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Two modes of microsatellite instability in human cancer: differential connection of defective DNA mismatch repair to dinucleotide repeat instability

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ABSTRACT

Microsatellite instability (MSI) is associated with defective DNA mismatch repair in various human malignancies. Using a unique fluorescent technique, we have observed two distinct modes of dinucleotide microsatellite alterations in human colorectal cancer. Type A alterations are defined as length changes of ≤6 bp. Type B changes are more drastic and involve modifications of ≥8 bp. We show here that defective mismatch repair is necessary and sufficient for Type A changes. These changes were observed in cell lines and in tumours from mismatch repair gene-knockout mice. No Type B instability was seen in these cells or tumours. In a panel of human colorectal tumours, both Type A MSI and Type B instability were observed. Both types of MSI were associated with *hMSH2* or *hMLH1* mismatch repair gene alterations. Intriguingly, *p53* mutations, which are generally regarded as uncommon in human tumours of the MSI$^+$ phenotype, were frequently associated with Type A instability, whereas none was found in tumours with Type B instability, reflecting the prevailing viewpoint. Inspection of published data reveals that the microsatellite instability that has been observed in various malignancies, including those associated with Hereditary Non-Polyposis Colorectal Cancer (HNPCC), is predominantly Type B. Our findings indicate that Type B instability is not a simple reflection of a repair defect. We suggest that there are at least two qualitatively distinct modes of dinucleotide MSI in human colorectal cancer, and that different molecular mechanisms may underlie these modes of MSI. The relationship between MSI and defective mismatch repair may be more complex than hitherto suspected.

INTRODUCTION

Microsatellites are repetitive DNA sequences comprising short reiterated motifs dispersed throughout the eukaryotic genome (1). Microsatellite lengths are highly polymorphic in human populations, but appear stable during the life span of the individual. Somatic instability of microsatellite sequences has initially been reported in human colorectal cancer (2,3), and particularly in the familial cancer-prone syndrome, hereditary non-polyposis colorectal cancer (HNPCC) (4,5). In 1993, mutations in one of the genes encoding proteins essential for DNA mismatch repair (MMR) were found in

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HNPCC individuals (6,7). MMR is an important editing system. It counteracts the base mismatches and strand misalignments that occur during DNA replication and recombination (8). Repetitive sequences such as those comprising microsatellites are particularly prone to polymerase slippage and, consequently, strand misalignment. If these errors remain uncorrected, the mutations are fixed during subsequent replication as addition or deletion of one (or more) repeat units. The phenomenon of unstable microsatellites, i.e. microsatellite instability (MSI), in which tumour cells accumulate this type of repeat length alterations in microsatellites, is considered to reflect MMR deficiency. The MSI^+ phenotype is frequently associated with various human malignancies (9). As defective MMR is regarded as a risk factor for familial predisposition or second malignancies, analyses of microsatellite instability have been prevalent, particularly in the field of oncology. However, the reported frequency for MSI^+ tumours in each malignancy differs widely in the literature (9).

Although analysis of MSI is now commonplace, a designation of MSI^+ may sometimes be a difficult decision. The 1997 National Cancer Institute (NCI) workshop, 'Microsatellite Instability and RER Phenotypes in Cancer Detection and Familial Predisposition', suggested that the variety of microsatellites used was a major cause of discrepancies among data from various laboratories, and recommended a panel of five microsatellites as 'working reference panel' (10). We believe that, in addition to selection of targets for analysis, methodological problems also account for some of the variability in results. Changes in microsatellite lengths are sometimes minor—as small as loss or gain of a single repeat unit. In addition, cells carrying changes in microsatellite sequences are not always major in a given sample. However, in an assay system using the conventional sequencing gel electrophoresis and autoradiography, it appears difficult to resolve microsatellite PCR products precisely and quantitatively. PCR itself has an intrinsic variability. The most widely used thermostable DNA polymerase (*Taq*) has a terminal deoxynucleotidyl transferase (TDT) activity, which adds one additional base to PCR products in a sequence-dependent manner. TDT activity of *Taq* polymerase is variably expressed, depending on the conditions used. This property, in addition to intrinsic strand misalignment during amplification of microsatellite repeats, increases the complexity of PCR products. In the conventional microsatellite analysis, intrinsic caution and the desire to avoid scoring false-positives may have led to an underestimate of the frequency of minor, more subtle microsatellite changes, such as alterations of limited numbers of repeat units. We have applied our fluorescent technique for microsatellite instability analysis (11) to address these problems. Here, we report that relatively subtle alterations in microsatellites are indeed generally associated with MMR deficiency. In contrast, most HNPCC tumours display much more extensive microsatellite changes. Our findings suggest that there are previously unrecognized aspects of microsatellite instability in human cancer.

MATERIALS AND METHODS

Cells and tissue specimens

Msh2$^{-/-}$ mouse embryonic fibroblast (MEF) cell line, RH95021 (12) and *Mlh1*$^{-/-}$ MEF cell line, MC2, were kindly provided by Dr Hein te Riele, Amsterdam Cancer Center and Dr Michael Liskay, University of Oregon, respectively. Cells were cultured in DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Samples of cancer tissues and the corresponding normal mucosa were obtained from 79 patients with colorectal carcinoma who underwent surgery in the Department of Surgery and Science, Kyushu University Hospital from 1996 to 1999. Written informed consent for studies using the tissues was obtained from each patient. Ethical approval was obtained from the IRB of Kyushu University. Specimens, taken immediately after resection, were placed in liquid nitrogen. High molecular weight DNA was extracted and subjected to microsatellite analyses.

Microsatellite instability

Microsatellite analysis using fluorescence-labelled primers and an automated DNA sequencer has been described in detail (11). Briefly, five human dinucleotide microsatellites, D2S123, D5S107, D10S197, D11S904 and D13S175, in genomic DNA from tissue specimens were amplified by PCR. 5′ primers were labelled with the fluorescent compound, ROX (6-carboxy-x-rhodamine) or HEX (6-carboxy-2′,4′,7′,4,7,-hexachloro-fluorescein). PCR reactions were done using TaKaRa *Taq* (TaKaRa Co. Ltd., Tokyo, Japan). T4 DNA polymerase was added to the PCR products, followed by incubation at 37°C for 10 min. To compare electrophoretic profiles between two samples, 1.2 μl of ROX-labelled product and 0.3 μl of HEX-labelled product were mixed. Samples were denatured and loaded onto the ABI 373A sequencer (Applied Biosystems, Foster City, CA, USA). The data were processed using the GeneScan software (Applied Biosystems). For mice, three dinucleotide microsatellites, D1Mit62, D6Mit59 and D7Mit91, were analysed.

DNA sequencing

All the exons and exon–intron junctions of *hMSH2* and *hMLH1* were amplified by PCR using *Taq* polymerase with 3′ exonuclease activity, TaKaRa *Ex Taq* (TaKaRa Co. Ltd., Tokyo, Japan). Primer sequences are the same as reported by Kolodner *et al.* (13,14), except that the additional sequence complementary for M13 universal primer was deleted, and that one-step PCR was mainly employed. PCR products were used as a template for cycle sequencing reactions using BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Mutations found in one PCR product were verified by reverse sequencing and finally confirmed in two independently amplified PCR products. Sequencing analyses of *p53* gene (exon 5–9) were performed using *p53* primers (Nippon Gene, Tokyo, Japan).

