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Genetic Alterations in Ulcerative Colitis-associated Neoplasia Focusing on *APC*, *K-ras* Gene and Microsatellite Instability

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The status of genetic alterations in ulcerative colitis (UC)-associated neoplasia (UCAN) was investigated focusing on microsatellite instability (MSI) which is seen in a certain fraction of colorectal carcinomas, and adenomatous polyposis coli (*APC*) gene and *K-ras* gene, in which mutations occur in the early stage of sporadic colorectal tumorigenesis. Thirty-one UCAN from 15 UC patients who had undergone colorectal resection at our institution were investigated. There were 8 lesions of invasive carcinoma, 15 high-grade dysplasia (HGD) and 8 low-grade dysplasia (LGD). DNA was extracted from each neoplastic lesion and corresponding non-neoplastic tissue by a microdissection method. MSI status at 9 microsatellite loci, loss of heterozygosity (LOH) at the *APC* locus, and *K-ras* codon 12 point mutation were examined. As for MSI, 4/31 (13%) UCAN (carcinoma: 1/8 (13%), HGD: 2/15 (13%), LGD: 1/8 (13%)) were MSI-high (3 or more unstable loci) and 12/31 (39%) UCAN (carcinoma: 3/8 (38%), HGD: 6/15 (40%), LGD: 3/8 (38%)) were MSI-low (1 or 2 unstable loci). LOH at the *APC* locus was not found in 9 UCAN from 6 informative (heterozygous) cases. The *K-ras* mutation rate of UCAN was 3/31 (9.7%) (carcinoma: 2/8 (25%), HGD: 1/15 (7%) and LGD: 0/8). MSI is relatively common in UCAN and is present at the early stage of tumorigenesis of UCAN, while the involvement of genetic alterations of the *APC* gene and *K-ras* gene is small. MSI may be one of the mechanisms of the increased neoplastic risk in UC, and UCAN may develop through a different carcinogenic pathway from sporadic carcinomas.

Key words: Ulcerative colitis — Dysplasia — Microsatellite instability — *APC* — *K-ras*

Ulcerative colitis (UC) is a risk factor for colorectal carcinoma, and the risk is high in patients with longstanding disease with total colorectal involvement.[1–3] Recent studies suggest a cumulative incidence of carcinoma of about 5–10% at 20 years and 12–20% at 30 years from the onset of UC.[4] UC-associated neoplasia (UCAN) consists of dysplasia limited to the mucosal layer and invasive carcinomas. Dysplasia is subdivided into high-grade dysplasia (HGD), low-grade dysplasia (LGD), and indefinite dysplasia (IND) according to the histological grade.[2]

Genetically, it can be predicted that the development of UCAN may be the result of some genetic alteration caused by chronic inflammation and repeated mucosal reproduction.[5, 6] Microsatellite instability (MSI) is found not only in neoplastic lesions,[7] but also in non-neoplastic mucosa of UC,[8–11] and MSI is regarded as one of the mechanisms of the increased neoplastic risk in UC. The prevalence of adenomatous polyposis coli (*APC*) gene mutations in UCAN is low or equal to that of sporadic carcinomas,[12–15] and loss of heterozygosity (LOH) at the *APC* locus is seen in 25 to 40% of UCAN.[12, 13, 16–18] UCAN has a low incidence of *K-ras* mutations,[19, 20] but there are some reports of a relatively high incidence of *K-ras* mutations among sporadic carcinomas.[13, 14, 21, 22] However, there is still little information about the genetic alterations in UCAN and no clear conclusion has been reached. Therefore, we investigated MSI, which is found in a certain fraction of colorectal carcinomas,[23, 24] and the state of *APC* and *K-ras* genes, in which mutations occur in the early stage of sporadic colorectal tumorigenesis.[25, 26]

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MATERIALS AND METHODS

Patients and specimens Specimens were obtained from 15 UC patients with UCAN (9 male, 6 female) who underwent resection at our institution from 1983 to 1998. The lesions were classified according to the 1983 Inflammatory Bowel Disease-Dysplasia Morphology Study Group Criteria (IBD-DMSGC),[2] and diagnoses were confirmed by two experienced gastrointestinal pathologists (TM, KM) (Table I). For each patient, several histologically distinct areas of neoplastic lesions were examined. In total, 31 UCAN, which consisted of 23 lesions of dysplasia and 8 lesions of invasive carcinoma, were examined. In the 23 lesions of dysplasia, there were 15 HGD and 8 LGD, and no IND was included in the subjects of this study (Table II).

