Frequent and Multiple Mutations at Minisatellite Loci in Sporadic Human Colorectal and Gastric Cancers—Possible Mechanistic Differences from Microsatellite Instability in Cancer Cells

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Minisatellites (MNs), composed of 5 to 100 nucleotide repeat units, range from 0.5 to 30 kb in length, and have been reported to be mutated in various human malignancies. In this study, frequencies of MN mutations in sporadic human colorectal (34 cases) and gastric cancers (24 cases) at various clinicopathological stages were assessed by multilocus DNA fingerprint analysis with three MN probes, Pc-1, 33.6 and 33.15. MN mutations were observed in both colorectal and gastric cancers, but at a significantly higher frequency in the former (56%) than in the latter (25%). Multiplicities of MN mutations were 1.50±1.81 and 0.46±1.10 in colorectal and gastric cancers, respectively, and the difference was also significant. Neither the presence nor multiplicity of MN mutations in either colorectal or gastric cancer cases had any correlation with the pathological stage, histological grading or the presence of microsatellite instability (MSI). Although the biological relevance of MN mutations still remains to be clarified, a subset of colorectal and gastric cancers could feature a new type of genomic instability, distinct from MSI.

Key words: DNA fingerprint analysis — Colorectal cancer — Minisatellite mutation — Minisatellite instability — Microsatellite instability

It has recently been proposed that at least two types of genomic instability underlie human colorectal cancers; namely, microsatellite instability (MSI) and chromosomal instability (CIS).[1, 2] Mutations in mismatch repair genes lead to MSI in hereditary nonpolyposis colorectal cancer and a subset of sporadic colorectal cancers,[1, 3] and mutations in genes involved in the mitotic checkpoint, such as *BUB1* and *hCHK2* genes, could result in CIS.[4, 5] Long tracts of mononucleotides in the *transforming growth factor-β type II receptor*, *BAX* and *insulin-like growth factor II receptor* genes are the targets of MSI, and they are frequently mutated.[6–8] Most of the mutations are of the frameshift type that result in inactivation of the gene products, potentially contributing to the proliferative properties of cancer cells. Recently, defects in mismatch repair were also demonstrated to promote telomerase-independent cell proliferation in yeast.[9]

Minisatellites (MNs), also called variable number of tandem repeats (VNTRs), are another type of repetitive sequence of which a few thousand copies exist per haploid genome of mammalian cells.[10] They are composed of 5 to 100 nucleotide repeat units, range from 0.5 to 30 kb in length and are dispersed throughout the entire genome of all vertebrates, being preferentially located on the telo-

meric sides of human chromosomes.[11] A subset of MN demonstrates considerably high mutation rates in germ cells, constituting hot spots for meiotic recombination,[12] even though most MNs are comparatively stable in somatic cells. Although several studies have already demonstrated alteration of MN (MN mutations) in various human neoplasms,[13–16] to our knowledge no extensive analysis has been performed with regard to the relation to MSI and the histopathological stage or differentiation grade of cancers.

In the present study, the frequencies of MN mutations in a series of sporadic human colorectal and gastric cancers with differing stages and grades were therefore analyzed using multilocus DNA fingerprint analysis with three MN probes, Pc-1, 33.6 and 33.15. Such fingerprinting carried out under low stringency conditions is a powerful method for screening multiple MN loci simultaneously, as described previously.[17, 18] Microsatellite length alterations at 12 loci were also analyzed to clarify whether there is any correlation between MSI and MN mutations.