Immunohistochemistry

Tissue specimens were fixed in buffered 10% paraformaldehyde and embedded in paraffin. Prior to the assay, the specimens were sectioned at 4 μm and deparaffinized using xylene. Immunohistochemistry was performed using the streptavidin–biotin–peroxidase complex method (Histofine SAB kit, Nichirei, Tokyo, Japan) using an automated stainer (VENTANA Discovery System, Ventana Medical Systems Inc., Tucson, AZ, USA). At least, two independent antibodies

were used to confirm the status of negative staining. Sections prepared from *Msh2*- and *Mlh1*-knockout mice were also used as negative controls. Antibodies used were as follows: anti-MSH2; NA27 and NA26 (Oncogene Research Products, Cambridge, MA, USA), anti-MLH1; PM-13291A (Phar Mingen, Hamburg, Germany), NA28 (Oncogene Research Products) and sc-581 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

RESULTS

Two modes of dinucleotide microsatellite instability in human cancer

We have established a sensitive fluorescent technique for microsatellite analysis (11). Application of this technique to human cancers revealed a number of previously unrecognized aspects of MSI. In particular, we observed two distinct patterns of alterations at dinucleotide microsatellites in human malignancies (15–17). Examples are shown in Figure 1. In some cases, length changes are relatively small and affect ≤6 bp (Type A, Figure 1A–D). In the other, more dramatic changes involving ≥8 bp are observed (Type B) (Figure 1E–H). Because Type B alterations involve large differences in microsatellite length, it can sometimes appear as if a 'third' allele is present in addition to the parental alleles (Figure 1E–H). Throughout the analyses using this technique, results were highly reproducible in several independent experiments. Neither additional peaks nor changes in the ratio between peaks were noted.

Microsatellite instability observed in mismatch repair gene-knockout mice

To analyse MSI in a defined genetic background, we used the *Msh2*$^{-/-}$ MEF cell line RH95021 (12). Alterations in the lengths of three dinucleotide microsatellites were analysed in RH95021 subclones. The majority of subclones (14/21) exhibited the same configuration at the D6Mit59 locus (exemplified by clone a, Figure 2A). In clones that deviated from this predominant pattern (Figure 2A, clones b–d), the microsatellite length was altered by ≤4 bp. In other words, the microsatellite changes were invariably Type A. A similar pattern of small-scale microsatellite changes was observed at

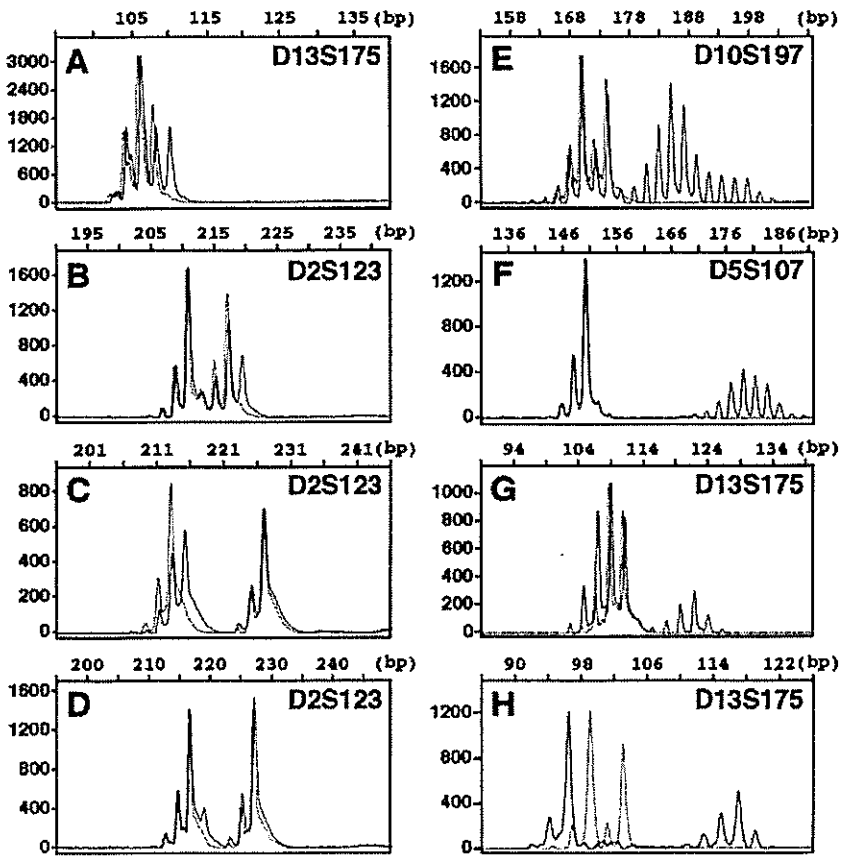

Figure 1. Type A and Type B microsatellite instability observed in human colorectal cancer. Using genomic DNA samples prepared from cancer and the corresponding normal mucosa, microsatellite sequences, indicated at the right top of each panel, were amplified by PCR with primers differentially labelled with fluorescence, then mixed and run on a same lane in an automated DNA sequencer. The amount of each DNA fragment was quantitatively detected and its size was estimated with accuracy of 1 bp, by standardization with size markers run in each lane. Results representative for each mode of microsatellite instability are shown: red lines, cancer; green lines, normal mucosa; Type A, (A) (IC678), (B) (IC810), (C) (IC721) and (D) (IC793); Type B, (E) (IC790), (F) (IC733), (G) (IC690) and (H) (NoTa). Patient codes in the parentheses correspond to those used in Table 1.