DNA extraction A 20-μm section was obtained from the formalin-fixed, paraffin-embedded block, and the neoplastic lesion was precisely dissected under a microscope with reference to the adjacent hematoxylin and eosin-stained section. The dissected lesion was about 2 to 4 mm^2, containing approximately 10 to 20 glands. In most cases, the corresponding lymph node was also dissected as a paired sample of genomic DNA. In exceptional cases with no lymph node in the sections, DNA obtained from histologically non-neoplastic mucosa was used as genomic DNA. DNA was extracted from each specimen after deparaffinization by treatment with sodium dodecyl sulfate-proteinase K and phenol-chloroform-isoamyl alcohol as described previously.[27] The DNA concentration was adjusted to 20 ng/μl. The possibility of error due to contamination with non-neoplastic cells, and hence masking of the presence of MSI or LOH, was considered negligible.

Detection of MSI We examined MSI according to the method described in the literature,[28] which has been established and widely acknowledged as a method of MSI diagnosis of colorectal carcinomas, with some modifications of polymerase chain reaction (PCR) primers and cycle parameters. Microsatellite markers in 10 loci, which were described in the literature, were as follows: BAT-25, BAT-26, BAT-40, D2S123 (AFM093xh3), D5S346 (*APC*), D10S197 (AFM119xh12a), D17S250 (Mfd15CA), D18S58 (AFM164xe31a), D18S69 (AFM248yf1), and MYCL1. Among these markers, MYCL1 was not utilized in this study because it has a relatively long repeating motif exceeding 100 bp and the efficiency of PCR was poor in amplifying the minimal amount of damaged DNA obtained by the microdissection method from formalin-fixed tissue. The remaining microsatellite markers at 9 loci were used for MSI diagnosis in this study. The DNA was amplified using a PCR9600 thermal cycler (Perkin-Elmer, Foster City, CA) in 10 μl reaction mixtures containing 20 ng of extracted tissue DNA, 0.5 p*M* each set of primers (fluorescence-labeled), 200 m*M* each deoxyribonucleoside triphosphate, 0.25 unit of *Taq* polymerase (AmpliTaqGold, Perkin-Elmer), and a 10% volume of attached buffer, according to the following protocol: 10 min at 95°C for polymerase activation, than 40 cycles at 94°C for 30 s, 56°C for 2 min, 72°C for 1 min, followed by an additional 3 min at 72°C. After denaturation by heating at 90°C for 5 min, the PCR product was evaluated with an ABI Prism 310 Genetic Analyzer (Perkin-Elmer), based on automated capillary electrophoresis and automated sizing of the alleles by GeneScan 2.0.2 and GenoTyper 2.1 software (Perkin-Elmer). Unequivocal extra bands in neoplastic samples that differed by a multiple of 2 base pairs in dinucleotide markers or 1 base pair in mononucleotide markers from their normal counterparts were scored as replication errors (Fig. 1). All results were confirmed by repeated experiments. The MSI phenotype was defined as MSI-high in cases with three or more unstable loci and as MSI-low in cases with one or two unstable loci, and lesions that showed no instability were classified as MSI-negative, according to the literature.[28, 29]

Detection of LOH at *APC* locus LOH at the *APC* locus was detected using D5S346, which is a highly polymorphic dinucleotide (CA)-repeat locus 30–70 kb downstream from the *APC* gene,[30, 31] and was one of the microsatellite markers for MSI diagnosis. If there was heterozygosity in the paired normal tissue and a more than 50% reduction in the intensity of one of the bands, a lesion was diagnosed as having LOH at the *APC* locus.

Detection of *K-ras* codon 12 point mutations *K-ras* mutations were examined focusing on codon 12, because *K-ras* mutations in sporadic colorectal neoplasms occur predominantly (77–82%) at this codon.[32, 33] The DNA was amplified and analyzed by the two-step PCR-restriction fragment length polymorphism method, which is highly sensitive and specific as previously described.[34, 35] Negative and positive controls (wild-type DNA and mutated DNA) were run with each analysis (Fig. 2).