MATERIALS AND METHODS

Tissue samples Surgical specimens from 34 colorectal and 24 gastric cancer patients were obtained at the National Cancer Center Hospital, and all cases were diagnosed as sporadic cancers on the basis of the absence of

segregation of cancer patients in their families. Paired samples of cancers and surrounding normal colon tissues were frozen in liquid nitrogen and stored at −80°C until use. Pathological TNM staging (pTNM) was determined according to the criteria approved by the American Joint Committee on Cancer.[19] Histological grading was performed following the WHO International Histological Classification of Tumors.[20, 21]

MN DNA probes The pPc-1 plasmid was kindly donated by Dr. Ryo Kominami (First Department of Biochemistry, Niigata University School of Medicine), and the p33.6 and p33.15 plasmids were from Dr. Alec J. Jeffreys (Department of Genetics, University of Leicester, UK). pPc-1 contains a murine MN, Pc-1, composed of 28 repeats of 5′-GGGCA-3′ flanked with locus-specific sequences at both ends.[22] The human MNs, 33.6 and 33.15, comprise 18 repeats of 37 nucleotides (5′-TGGAGGAAGGG-CTGGAGGAGGGCTCCGGAGGAAGGGC-3′) and 29 repeats of 16 nucleotides (5′-GAGGTGGGCAGGTGGA-3′), respectively, also flanked with locus-specific sequences.[17, 18] MN probes for DNA fingerprint analysis were prepared as follows. The pPc-1 plasmid was digested with SalI and EcoRI, and the insert was isolated after being size-fractionated in a 2% agarose gel, and labeled with [α-^{32}P]dCTP using the multiprime DNA labeling system (Amersham Pharmacia Biotech, Buckinghamshire, UK). p33.6 was digested with BamHI and EcoRI, p33.15 with BamHI and KpnI, and each MN insert was isolated and labeled likewise.

DNA fingerprint analysis Genomic DNA was extracted and purified as described elsewhere[23] from approximately 0.5 g of cancer and surrounding normal tissues. Five micrograms aliquots of genomic DNA were digested with an excess amount of HinfI (40 units) for 12 h, followed by serial digestion with the same amount of the enzyme for another 12 h to ensure complete breakdown of the sample DNA. This was followed by electrophoresis through 1.2% agarose gels of 24 cm length in 1× TAE buffer (40 mM Tris·HCl/40 mM acetic acid/1 mM EDTA, pH 8.0), blotting and fixation onto nylon membranes. Hybridization was performed in 4× SSC (1× SSC is 150 mM NaCl/15 mM sodium citrate)/1% SDS containing 25 ng of ^{32}P-labeled MN probe at 65°C overnight. The membranes were washed twice at 65°C in 2× SSC/0.5% SDS for 60 min and used to expose Kodak XAR film (Eastman Kodak Co., New York) at room temperature for 1 to 6 days. Fingerprint analysis for each sample was repeated at least twice to confirm the reproducibility of the results.

Microsatellite mutation analysis Microsatellite length alterations at 12 loci, D1S158, D2S123, D5S82, D5S346, D10S89, D10S197, D11S904, D17S261, DXS538, BAT-25, BAT-26, and BAT-40 were examined by the PCR-simple sequence length polymorphism (PCR-SSLP) method, as described previously.[24] All primer sets were purchased from Research Genetics (Huntsville, AL). The repeat unit is (CT)$_n$ in D1S158; (AT)$_l$ (AC)$_m$ (AT)$_n$ in DXS538; (CA)$_n$ in D2S123, D5S82, D5S346, D10S89, D10S197, D11S904, and D17S261; (T)$_n$ in BAT-25 and BAT-40; (T)$_m$ (A)$_n$ in BAT-26. PCR was performed with 200 ng of template DNA, 0.6 μM of each primer set, 175 μM dATP, dGTP and dTTP, 17.5 μM dCTP, 1× PCR buffer (Takara, Tokyo), 0.24 pmol of [α-^{32}P]dCTP and 0.25 U of Taq polymerase (Takara). The cycling program for each primer set was as follows; 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min for D5S82, D5S346, D10S89, D17S261, BAT-25, BAT-26, and BAT-40; 94°C for 3 min followed by 40 cycles of 94°C for 30 s, 60°C for 2 min for D1S158 and DXS538; 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 60°C for 2 min for D2S123, D10S197, and D11S904. Out of 12 loci analyzed for each case, 11 or 12 gave informative results.