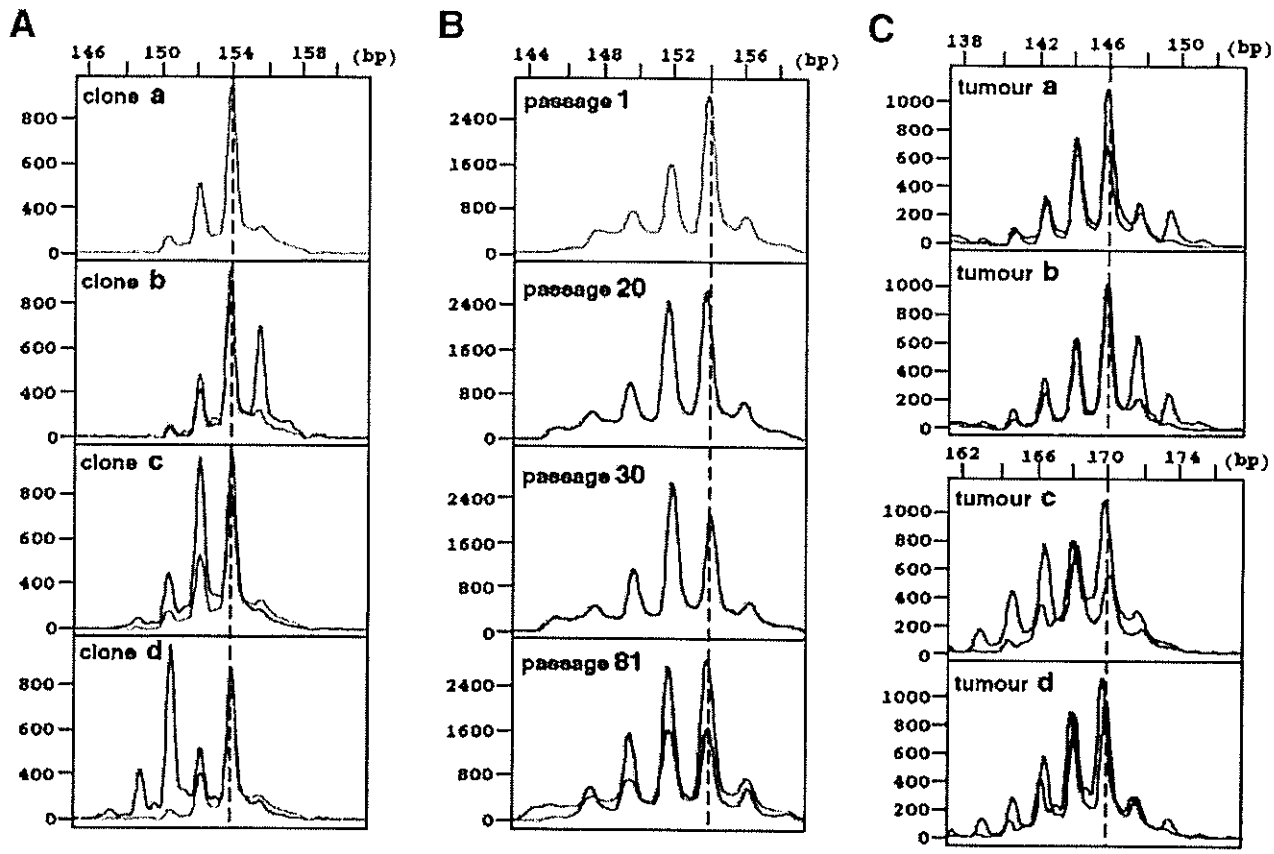

Figure 2. Microsatellite changes in *Msh2*$^{-/-}$ mouse embryonic fibroblast (MEF) cells and in tumours that arose in *Msh2*-knockout mice. (A) More than 20 subclones were isolated from RH95021 (*Msh2*$^{-/-}$) MEF cells and microsatellite changes were compared among these subclones. The majority exhibited the same configuration (clone a) and a few deviated from this predominant pattern (clone b–d). In each clone, the pattern of clone A has been superimposed with green lines, to facilitate comparison. Results obtained in D6Mit59 microsatellite are shown. (B) RH95021 cells were continuously cultured and sampled at different passages. Results obtained in D6Mit59 microsatellite at passage 1, 20, 30 and 81 are shown. In passage 81, the initial profile at passage 1 has been superimposed with a green line. (C) Tumours that arose *in vivo* in *Msh2*-knockout mice were analysed. Representative results obtained in D7Mit91 (tumours a and b) and D6Mit59 (tumours c and d) microsatellites are shown: red lines, tumour; green lines, the corresponding normal tissue.

Table 1. *hMSH2* and *hMLH1* alterations found in tumours exhibiting Type A and Type B MSI

PATIENT	MSI A/B	NUCLEAR EXPRESSION hMSH2	NUCLEAR EXPRESSION hMLH1	SEQUENCE *hMSH2* exon 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16	SEQUENCE *hMLH1* exon 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19	SUMMARY
IC669	A	P	N			cytoplasmic hMLH1
IC678	A	P	P		●	L521I (CTC to ATC)
IC692	A	P	P	□		G132G (GGC to GGA)
IC721	A	P	P	●		L390F (CTT to TTT)
IC724	A	P	P	●		G178R (GGA to AGA)
IC759	A	P	P			
IC793	A	P	N			*hMLH1* silencing
IC807	A	P	P		△	I219V (ATC to GTC)
IC810	A	P	P	●	△	*hMSH2* ; L390F / *hMLH1* ; I219V
IC815	A	P	P			
IC824	A	P	P			
IC860	A	P	P			
IC622	B	P	P			
IC653	B	P	P			
IC676	B	P	P			
IC690	B	P	N		●	R226Q (CGA to CAA) / cytoplasmic hMLH1, HNPCC
IC698	B	P	P		△	I219V (ATC to GTC)
IC733	B	P	P			
IC790	B	P	P			
IC853	B	P	P	□		G132G (GGC to GGA)
IC873	B	P	P		□	Q604Q (CAA to CAG)
NoTa	B	P*	N*			*hMLH1* silencing, HNPCC

MSI, microsatellite instability; P, positive nuclear staining in immunohistochemistry; N, negative; *, determined by immunoblotting; Closed circle, base substitution with amino acid change; open triangle, possible polymorphism; Open rectangle, base substitution without amino acid change.

two other dinucleotide microsatellites, D1Mit62 and D7Mit91 (data not shown). To investigate whether Type B variations might simply reflect the accumulation of numerous smaller alterations over many generations, RH95021 cells were continuously cultured, and sampled periodically at different passages. As shown in Figure 2B, even after undergoing 81 population doublings (2^{81} corresponds to 10^{24}), there was no detectable appearance of shorter or longer D6Mit59 alleles. Similar patterns were observed at the two other microsatellite loci (data not shown). Thus, there was no evidence of the accumulation of changes consistent with Type B instability at any of the three microsatellites in these MMR-defective MEFs. Similar data were obtained using a second MMR-deficient MEF cell line, MC2, which derived from an *Mlh1*$^{-/-}$ mouse (data not shown).

We also examined microsatellite instability in lymphomas and adenocarcinomas that arose in various organs of *Msh2*$^{-/-}$mice. Among 16 tumours that were analysed at the three microsatellite loci, each contained alterations at one or more locus. In all cases, changes were limited to ≤6 bp (examples are shown in Figure 2C) and no Type B alteration was observed. Intriguingly, an examination of published microsatellite changes in cells of MMR gene-knockout mice clearly indicates that most changes are of Type A (12,18,19).

Analysis of microsatellites in MEFs and tumours from MMR-defective animals therefore indicates that Type A MSI is a direct consequence of defective MMR. The absence of more extensive microsatellite length changes may indicate further that an *Msh2* or *Mlh1* defect is insufficient for the development of Type B instability.