RESULTS

The clinicopathological characteristics of the 15 investigated patients with UC who underwent colorectal resection because of UCAN are reviewed in Table I. The UC duration from the onset to the diagnosis of UCAN was 14.9±5.7 (mean±SD) years. There was one case of left-

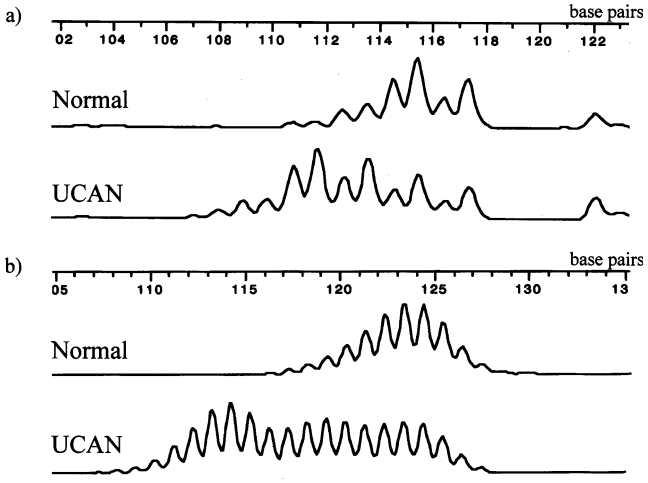

Fig. 1. a) An example of replication error in a dinucleotide marker (D18S69). b) An example of replication error in a mononucleotide marker (BAT40). Unequivocal extra bands in neoplastic samples that differed by a multiple of 2 base pairs in the dinucleotide marker or 1 base pair in the mononucleotide marker from their normal counterparts were observed.

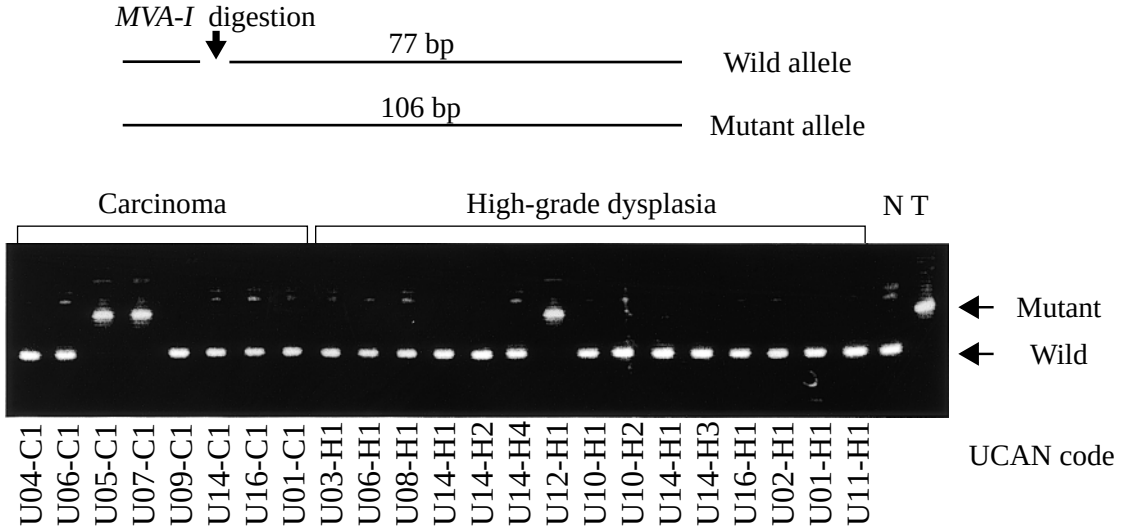

Fig. 2. Detection of *K-ras* codon 12 point mutations by two-step PCR-restriction fragment length polymorphism (RFLP) method. N, normal tissue for negative control; T, tumor with *K-ras* codon 12 point mutation for positive control. The arrow indicates the *MVA*-I digestion site in the second PCR product of the wild-type allele, dividing the 106 base pair product into two fragments of 77 and 29 base pairs.