Statistical analysis Statistical analyses were performed using the χ^2 or Mann-Whitney U tests with an SPSS package (SPSS, Tokyo) on a Macintosh computer. Student's t tests were also carried out to determine the statistical significance of the early-stage preference for MN mutations and the reverse correlation of MN mutations with MSI. Differences were considered significant when the P-value was less than 0.05.

RESULTS

MN mutation frequency in sporadic colorectal and gastric cancers Representative results of DNA fingerprint analysis with three MN probes of samples obtained from cancer tissues and their surrounding normal tissues are shown in Fig. 1. Approximately 10 to 20 bands were observed with each DNA sample within the range of 1.5–20 kb in size with the Pc-1 probe, and 30 to 40 bands with the 33.6 and 33.15 probes. Band patterns were compared between cancers and normal tissues, and band alterations in cancers were categorized into three types—new band, band deletion and shifted band (Fig. 1). Cases with one or more mutations detected by at least one of the three probes were classified as MN mutation-positives. Data for the numbers of MN mutation-positive cases with the respective probes are summarized in Table I. MN mutations were frequently observed in both colorectal and gastric cancer cases. The number of cases with MN mutations detected by either Pc-1 or 33.15, and the number of cases with mutations detected by at least one of the three probes, were significantly greater in colorectal cancers than those in gastric cancers ($P<0.05$, Table I).

Multiple MN mutations in a subset of colorectal and gastric cancers We further analyzed the total number of distinct MN mutations detected with the three probes in each tumor sample. Fig. 2 shows a representative case with multiple MN mutations. Since the three MN probes

feature GC-rich sequences in the repeat units and share sequence similarities to some extent,[17, 18, 22] some of the MN mutations were detected more than once with differ-

ent probes. Therefore, DNA fragments detected as being of the same length with two or more probes were regarded as identical bands. As is clearly shown in Fig. 2, five
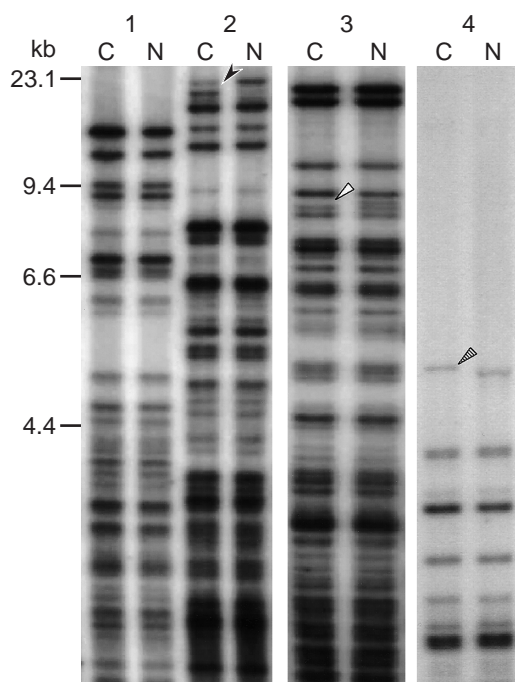

Fig. 1. DNA fingerprint analysis of surgical specimens. Cases 1, 2, and 4 are colon cancers, and case 3 is a gastric cancer. The 33.15 probe was used for cases 1, 2 and 3, and the Pc-1 probe for case 4. C, cancer; N, surrounding normal tissue. Identical band patterns of C and N were observed in case 1, so there was no MN mutation in this case. In contrast, a new band (solid black arrowhead), a band deletion (white triangle), and a shifted band (hatched triangle) were detected in cases 2, 3 and 4, respectively.
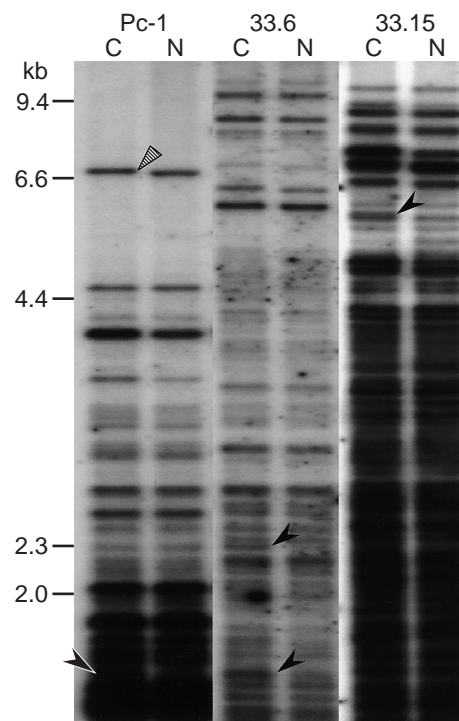