Mismatch repair gene inactivation is associated with both Type A and Type B MSI in human colorectal cancer

HNPCC patients inherit mutations in MMR genes (6,7). More than 90% of HNPCC tumours are MSI$^+$ (20). The MSI$^+$ phenotype is also frequent among sporadic colorectal carcinomas (2–4,6,21). Inspection of published data derived from the conventional microsatellite analysis reveals that microsatellite changes thus far reported in various tumours, including those in HNPCC individuals, are largely Type B (2–4,20–22). We considered the possibility that the more subtle Type A MSI might have remained undetected in some cases. Using our fluorescent assay with a panel of five dinucleotide repeat microsatellites, we found that the frequencies of Type A and Type B MSI among 79 colorectal carcinomas were 30% and 17%, respectively. In agreement with previous observations that Type B instability is common in HNPCC colon tumours, the IC690 tumour and the colorectal carcinoma cell line, NoTa (Table 1), both of which were derived from patients who fulfilled the Amsterdam Criteria II for HNPCC (23), exhibited Type B instability (Figure 1G and H). Our finding that 17% of colorectal tumours display Type B MSI is consistent with the generally reported figure of around 20% for MSI among colorectal carcinomas (20,24,25). The observation that a further 30% of tumours displayed Type A MSI suggests that the frequency of MSI, at least in colorectal carcinomas, has previously been underestimated.

The relationship between MSI and defective MMR in our set of colorectal tumours was investigated further. *hMSH2* and

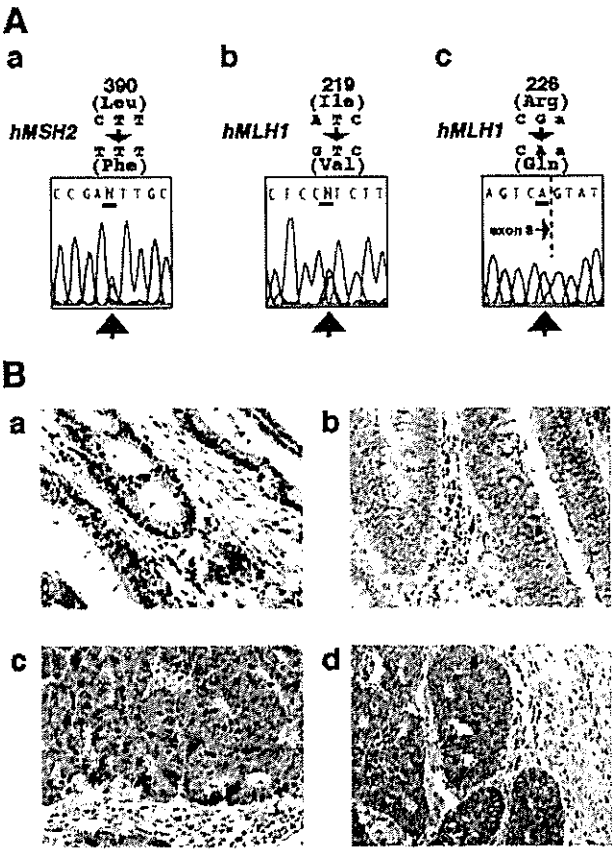

Figure 3. *hMSH2* and *hMLH1* alterations in tumours exhibiting Type A or Type B microsatellite instability. (A) Sequences for all the exons including exon–intron boundaries of *hMSH2* and *hMLH1* were determined using an automated sequencer. Sequence alterations found at (a) codon 390 of *hMSH2* (patient IC810), (b) codon 219 of *hMLH1* (patient IC810) and (c) codon 226 of *hMLH1* (patient IC690) were shown. (B) Abnormal expression of hMLH1 proteins observed in a panel of human colorectal carcinomas. (Panel a) A typical result with a strong nuclear staining implying normal hMLH1 expression (IC853). (Panel b) Complete loss of hMLH1 expression in tumour cells, which suggest a possible epigenetic silencing (IC793). (Panels c and d) Results without evident nuclear staining, but with an accumulation of hMLH1 antigens in the tumour cytoplasm, which may suggest an abnormal intracellular distribution of this protein (IC690 and IC669).

hMLH1 MMR genes of 12 tumours with Type A and 9 with Type B MSI were sequenced. The same genes in the NoTa cell line were also sequenced (Figure 3A and Table 1). Sequence alterations causing amino acid substitutions were identified in 5 of the 21 tumours. Four of these (80%) were associated with Type A MSI. In addition, one patient with Type A MSI (IC793) in whom no mutation was identified was negative for immunohistochemical staining of hMLH1 (Figure 3B, panel b). This is consistent with a possible epigenetic *hMLH1* gene silencing (26–28). In one other Type A case (IC669) and one Type B case (IC690), there was an abnormal intracellular distribution of hMLH1 which remained predominantly cytoplasmic (Figure 3B, panels c and d). Among the 10 tumours displaying Type B MSI, there was an example of base substitutions causing amino acid change in *hMLH1*. In this case, IC690, the failure of hMLH1 to localize to the nucleus was associated with the codon 226 mutation in exon 8 (Figure 3A,

Table 2. *p53* mutations found in 79 colorectal carcinomas

No.	Patient	EX05	EX06	EX07	EX08	EX09	Codon change	Base substitution	(type)	AA change	MSI
1	IC628				**273**		CGT → CAT	G:C → A:T	TS	Arg → His	N
2	IC630		196				CGA → TGA	G:C → A:T	TS	Arg → stop	A
3	IC634				306		CGA → TGA	G:C → A:T	TS	Arg → stop	N
4	IC668		193				CAT → CGT	A:T → G:C	TS	His → Arg	N
5	IC669	175					CGC → CAC	G:C → A:T	TS	Arg → His	A
6	IC673	176					TGC → AGC	A:T → T:A	TV	Cys → Ser	N
7	IC674				285		GAG → AAG	G:C → A:T	TS	Glu → Lys	N
8	IC680		ND	255			ATC → ACC	A:T → G:C	TS	Ile → Thr	A
9	IC693	179					CAT → CTT	A:T → T:A	TV	His → Leu	A
10	IC694				273		CGT → CAT	G:C → A:T	TS	Arg → His	A
11	IC711			239			AAC → GAC	A:T → G:C	TS	Asn → Asp	N
12	IC721	175					CGC → CAC	G:C → A:T	TS	Arg → His	A
13	IC748		190			ND	CCT → CTT	G:C → A:T	TS	Pro → Leu	A
14	IC754		196				CGA → CCA	G:C → C:G	TV	Arg → Pro	N
15	IC763	151					CCC → CAC	G:C → T:A	TV	Pro → His	N
16	IC772	175					CGC → CAC	G:C → A:T	TS	Arg → His	A
17	IC778	175	ND				CGC → CAC	G:C → A:T	TS	Arg → His	N
18	IC784		214		ND	ND	CAT → CGT	A:T → G:C	TS	His → Arg	A
19	IC808		205				TAT → GAT	A:T → C:G	TV	Tyr → Asp	A
20	IC812		190				CCT → CTT	G:C → A:T	TS	Pro → Leu	N
21	IC816				273		CGT → CAT	G:C → A:T	TS	Arg → His	A
22	IC819			248			CGG → CAG	G:C → A:T	TS	Arg →Gln	N
23	IC860				273		CGT → CAT	G:C → A:T	TS	Arg → His	A

MSI, microsatellite instability; N, negative; A, Type A MSI; TS, transition; TV, transversion; ND, not determined. Bold codon numbers indicate the acknowledged hot-spots for mutation.

panel c). These findings suggest that Type A instability, as well as Type B, is indeed associated with MMR defects.