Table I. Clinicopathological Characteristics of Investigated Cases

Patient code	Age at resection (years)	Age at onset of UC (years)	Suffering duration (years)	Sex[a)]	Clinical type[b)]	Clinical course[c)]	Main tumor[d)]	Loc.[e)]	Invasion[f)]	Stage[g)]	Histology[h)]	Dys.[i)]	Prognosis
U01	42	29	13	F	total	RR	ca.	S	s	C2	sig.	+	dead
U07	40	27	13	M	total	RR	ca.	D	s	C2	muc.	+	dead
U05	44	31	13	F	total	RR	ca.	D	ss	B2	well	+	alive
U06	46	20	26	M	total	RR	ca.	R	ss	B2	poor	+	alive
U04	49	35	14	M	total	RR	ca.	C	mp	B1	well	+	alive
U14	41	28	13	F	total	RR	ca.	R	sm	A	well	+	alive
U16	63	45	18	M	total	RR	ca.	D	sm	A	well-mod.	+	alive
U09	37	15	22	F	left sided	RR	ca.	R	sm	A	poor	+	alive
U13	27	21	6	M	total	RR	HGD	R	m		well	+	alive
U03	52	45	7	M	total	RR	HGD	R	m		well	–	alive
U10	59	48	11	M	total	CC	HGD	R	m		well	+	alive
U02	28	16	12	F	total	RR	HGD	R	m		well	+	alive
U12	66	53	13	M	total	RR	HGD	R	m		well	+	alive
U08	66	46	20	F	total	RR	HGD	S	m		well	+	alive
U11	53	30	23	M	total	RR	HGD	D	m		well	+	alive
	47.5±12.4	32.6±12.2	14.9±5.7	(mean±SD)									

a) M, male; F, female.
b) total, total colitis type; left sided, left-sided colitis type.
c) RR, relapse-remitting type; CC, chronic continuous type.
d) ca., invasive carcinoma; HGD, high-grade dysplasia.
e) Location of the main tumor: C, cecum; D, descending colon; S, sigmoid colon; R, rectum.
f) s, serosa; ss, subserosa; mp, muscularis propria; sm, submucosa; m, mucosa.
g) Astler-Coller's modification of Dukes' staging.
h) well, well differentiated; mod., moderately differentiated; poor, poorly differentiated; sig., signet ring cell; muc., mucinous.
i) Existence of accompanying dysplasia.

sided colitis type and all remaining cases were of total colitis type. The clinical course was of chronic continuous type in one case and all remaining cases were of relapse-remitting type. There were 8 cases with invasive carcinoma, and the remaining 7 cases had only dysplasia. There was no case with more than two invasive carcinomas, but all cases with invasive carcinoma were associated with one or more other histologically distinct area(s) of dysplasia. All other cases except one had multiple areas of dysplasia.

The details of the genetic status of each UCAN are shown in Table II. As for MSI in UCAN, 4 lesions (13%) were MSI-high, 12 lesions (39%) were MSI-low, and the remaining 15 lesions (48%) were MSI-negative (Table III). The most unstable lesions were an advanced carcinoma and an HGD, both of which showed instability at 4 of 9 loci tested, and there was no lesion having 5 or more unstable loci. There were no significant differences in clinicopathological features between MSI-low, MSI-high and MSI-negative UCAN. The tendency of instability was not similar among the lesions obtained from each patient, in spite of having the same genetic and environmental background. The most unstable marker in this study was D10S197, which showed instability in 22.6% (7/31) of UCAN (Table IV). In contrast, BAT26, which showed instability in about 30% of sporadic colorectal carcinomas,[36] was unstable only in one UCAN. In the results on LOH at the *APC* locus, none of the 9 UCAN in 6 informative (heterozygous at D5S346) cases had allele loss (Tables II and III). *K-ras* mutations at codon 12 were present in 2 of 8 (25%) invasive carcinomas and 1 of 15 (7%) HGD, but none was found in LGD. Overall, the mutation rate in UCAN was 9.7% (3/31) (Table III).