Fig. 2. Colon cancer case with multiple MN mutations. Paired samples of a cancer and surrounding normal colon tissue (case 8) were electrophoresed side by side, blotted onto a membrane, and hybridized with three probes separately to identify independent mutations. Solid black arrowheads below 2 kb indicate new bands of the same length detected with Pc-1 and 33.6. These two were regarded as identical, and were counted as one MN mutation. In this case, the number of distinct MN mutations was determined as four; namely, one shifted band (hatched triangle) and three new bands (solid black arrowheads).

Table I. Frequency of Minisatellite Mutations in Sporadic Human Colorectal and Gastric Cancers

| | The number of cases with MN mutation(s) (%) | | | |
| | MN probes used | | | Total[a] |
	Pc-1	33.6	33.15	
Colorectal cancer (n=34)	7 (21)*	9 (26)	14 (41)*	19 (56)*
Gastric cancer (n=24)	0 (0)	2 (8)	4 (17)	6 (25)

a) Since seven colorectal cancer cases had mutations detected by two different probes and in two cases by three probes, simple summation of MN mutation-positives with each probe does not match the total number of positives.
* Difference between colorectal and gastric cancers is significant by χ^2-analysis ($P<0.05$).

Table II. Multiplicity of Minisatellite Mutations in Sporadic Human Colorectal and Gastric Cancers

| | Number of distinct MN mutations in each case (%) | | | Multiplicity of MN mutations |
	0	1	2≤	
Colorectal cancer (n = 34)	15 (44)	8 (24)	11 (32)*	1.50±1.81**
Gastric cancer (n = 24)	18 (75)	4 (17)	2 (8)	0.46±1.10

* Difference between colorectal and gastric cancers is significant by χ^2-analysis ($P<0.05$).
** Difference between colorectal and gastric cancers is significant by Mann-Whitney U test ($P<0.05$).

Table III. Micro- and Mini-satellite Mutations and Clinicopathological Features of Colorectal Cancers

Case	Age	Sex	Location[a]	Stage	Grade[c]	Micro-satellite mutations[d]	No. of MN mutation
1	66	F	Ca	IV	L	1/12	0
3	79	F	R	II	H	1/11	3
4	29	M	R	IV	NA	0/12	0
6	42	M	Cs	IV	H	0/12	0
7	63	F	Cs	IV	L	0/12	0
8	66	M	Cs	IV	L	0/12	4
9	62	M	R	I	L	0/12	0
10	52	M	Cd	IV	L	0/12	4
11	72	M	Ca	II	L	7/11[e]	0
12	83	M	Ca	IV	L	0/12	1
13	50	F	Ce	II	L	0/12	1
14	76	F	Cs	III	L	0/12	1
15	57	F	R	I	L	0/12	0
16	59	F	R	II	L	0/12	1
17	68	M	R	II	L	0/11	5
18	60	M	R	III	L	0/12	0
19	74	M	Ca	III	NA	8/11[e]	4
20	62	F	R	II	L	0/12	5
21	75	F	NA[b]	NA	NA	1/12	1
22	80	M	R	III	L	0/12	0
23	66	M	Cs	III	L	0/12	0
24	70	M	Cs	II	H	0/11	1
25	71	M	Ca	II	L	1/12	0
26	49	M	Cs	III	L	0/12	4
27	46	F	R	III	L	0/12	5
28	67	M	R	II	L	0/12	0
29	61	M	R	III	L	0/12	4
30	45	M	R	I	L	0/12	3
31	62	F	Ct	IV	L	0/12	0
32	67	F	Cs	II	L	0/12	0
33	59	F	R	IV	NA	0/12	1
34	64	F	R	III	L	0/12	0
35	76	M	R	IV	L	0/12	2
36	60	F	Cs	III	L	0/12	1