p53 mutation is strongly associated with Type A MSI in human colorectal cancer

One view of the involvement of MMR defects in cancer development is that the 'microsatellite mutator phenotype (MMP)' (29,30) in mismatch repair-defective cells offers an alternative to chromosomal instability as a mechanism for genetic instability in cancer (31). On this model, MSI and chromosomal instability represent mutually exclusive pathways of tumour development. This reasoning is based partly in the observation that *p53* mutations, commonly associated with chromosomal instability, are infrequent among MSI^+ tumours (2,32–34). To examine the relationship between Type A/B instability and *p53* mutation, we sequenced the *p53* gene in our panel of 79 colorectal tumours. *p53* mutations resulting in an amino acid substitution were detected in 23 tumours (29.1%). The mutations were predominantly transitions in acknowledged hot spots; codons 175, 248 and 273 (Table 2). Of the *p53* mutations that were found in MSI^+ tumours, all were associated with Type A MSI (Tables 2 and 3). No *p53* mutations were detected among the 14 Type B tumours. Among Type A tumours, the frequency of *p53* mutation approached 50% (12/25). These findings confirm that *p53* mutations are rare in tumours with Type B MSI. More importantly, they suggest that, in contrast to prevailing opinion, defective MMR is significantly associated with *p53* mutation, at least in human colorectal cancer.

Table 3. *p53* mutation highly correlates with Type A MSI

		MSI Type A	Type B	Negative	Subtotal
p53	Wild type	13	14	29	56
	Mutant	12	0	11	23
	Subtotal	25	14	40	79

$p = 0.006$. MSI: Microsatellite instability.

DISCUSSION

The fluorescent technique we used here allows the unequivocal designation of Type A and Type B MSI, and has revealed a previously unrecognized complexity in the relationship between dinucleotide MSI and defective DNA MMR in human cancer. Type A MSI (changes $\leq$ 6 bp) is clearly linked with MMR inactivation in both mice and humans, which implies that Type A MSI is a direct consequence of defective MMR. Since we found no evidence of Type B instability (changes $\geq$ 8 bp) in MMR-defective animals, it is possible that changes in addition to repair deficiency contribute to, or are responsible for, Type B MSI. One important finding of this study is that Type A instability is frequent among human tumours. Type A MSI predominated in our large panel of colorectal carcinomas. We suggest that, because the changes associated with Type A instability are more subtle, the frequency of MSI among colorectal tumours may have been considerably underestimated. Our findings also reveal a hitherto unrecognized association between defective MMR and *p53* mutation. Significantly, Type A MSI was strongly associated with *p53* mutation in human colorectal tumours. Since Type A instability is unequivocally associated with MMR deficiency, this novel finding implies that, in contrast to prevailing opinion, *p53* mutations are common in MMR-defective tumours, at least in human colorectal cancer.

Established guidelines for classification of MSI utilize the frequency of changes in a defined set of microsatellite

markers, i.e. MSI-H and -L (10). However, qualitative differences in microsatellite changes are not widely discussed. In one of the earliest report of the MSI phenomenon, however, Thibodeau *et al.* divided microsatellite changes into two categories; Type I and II mutations (3). The former was defined a 'significant increase (expansion) or decrease (deletion) in the apparent fragment size' and the latter as a 'single 2 bp change'. This distinction has received little attention since then. Our data indicate that Type A MSI, which appears similar to their Type II mutation, is more frequent than hitherto suspected, and suggest that it represents the bona fide MMR-deficient phenotype. On the other hand, Type I mutations may correspond to our Type B instability. The problem is that mutations in MMR genes have been reported in tumours displaying this type of instability. More than 90% of HNPCC tumours are MSI^+ (20), and this type of MSI can be categorized as Type B/Type I (2–4,20–22). However, the frequencies of mutation in the two major MMR genes, *hMSH2* and *hMLH1*, in HNPCC kindred are not high in some reports (35–40). Among the panel of tumours displaying Type B MSI, we found a base substitution mutation in *hMLH1* and one case with a possible *hMLH1* silencing. This incidence of MMR gene inactivation in the Type B group is not unduly low, compared with the reported frequencies in the literature (25,36,41–43). The relationship between Type B MSI and defective MMR is probably more complex than hitherto suspected.

Type B MSI may involve molecular abnormalities in addition to defective MMR. We suggest that whereas Type A MSI probably reflects the uncorrected DNA polymerase slippage events that accumulate in MMR-defective cells, inappropriate processing of damage by recombinational DNA repair may contribute to Type B MSI. This hypothesis may be supported partly by our observation that there was no evidence of the emergence of Type B instability in MMR gene-knockout animals. It is known that microsatellite alterations occur via several independent mechanisms, including recombination (44–46), and MMR counteracts incorrect strand alignment during homologous recombination (47). The drastic microsatellite changes in Type B MSI may be more consistent with dynamic events, such as recombination, than with replication slippage. In this context, defective MMR might be a promoting, and consequently highly coincidental, but insufficient factor for Type B changes. Connection between MSI and the recombinational repair pathway in tumours, particularly in HNPCC, may warrant attention.

Type A MSI is also strongly associated with *p53* mutation in human colorectal tumours. This observation may be compatible with several recent reports (36,48,49) that have shown a connection between *p53* mutation and the MSI-L phenotype, since in colorectal cancer Type A MSI tends to be observed in a limited number of markers and, consequently, categorized as MSI-L. This finding may also provide an insight to the mechanism of genetic instability in tumours. Genetic instability in tumours has been regarded as deriving from two mutually exclusive pathways, chromosomal instability (CIN)—frequently associated with mutations in various oncogenes or tumour suppressor genes such as *p53*—and 'microsatellite mutator phenotype (MMP)' (29,30), in which *p53* mutations are rare and, instead, mutations are found in mononucleotide repeats within genes of a different variety. Several recent reports suggest that there might be an oversimplification in this distinction (50–53). From our observations, dinucleotide MSI in tumours can be divided into two modes, Type A and Type B, and Type A instability is the direct consequence of defective MMR. A close association of Type A MSI with *p53* mutation may suggest a hitherto unrecognized causal relationship between *p53* mutation and defective MMR. *p53* mutations may derive from a state with an elevated mutation rate, i.e. MMR-deficient phenotype, as initially suspected. Thus, our observations suggest added complexities to the relationship between MMR defects and MSI, and also shed light on previously unrecognized fundamental processes in the molecular mechanisms of genetic instability underlying tumour development.