Table II. Genetic Status of Each UCAN

UCAN code	Grade[a]	MSI		*APC*[c]	*K-ras*[d]
		Freq.[b]	Phenotype		
U04-C1	ca.	0/9	negative	n.i.	–
U06-C1	ca.	0/9	negative	n.i.	–
U05-C1	ca.	0/9	negative	n.i.	+
U07-C1	ca.	0/9	negative	n.i.	+
U09-C1	ca.	1/9	low	–	–
U14-C1	ca.	1/9	low	n.i.	–
U16-C1	ca.	2/9	low	n.i.	–
U01-C1	ca.	4/9	high	–	–
U03-H1	HGD	0/9	negative	n.i.	–
U06-H1	HGD	0/9	negative	n.i.	–
U08-H1	HGD	0/9	negative	–	–
U14-H1	HGD	0/9	negative	n.i.	–
U14-H2	HGD	0/9	negative	n.i.	–
U14-H4	HGD	0/9	negative	n.i.	–
U12-H1	HGD	0/9	negative	–	+
U10-H1	HGD	1/9	low	n.i.	–
U10-H2	HGD	1/9	low	n.i.	–
U13-H1	HGD	1/9	low	n.i.	–
U14-H3	HGD	1/9	low	n.i.	–
U16-H1	HGD	2/9	low	n.i.	–
U02-H1	HGD	2/9	low	–	–
U01-H1	HGD	3/9	high	–	–
U11-H1	HGD	4/9	high	–	–
U04-L3	LGD	0/9	negative	n.i.	–
U09-L1	LGD	0/9	negative	–	–
U16-L2	LGD	0/9	negative	n.i.	–
U16-L3	LGD	0/9	negative	n.i.	–
U04-L1	LGD	1/9	low	n.i.	–
U04-L2	LGD	1/9	low	n.i.	–
U16-L1	LGD	2/9	low	n.i.	–
U08-L1	LGD	3/9	high	–	–

a) ca., invasive carcinoma; HGD, high-grade dysplasia; LGD, low-grade dysplasia.
b) Number of loci which showed replication errors.
c) LOH at *APC* locus: –, negative for LOH; n.i., not informative.
d) *K-ras* codon 12 point mutation: +, positive for mutation; –, negative for mutation.

DISCUSSION

About 10–20% of sporadic colorectal carcinomas and nearly all colorectal carcinomas in hereditary nonpolyposis colorectal cancer (HNPCC) patients have DNA mismatch

Table III. Grade of UCAN and MSI Status, *K-ras* Codon 12 Point Mutation, and LOH at *APC* Locus

Grade[a]	MSI[b]			*APC*[c](%)	*K-ras*[d](%)
	Negative(%)	Low(%)	High(%)		
ca.	4/8 (50)	3/8 (38)	1/8 (13)	0/2 (0)	2/8 (25)
HGD	7/15 (47)	6/15 (40)	2/15 (13)	0/5 (0)	1/15 (6.7)
LGD	4/8 (50)	3/8 (38)	1/8 (13)	0/2 (0)	0/8 (0)
total	15/31 (48)	12/31 (39)	4/31 (13)	0/9 (0)	3/31 (9.7)

a) ca., invasive carcinoma; HGD, high-grade dysplasia; LGD, low-grade.
b) MSI-high, unstable in 3 or more loci; MSI-low, unstable in 1 or 2 loci; MSI-negative, stable in all loci.
c) LOH at *APC* locus.
d) *K-ras* codon 12 point mutation.

Table IV. Grade of UCAN and Frequency of Instability in Each Microsatellite Marker

Microsatellite marker	Grade[a)]			Total(%)	
	ca.	HGD	LGD		
BAT-25	0/8	1/15	1/8	2/31	(7)
BAT-26	1/8	0/15	0/8	1/31	(3)
BAT-40	0/8	2/15	1/8	3/31	(10)
D5S346	1/8	2/15	1/8	4/31	(13)
D10S197	1/8	4/15	2/8	7/31	(23)
D17S250	2/8	1/15	1/8	4/31	(13)
D18S58	0/8	2/15	1/8	3/31	(10)
D18S69	1/8	1/15	1/8	3/31	(10)
D2S123	2/8	3/15	1/8	6/31	(19)

a) ca., invasive carcinoma; HGD, high-grade dysplasia; LGD, low-grade dysplasia.