a) Ce, cecum; Ca, ascending colon; Ct, transverse colon; Cd, descending colon; Cs, sigmoid; R, rectum.
b) NA, data not available.
c) H, high-grade cancer; L, low-grade cancer.
d) Out of 12 loci analyzed, 5 cases gave informative results at 11 loci.
e) Cases with microsatellite instability (MSI+).

bands were altered in the cancer lane, while new bands below 2 kb in lanes Pc-1 and 33.6 demonstrated identical mobility. In contrast, the shifted band around 6.6 kb in lane Pc-1 and two new bands around 6 kb in lane 33.15 and around 2.3 kb in lane 33.6 were not detected by other probes. The number of distinct MN mutations (multiplicity of MN mutation) in this case was determined to be four; namely, one shifted band and three new bands. Eleven (32%) colorectal cancer cases and 2 (8%) gastric cancers had multiple (two or more) MN mutations, and the multi-

plicity of MN mutations was significantly higher in colorectal cancers than in gastric cancers, the values being 1.50±1.81 and 0.46±1.10, respectively (Table II, $P<0.05$). **Status of MN mutations with respect to histopathological stage and grade of the tumors** In the case of colorectal cancers, seven of 13 (54%) stage I or II lesions, and eleven of 20 (55%) stage III or IV tumors harbored MN mutations (Table III). In addition, four (31%) cases in stages I or II, and seven (35%) in stages III or IV had multiple MN mutations. In gastric cancers, five of ten (50%)

Table IV. Micro- and Mini-satellite Mutations and Clinico-pathological Features of Gastric Cancers

Case	Age	Sex	Stage	Grade[a]	Micro-satellite mutations	No. of MN mutation
1	85	M	IA	H	11/12[c]	0
2	44	M	II	H	0/12	0
3	72	M	IIIA	NA[b]	0/12	0
4	46	M	II	H	12/12[c]	0
6	64	F	IIIA	H	0/12	0
7	79	F	IB	L	12/12[c]	1
8	54	M	IV	H	1/12	0
9	59	M	IV	H	1/12	0
10	68	F	IIIA	L	0/12	0
11	80	F	IIIA	H	0/12	1
13	48	F	IV	L	0/12	0
14	74	M	II	H	0/12	0
15	66	F	IIIA	L	12/12[c]	0
16	48	F	IIIA	L	0/12	0
17	66	M	IV	H	2/12	0
18	84	M	II	L	0/12	1
19	50	M	IV	L	0/12	0
20	69	M	II	H	11/12[c]	1
21	62	M	IIIB	L	0/12	0
22	61	M	II	L	0/12	2
23	62	M	IB	H	0/12	5
24	74	M	IV	L	0/12	0
25	55	M	II	H	0/12	0
26	58	M	IV	NA	0/12	0

a) H, high-grade cancer; L, low-grade cancer.
b) NA, data not available.
c) Cases with microsatellite instability (MSI+).

stage I or II lesions, and only one of 14 (7%) stage III or IV tumors harbored MN mutations (Table IV). Two cases, cases 22 and 23, which harbored multiple MN mutations, were in stage II and stage IB, respectively, and none of 14 cases in stage III or IV harbored multiple MN mutations. No correlation was observed between the histopathological grades and the status of MN mutations in both gastric and colorectal cancers.