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ELECTRONIC LETTER

A variant form of *hMTH1*, a human homologue of the *E coli mutT* gene, correlates with somatic mutation in the *p53* tumour suppressor gene in gastric cancer patients

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Oxidative damage to diverse physiological molecules, including proteins, lipids, and nucleic acids, is an inevitable outcome of various cellular activities in living organisms. In particular, some oxidised forms of nucleotides cause miscoding of genetic information, and have therefore been present as a major threat for cells. Multigene systems to counteract such oxidative damage have evolved in diverse organisms. In *E coli*, several mutants designated 'mutator' have been isolated, and in these cells the mutation rate is significantly elevated, due to disruption of genes regulating the spontaneous mutation rate on the genome. Previous studies using these mutators have identified three genes that function in the system to counteract mutagenic oxidative damage. The *mutT*⁻ strain is one of the mutators that exhibit the highest spontaneous mutation rate. Maki H *et al* have shown that the product of the *mutT* gene hydrolyses an oxidised form of guanine nucleotides, 8-oxo-2'-deoxyguanosine 5'-triphosphate (8-oxo-dGTP).[1] 8-oxo-dGTP incorporated into the genome stably pairs with adenine as well as cytosine in the template strand, accumulation of this oxidised form of guanine nucleotides leads to an increase in base substitution mutations—that is, A:T to G:C and G:C to T:A transversions. In the *mutT*⁻ strain, the rate for A:T to C:G transversion is indeed elevated 1000 fold over the wild type level.[2] The other two genes that function in cooperation with *mutT* are *mutM (fpg)* and *mutY*, both of which encode a DNA glycosylase to excise deleterious bases on the genome.[3] The former excises 8-oxo-guanine in the opposite site of cytosine on the genome, the latter removing adenine that pairs with 8-oxo-guanine. Thus, even in cells lacking *MUT*, G:C to T:A transversions are suppressed low.[4] Multiplicity of cellular anti-mutagenic systems guarantees the spontaneous mutation rate on the genome at an extremely low level.

Several mammalian counterparts of these *E coli* genes are now known. *MTH1* is the first identified mammalian homologue of *E coli mutT*.[5] The human *MTH1* gene, *hMTH1*, has been described in detail.[6] Altered function of *hMTH1* and consequent elevation of the mutation rate may be an attractive hypothesis for various human diseases, particularly cancer. In several human diseases, including some common malignancies, the nucleotide sequence of *hMTH1* has been explored.[6] However, no apparent mutations were found. Instead, a single nucleotide polymorphism (SNP) at the first nucleotide of codon 83, which results in amino acid change from valine (V83: GTG) to methionin (M83: ATG), has been found, and the incidence of the M83 allele is relatively frequent in healthy controls (allele frequency = 0.09).[7] In mice, an *mutT* homologue has been identified and designated *Mth1*. *MTH1*-null mice, in which *Mth1* is homozygously disrupted, have been reported.[8] Although the estimated increase in the mutation rate was only twice that in wild type counterparts, relatively frequent tumourigenesis was observed in the liver, lung, and stomach of the animals.[9] In patients with liver and lung cancer, sequence of *hMTH1* has been determined.[7] However, no relevant sequence alterations were found, and the M83 allele is not also frequent in patients with these malignancies.

Gastric cancer is one of the most common malignancies in human populations, and in this cancer mutations are frequently found in various genes, including proto-oncogenes and tumour suppressor genes, as in other cancers.[10] It still remains controversial whether increase in mutation rate plays an important role in tumourigenesis, and the sources of mutations in cancer are unknown.

Increased occurrence of gastric tumours in *MTH1*-null mice prompted us to explore the structure of this gene in a patient population. Here we report that the minor polymorphic base alteration at codon 83 is significantly more frequent in gastric cancer patients than in healthy controls, and correlates with mutation in *p53* tumour suppressor gene.

Key points

- *hMTH1* is a human homologue of the *E coli mutT* gene, which encodes a polypeptide that hydrolyses a potent mutagenic substrate for DNA replication, 8-oxo-dGTP. Altered function of *hMTH1* has been suspected in various pathological states.
- In some common malignancies, the nucleotide sequence of *hMTH1* has been explored. However, no mutations have been found. Instead, G:C to A:T transition at the first nucleotide of codon 83, GTG(Val) to ATG(Met), has been reported as a polymorphism.
- We show here that this polymorphic variation (V83M) is significantly more frequent in gastric cancer patients than in healthy controls, and correlates with mutation in the *p53* tumour suppressor gene.
- V83M variation of hMTH1 correlated with mutation in *p53* suppressor gene. The frequency of *p53* mutation is significantly higher in tumours harbouring the M83: ATG allele (6/14, 42.9%) than those without this variant allele (9/58, 15.5%).
- Connection between variant *hMTH1* and mutation in a common tumour suppressor gene may suggest a possible mechanism for a mutator phenotype underlying the pathogenesis of tumours.

MATERIALS AND METHODS

Patients and tissue specimens

Samples were collected from 72 gastric cancer patients who underwent surgery in our department from January 1990 to December 1997. Men and women numbered 47 and 25,

respectively; age ranged from 35 to 86 years, with a mean of 64 years. Written informed consent for studies using the tissues was obtained from each patient. Ethical approval was obtained from the IRB of Kyushu University. Specimens were taken immediately after resection and placed in liquid nitrogen.

Reverse transcription-polymerase chain reaction (RT-PCR) for *hMTH1* gene

Total RNA was extracted from cancerous and corresponding normal tissue specimens using ISOGEN (Nippon Gene, Toyama, Japan), according to the manufacturer's instructions. The first strand of cDNA was synthesised using 2 µg of total RNA, random hexadeoxynucleotide primer (TaKaRa, Tokyo, Japan), and RAV-2 reverse transcriptase (TaKaRa). After the first strand synthesis, PCR was performed using a GeneAmp 9600 thermal cycler (Applied Biosystems, Foster City, CA, USA.). A 50 µl reaction mixture contained 0.2 µM oligonucleotide primer, 25 ng cDNA, 1 X PCR buffer (TaKaRa), 250 µM dNTP, and 2.5 U *ExTaq* polymerase (TaKaRa). The oligonucleotide primers used to amplify the *hMTH1* sequence were 5'-ACCTGCCCCACCAATTACA-3' (forward), and 5'-GCCTCTTGTAAAGACTGGTT-3' (reverse) (fig 1). The thermal conditions were as follows: 35 cycles at 95°C for three seconds, 55°C for 45 seconds, and 72°C for 60 seconds; and one cycle at 72°C for five minutes. The PCR products were purified using a microcon-100 microconcentrator (Amicon, Beverly, MA, USA.).

Genomic PCR for *p53* gene

Genomic DNA was extracted from tumour specimens. Tissue was ground in liquid nitrogen and lysed in digestion buffer (10 mM Tris.Cl, pH 8.0; 0.1 M EDTA, pH 8.0; 0.5% SDS; and 20 µg/ml pancreatic RNase). After treatment with proteinase K and extraction with phenol, DNA was precipitated with ethanol, and then dissolved in 1 X TE (10 mM Tris-Cl, pH 7.5; and 1 mM EDTA). To amplify the sequences corresponding to *p53* exon 5 to exon 9, PCR was carried out using a GeneAmp 2400 thermal cycler (Applied Biosystems). A 100 µl of reaction mixture contained 100 ng genomic DNA, 10 µM of each primer, 200 µM dNTP, and 2.5 U *Taq* DNA polymerase (TaKaRa). The primer sequences were for exon 5: 5'-TCTGTTCACTTGTGCCCTGAC-3'(forward), and 5'-ATCAGTGAGGAATCAGAGGCC-3'(reverse); for exon 6: 5'-GCGCTGCTCAGATAGCGATG-3'(forward), and 5'-GGAGGG CCACTGACAACCA-3'(reverse); for exon 7: 5'-TGCCACAG GTCTCCCCAAGG-3'(forward), and 5'-GCACAGCAGGCCA GTGTGCA-3'(reverse); for exons 8 and 9: 5'-TTGGGAGTA GATGGAGCCT-3'(forward), and 5'-AGTGTTAGACTGGAAA CTTT-3'(reverse). The thermal conditions were as follows: one cycle at 95°C for 10 minutes; 35 cycles at 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 45 seconds. PCR products were purified using a microcon-100 microconcentrator (Amicon).

