumor suppressor	+	0	1	n.s.
		–	12	53	

n.s.: Non-significant. *Fisher's test.

Kanazawa *et al*. reported higher concentrations of serum bFGF in patients with inflammatory bowel disease with a positive correlation to disease activity. They also confirmed the overexpression of bFGF in the epithelium of patients with colitis (23). Yamagata *et al*. reported the variability of concentration of bFGF in a single patient with colitic cancer and showed a higher bFGF-positive cell count in stenotic sites than that in non-stenotic sites (25). These reports imply that bFGF and its pathway play key roles in activated inflammation induced by immune dysfunction. Our study supports this speculation. We determined that *FGFR2* mutation during UC carcinogenesis was already present in non-dysplastic mucosa. This alteration affects mucosal healing and degeneration and induces further inflammation and, as a result, higher levels of bFGF are recorded. As far as we know, this is the first report showing *FGFR2* mutation in UC.

We also revealed that *CDH1* mutation occurred not only in the dysplastic epithelium but also in non-dysplastic mucosa in patients with colitic cancer. *CDH1* encodes E-cadherin, a glycoprotein that mediates intercellular connections by way of the *zonula adherens*. The alteration of CDH1 contributes to epithelial–mesenchymal transition, the biological phenomenon allowing epithelial cells to transition into fibroblastic cells with an ability to migrate and invade into the extracellular matrix (26, 27).

Because this molecule is responsible for adherens junctions between epithelial cells, it is implicated in tumor progression in carcinogenesis (28, 29). A meta-analysis revealed that the polymorphism in CHD1 rs9929218 was associated with CRC (30). Moreover, the loss of *CDH1* expression was associated with infiltrative tumor growth and lymph node metastasis (31). These results suggested that the loss of CDH1 is a core factor for sporadic CRC progression.

In contrast, a study by Sommeren *et al*. revealed this to be a core molecule in pathogenesis of UC (12). Another study revealed a higher hypermethylation rate of the *CDH1* promoter region in UC (32); this was positively associated with the severity of inflammation, age, and Mayo Endoscopic Scores (33). We consider that severe and longstanding inflammation in colorectal mucosa in UC causes hypermethylation of *CDH1*, which in turn confers invasive potential on epithelial cells. These reports suggest a novel implication concerning the progression of UC; however, several facts had not been clarified.

Firstly, in the previous study about the relationship of CDH1 and colitis, the alteration was assessed by hypermethylation in the promoter region not by mutational analysis. Secondly, the previous report described the association only among the characteristics of colitis and CHD1 and did not comment on colitic carcinogenesis.

We consider that our study complements these previous findings. We examined the mutational rate of *CDH1* in colitic cancer and showed that it did not differ from that of sporadic CRC. Moreover, our case with *CDH1* mutation in colitic cancer cells also showed mutation in non-neoplastic mucosa. Taking our results together with those of previous studies, we note that the mutational change in *CHD1* had already occurred in non-neoplastic mucosa in UC. In contrast, as described in the previous report, during sporadic colorectal carcinogenesis, the adenoma did not exhibit mutation, whereas in our study, we found colitic cancer to have a similar mutation rate to sporadic CRC. These results suggest that *CDH1* mutation occurs while the adenoma progresses to carcinogenesis in sporadic CRC. This leads to the conclusion that *CDH1* mutation would appear to occur at a much earlier stage during carcinogenesis in colitic cancer than it does in sporadic CRC. The early acquisition of invasive potential conferred by *CDH1* mutation explains why colitic cancer is morphologically different from sporadic CRC, invading into deeper layers when the tumor size is still relatively small.

The severity of inflammation of UC mucosa a known risk factor for colitic cancer, and CDH1 is reported as a predictor for the grade of inflammation (11, 12, 34). Therefore, we consider that *CDH1* mutation might be a risk factor for UC carcinogenesis, and this may extend to a therapeutic strategy for colitic cancer. An *in vivo* study by Inoue *et al*. gives indications of future developments of this hypothesis (35). They artificially developed a chronic colitis model by administering 1,2-dimethylhydrazine and 3% dextran sulfate sodium and examined tumorigenesis in mice sacrificed on the 28th day after administration. They observed fewer tumor nodules in mice that had been administered with R-etodolac, compared with those without it. R-etodolac is known to up-regulate *CDH1*. This result suggests that the induction of *CDH1* potentially suppresses colitic cancer and implies that the induction of *CHD1* would be a possible key to the prevention or suppression of colitic cancer.

Conclusion

Colitic cancer presented a lower mutational rate of *KRAS* and *APC* than did sporadic cancer, in line with previous reports. We also showed that the mutation of *FGFR2* had already occurred in non-dysplastic mucosa, which may affect mucosal healing and induce immune dysfunction. Moreover, the mutational rate of *CHD1*, a key molecule in UC carcinogensis, was similar to that of *KRAS* and *APC* in UC carcinogenesis, and occurs at an earlier stage than it does in sporadic CRC.

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