MSI in colorectal and gastric cancers To characterize the nature of MN mutations with respect to the possible defects in mismatch repair systems, microsatellite (MS) length alterations in both colorectal and gastric cancers were examined at 12 loci. Six of 34 colorectal cancers (18%) and 8 of 24 gastric cancers (33%) demonstrated MS mutations (Tables III and IV). In particular, two cases (6.3%) of colorectal cancers (cases 11 and 19) and five (21%) of the gastric cancers (cases 1, 4, 7, 15 and 20) harbored more than seven mutated loci out of 11 or 12 which were informative, and were therefore classified as harboring microsatellite instability (MSI), based on the International Criteria recently proposed by Boland *et al.*[25] In

addition, three of six (50%) colorectal cancers and six of 8 (75%) gastric cancers with MS mutations harbored no MN mutations. Furthermore, nine of 11 (82%) colorectal cancers and both (100%) gastric cancers with multiple MN mutations showed no MS mutations. The presence of MN mutations did not correlate positively with the presence of MSI in either colorectal or gastric cancers. Indeed, in the case of colorectal cancers, a reverse correlation was detected by Student's *t* test ($P < 0.01$).

DISCUSSION

In the present study, multi-locus DNA fingerprint analysis was conducted to investigate MN mutations in colorectal and gastric cancers. Although the data presented here should be interpreted with caution because of the limitations of DNA fingerprint analysis, MN mutations occur in both colorectal and gastric cancers, but significantly more frequently in the former. While the molecular mechanisms underlying the MN mutations remain to be clarified, several possibilities can be considered. Since losses of heterozygosity (LOHs) at various chromosomal loci are commonly observed in human colorectal and gastric cancers, and nearly all colorectal cancers with metastases have LOHs,[26, 27] one possible interpretation is that some altered bands, particularly deleted bands, could be due to the presence of LOH. However, the fact that five out of ten colorectal cancers with metastases (cases in stage IV) had no MN mutation and that multiple MN mutations were observed in early stages (stages I and II) as frequently as in more advanced tumors (stages III and IV), suggests that these altered bands cannot be simply explained by LOH.

Another intriguing scenario is that a certain molecular mechanism which stabilizes genomic integrity at MN loci could be altered in colorectal cancers. Recombination-based strand-break repair systems could conceivably be involved.[28] Highly frequent MN mutations in human cancers, especially in colorectal cancers, might thus indicate the presence of a novel type of genomic instability, which could be referred as MN instability (MNI), as observed in *scid* fibroblasts.[29] To date, two types of genomic instability have been proposed to underlie the development of human colorectal cancers, microsatellite instability (MSI) and chromosomal instability (CIS). Then, the question that arises is whether MNI could be related to the presence of MSI or CIS. The incidence of MSI has been reported to be approximately 15% in sporadic colorectal cancers[25, 30, 31] and 30–50% in sporadic gastric cancers.[32–34] Although the number of cases with MSI was relatively small in this study, the lack of correlation between the presence of MN mutations and MSI was in good agreement with what was observed in NIH3T3 cells treated with okadaic acid and in *scid* fibroblasts in our previous

investigations.[29, 35] Alteration in mismatch repair systems is therefore unlikely to be a causative genetic event responsible for the induction of MN mutations or MNI. With regard to the correlation between MN mutations and CIS, the latter was earlier found to show a reverse correlation with the presence of MSI. In our present study, no reverse correlation between MN mutations and MSI was observed in gastric cancers. Although the reverse correlation was observed in colorectal cancers, the number of cases with MSI was too small, being only two. Taking the available information together, we hypothesize that a novel molecular mechanism is involved in the induction of MN mutations or MNI.

The biological consequence of MN mutations in cancer cells remains essentially unclear. One possibility is that alterations of MNs, located upstream or downstream of genes, could affect their expression levels.[36, 37] In order to clarify further the biological relevance of MN mutations in carcinogenesis, studies should be conducted to identify specific genes, whose structure and/or expression are altered by MN mutations in cancer cells.

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