DNA sequencing

Labelled dideoxynucleotide terminator cycle sequencing reactions were performed using ABI Prism Dye Terminator Sequencing Kits (Applied Biosystems) and an ABI Prism 310 Genetic Analyser (Applied Biosystems). For each of PCR products, the same primers were used as a sequencing primer. Data were analysed using the ABI sequence software, DNA Sequencing Analysis, ver. 3.0 (Applied Biosystems). In each analysis, electropherograms were checked visually, not to misread minute sequence alterations. Sequence alterations were designated by comparison with the data deposited in the NCBI database sequence alterations found in one PCR product were verified by reverse sequencing and finally confirmed in more than two independently amplified PCR products.

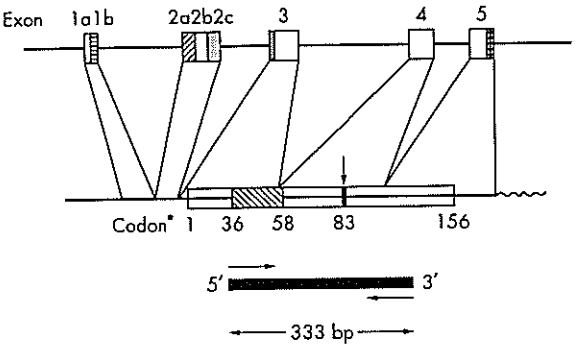

Figure 1 Schematic representation of *hMTH1* gene structure and sequencing strategy. Genomic and cDNA structures are shown. The position of codon 83 is indicated by a vertical arrow. The region corresponding to the 'phosphohydrolase module' is shown by a hatched box on mRNA. The 333-bp region, including codon 10–107, was amplified by RT-PCR. PCR primers are indicated by horizontal arrows. Another polymorphism in the 5' untranslated region is also shown (asterisk).[28]

RESULTS

A variant form of *hMTH1* (M83) is more frequent in gastric cancer patients

In a panel of 72 gastric cancer patients, the nucleotide sequence of the *hMTH1* gene was determined using reverse transcript-polymerase chain reaction (RT-PCR) products. The 333-bp cDNA fragment that encompasses a region coding the 'phosphohydrolase module', the catalytic domain of oxidised purine nucleoside triphosphatase, was amplified and sequenced (fig 1). In agreement with previous reports,[6] the minor M83 allele that encodes methionin (ATG), instead of valine (GTG), at codon 83 was found in several patients. According to the sequence data, the subject population was divided into three allelotypes, V83: GTG/V83: GTG, V83: GTG/M83: ATG, and M83: ATG/M83: ATG (table 1). In gastric cancer patients, the M83 allele appeared more frequent than that in healthy controls (χ_2 test, $p = 0.007$). Among 72 gastric cancer patients, 14 (19.5%) carried the M83 allele. Four patients (5.6%) were homozygous for this variant allele. The subject population was next divided into two subgroups, one homozygous for this variant allele and the other. Another χ_2 test revealed that the allelotype homozygous for the variant *hMTH1* allele (M83) was significantly more frequent in gastric cancer patients than in healthy controls ($p = 0.01$) (table 1). Thus, we conclude that the variant form of *hMTH1* (M83) is more frequent in gastric cancer patients. These sequence alterations were also confirmed by sequencing PCR products of genomic DNA extracted from tumour specimens and corresponding normal tissues (data not shown). No other sequence alterations were found within the region examined.

We statistically tested whether this V83M variation of *hMTH1* correlates with clinicopathological features of the patients. However, no statistically significant correlation was found between the presence of this variant allele and any of the common clinicopathological features of patients (data not shown).

V83M variation of *hMTH1* correlates with mutation in *p53* gene

hMTH1 encodes an enzyme that hydrolyses mutagenic oxidised purine nucleoside triphosphates such as 8-oxo-dGTP. Variation of a cellular component functioning in the

Table 1 The frequency of single nucleotide polymorphism at codon 83 in the *hMTH1* gene: a comparison between gastric cancer patients and healthy controls

hMTH1 allelotype	Gastric cancer (n=72)	Controls (n=400)[7]	
V83: GTG/V83: GTG	58(80.5%)	330(83.5%)[7]	
V83: GTG/M83: ATG	10(13.9%)	67(16.8%)[7]	
M83: ATG/M83: ATG	4(5.6%)	3(0.8%)[7]	
			p=0.0073
V83: GTG/V83: GTG, V83: GTG/M83: ATG	68(94.4%)	397(99.2%)[7]	
M83: ATG/M83: ATG	4(5.6%)	3(0.8%)[7]	
			p=0.01

anti-mutagenic systems may affect the sequence stability maintained throughout the genome. Carcinogenesis is thought to comprise stepwise alterations in proto-oncogenes or tumour suppressor genes. We therefore examined the relationship between *hMTH1* variation and mutation in *p53* gene, a tumour suppressor gene most commonly mutated in various human malignancies. In our panel of 72 patients with gastric cancer, *p53* mutations resulting in an amino acid substitution were detected in 15 tumours (20.8%). The mutations were predominantly G:C to A:T transitions in codons, including the acknowledged hot spots (data not shown). Intriguingly, *p53* mutation correlated with the variant form of *hMTH1* (table 2). The frequency of *p53* mutation was significantly higher in tumours harbouring at least one M83 allele than in those without M83 allele (Fisher's exact test, p = 0.034). However, in a comparison between tumours homozygous for M83 allele and others, difference in the incidence of *p53* mutation was not statistically confirmed, probably due to a paucity of M83-homozygous tumours. Among nine *p53*-mutant tumours without the M83 allele, G:C to A:T and A:T to G:C transitions were noted in three (3/9, 33.3%) and two (2/9, 22.2%), respectively. G:C to T:A transversion was also shown in three tumours (3/9, 33.3%) (table 3). On the other hand, among six tumours with *p53* mutations that possessed at least the M83 allele, G:C to A:T transitions were observed in four (66.7%) (table 3). However, this partiality of mutation to G:C to A:T transition in tumours harbouring a M83 allele was not statistically confirmed. A:T to C:G transversion was not found in neither.

DISCUSSION

Altered function of *hMTH1* has been suspected in various pathological states, particularly in tumourigenesis, in human bodies. However, in spite of efforts to explore *hMTH1* gene in various human diseases,[6] no mutations have been found. Single nucleotide polymorphism (SNP) at codon 83, V83: GTG to M83: ATG, was not clearly associated with lung or liver cancers.[7] However, in the present study, we have shown that in gastric cancer patients this polymorphic variation of *hMTH1* is significantly more frequent than in healthy controls. In this context, this is the first report that has shown a connection between V83M variation of *hMTH1* and human malignancies.