repair defects, leading to replication errors.[23, 24)] It is supposed that DNA mismatch repair defects accelerate the accumulation of genetic alterations. Tumors that have a phenotype of replication errors are depicted as MSI-positive. Genes such as the type II transforming growth factor β2 receptor are rich in mononucleotide or dinucleotide repeat sequences, and therefore, may be prone to mutation in MSI-positive tumors.[37)] It has been demonstrated that UCAN exhibits MSI to some degree,[7–11)] and MSI is regarded as one of the mechanisms of the increased neoplastic risk in UC. However, there is no clear conclusion about how and when MSI is involved in the tumorigenesis of UCAN. Therefore, we investigated MSI status according to the grade of UCAN. In our present study, the proportion of UCAN that was diagnosed as MSI-high (unstable in more than 3 or more loci) was 13% (Table III). It was similar to that in sporadic colorectal carcinomas, which had been reported to be about 15%.[24)] The proportion of UCAN with at least one unstable locus was 52%, and it was relatively higher than that in non-neoplastic mucosa of UC patients, which has been reported to be 13 to 50%.[8, 10)] The most unstable lesion showed instability at 4 of 9 loci tested (Table II). The finding that LGD, HGD and invasive carcinomas had almost the same prevalence of MSI-high suggests that MSI is present at the early stage of tumorigenesis of UCAN. These results are consistent with the concept that chronic inflammation and repeated mucosal reproduction may be the cause of MSI, which subsequently accelerates the neoplastic transformation of UC mucosa to dysplasia or carcinoma. It is not clear that whether the low frequencies of MSI at BAT25 and BAT26 loci are characteristic of UCAN or are due to the small number of specimens. Therefore, it is necessary to collect more specimens of UCAN to allow a conclusion.

Most sporadic colorectal carcinomas develop through the adenoma-carcinoma sequence[38, 39)] in accordance with the model of genetic alteration proposed by Fearon *et al*.[25, 26)] In this model, *APC* mutations occur at the initial step of adenoma formation,[40)] followed by point mutations of the *K-ras* gene, paralleling increases in adenoma size and grade of atypia, as well as mutations of various other tumor suppressor genes which accumulate during tumor development. Unlike them, UCAN is considered to be another type of colorectal neoplasia arising in UC with chronic inflammation and repeated mucosal reproduction, and it is morphologically characterized by a high ratio of superficial or sessile tumors. Consequently, it has been considered that the state of genetic alterations, such as *APC* or *K-ras* mutations, may be different to some extent from that of sporadic colorectal carcinomas. Several investigations have been performed, focusing on *APC* or *K-ras* gene in UCAN,[12–22)] but no conclusion has yet been reached. Therefore, we decided to examine the state of *APC* and *K-ras* genes in UCAN.

Because the *APC* gene is a tumor suppressor gene and 20–50% of colorectal carcinomas and about 30% of colorectal adenomas are accompanied by LOH,[41, 42)] we examined LOH at this locus. In our present study, none of the 9 UCAN in 6 informative (heterozygous) cases had allele loss (Table III). This ratio is lower than that in sporadic colorectal invasive carcinomas. This result suggests non-significant involvement of the *APC* gene in neoplastic development in cases of UC. The possibility that the non-existence of LOH at the *APC* locus was caused by the small number of the specimens could not be ruled out. Therefore, it is necessary to collect more specimens of UCAN to obtain a definitive conclusion.

K-ras gene mutations have been found in nearly 50% of polypoid adenomas larger than 1 cm in diameter[25)] and in 44/92 (48%) of sporadic colorectal carcinomas resected at our institution from 1991 to 1995 using the same method. In our present study, the mutation rate of *K-ras* in UCAN was only 3/31 (9.7%) (Table III) and it was significantly lower than that in sporadic colorectal carcinomas (Fisher's exact test, $P<0.0001$). This finding suggests that the involvement of *K-ras* mutation in UCAN may be small.

In summary, we demonstrated that MSI is relatively common in UCAN and may be present at the early stage of tumorigenesis of UCAN, and that the involvement of genetic alterations of *APC* gene and *K-ras* gene is small. It is hypothesized that MSI may act as one of the mechanisms for the increased neoplastic risk in UC, and that UCAN may develop through some other carcinogenic pathway than sporadic carcinomas, based on the finding of low prevalence of *APC* and *K-ras* alterations in UCAN.

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