Mutations are found in various genes in tumours. One may presume a state with an elevated mutation rate in some steps of tumourigenesis. However, it still remains controversial whether a state with an elevated mutation rate plays an important role in tumourigenesis. Bodmer and colleagues[11 12] pointed out that tumour cells that harbour mutations in tumour suppressor genes or proto-oncogenes can be selected merely by phenotypical advantage, without an elevated mutation rate. On the other hand, a line of evidences suggests an existence of a state with an elevated mutation rate in tumours, and this state is now referred to as 'mutator phenotype'. The critical role of mutator phenotype in tumourigenesis has been emphasised by Loeb and colleagues.[13 14] Spontaneous mutation rate on the genome is invariably regulated. Previous studies using *E coli* mutators suggest that there are several cellular systems, the failure of which will lead to a significant increase in the mutation rate.

The first example of mutator phenotype derived from a disruption of such cellular systems is defective DNA mismatch repair in a cancer prone syndrome, hereditary non-polyposis colorectal cancer (HNPCC).[15 16] Deficiency in mismatch repair is also associated with other human malignancies.[17] One may expect that the spontaneous mutation rate is elevated in mismatch repair defective cells. Indeed, in mismatch repair gene knock out mouse cells, the mutation rate was 15-fold higher than in wild type cells.[18] However, mutations in representative tumourigenic genes, such as *p53* and *ras*, were not found in mismatch repair defective human tumours. Instead, insertion/deletion mutation in repetitive sequences such as microsatellites was observed. This phenomenon is now referred to as 'microsatellite instability' (MSI), and the cellular phenotype with MSI has been designated 'microsatellite mutator phenotype' (MMP).[19 20] The discrepancy between knock out animals and

Table 2 Relationship between *p53* gene status and V83M variation of *hMTH1*

	p53 gene status	
hMTH1 allelotype	wt (n=57)	m (n=15)
V83: GTG/V83: GTG	49(84.5%)	9(15.5%)
V83:GTG/M83:ATG, M83: ATG/M83: ATG	8(57.1%)	6(42.9%)
		p=0.034

Table 3 Spectra of *p53* mutations in gastric tumours with and without the M83 *hMTH1* allele

p53 mutation	V83:GTG/ V83:GTG (n=9)	V83:GTG/M83:ATG, M83:ATG/M83:ATG (n=6)
Transition		
G:C → A:T	3(33.3%)	4(67.4%)
A:T → G:C	2(22.2%)	0
Transversion		
G:C→T:A	3(33.3%)	1(16.7%)
G:C→C:G	0	0
A:T→T:A	1(11.1%)	1(16.7%)
A:T→C:G	0	0

human tumours may suggest an oversimplification in our discussions.

The second example is failure in the systems counteracting mutagenic oxidative damage in cells. In *E coli*, MutT, MutY, and MutM cooperatively function to minimise the effects of an oxidised form of guanine nucleotides, 8-oxo-dGTP, in the pool and on the genome.[4] Mammalian homologues of these proteins and mice in which their genes are homozygously disrupted have been reported.[9 21–23] As mentioned above, the failure in this system that is clearly associated with human diseases has not been found. However, in *MTH1*-null ES cells, the mutation rate is elevated twofold higher, compared with the wild type counterparts.[9] Altered function of *hMTH1* may also lead to accumulated mutations in various genes in human cells.

Connection between V83M variation and mutation in other genes, particularly proto-oncogenes or tumour suppressor genes, has not thus far been addressed. This is the first report of a connection between the variant *hMTH1* and *p53* mutation.

E coli MutT hydrolyses an oxidised nucleotide, 8-oxo-dGTP, into its monophosphate form.[1] 8-oxo-dGTP incorporated in the genome pairs with adenine, as well as cytosine, which results in G:C to T:A or A:T to C:G transversions. In an *E coli* strain lacking MutT, the spontaneous occurrence of A:T to C:G transversion increases 1000-fold higher than in wild type strains.[2] In the *mutT*$^-$ strain, G:C to T:A transversions are suppressed low by compensatory activities of MutM and MutY glycosylases.[4]

Frequent variation of *hMTH1* in gastric cancer may suggest a biased spectrum in observed mutations. However, the majority of mutations found in *p53* was G:C to A:T transitions, albeit this bias has not been statistically confirmed (table 3). No A:T to C:G transversions were found. In fact, the biological significance of V83M variation has not been determined. Yakushiji H *et al*[24] reported that recombinant M83-variant hMTH1 exhibited an increased heat lability in both structure and catalytic function; however, within a physiological range of temperature, difference in the activity to hydrolyse 8-oxo-dGTP was not confirmed.

Recently, other forms of oxidised nucleotides have been found as a mutagenic substrate for DNA replication. 2-hydroxy-2′-deoxyadenosine 5′-triphosphate (2-OH-dATP) is known to be formed in cells by an oxidative stress[25] and, once incorporated into the genome, pairs with various bases including cytosine.[26] Pairing of cytosine with an incorporated adenine nucleotide results in G:C to A:T transitions. Intriguingly, hMTH1 efficiently hydrolyses this oxidised adenine nucleotide as well as 8-oxo-dGTP, whereas *E coli* MutT does only the latter.[6 27] Difference in the activity to hydrolyse 2-OH-dATP between M83-variant hMTH1 and its authentic form is now being approached in our laboratories.

V83M variation may conceal a previously unrecognised biological significance. H Oda *et al*[28] reported that the polymorphism at codon 83 is tightly linked with another SNP, exon2b(GT) to exon2b(GC) at the 5′-splicing site of exon2b segment that is an exonic intron and spliced out by alternative splicing (fig 1). In the exon2b(GC) allele, an alternative translation initiation occurs on *hMTH1* transcripts, resulting in hMTH1, a protein that possesses a functional mitochondrial targeting sequence (Sakai and Nakabeppu, in preparation). Although this polymorphism has not been addressed in this study, a tight linkage between M83 allele and the exon2b(GC) allele may suggest the existence of another molecular variety with different biological functions. This present study suggests that a functional alteration of hMTH1 due to these polymorphisms may occur in vivo, and that an imbalance among these molecular varieties may relate to tumourigenesis, at least in the stomach.

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Frequent Loss of Heterozygosity but Rare Microsatellite Instability in Oesophageal Cancer in Japanese and Chinese Patients

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Key Words
Squamous cell carcinoma · Replication error · DNA mismatch repair · Fluorescent dye

Abstract
Reported frequencies for microsatellite instability (MSI) in oesophageal cancer differ widely in the literature, perhaps due to the high incidence of loss of heterozygosity (LOH) in this cancer. Using high-resolution fluorescent microsatellite analysis (HRFMA), we analysed microsatellite alterations in detail in 50 Japanese and 50 Chinese patients with squamous cell carcinoma in the oesophagus. In HRFMA, several devices have been developed to improve the detection characteristics, reproducibility of polymerase chain reaction and the migration accuracy of electrophoresis. All the alterations observed were separable into MSI, LOH and alterations ambiguous for both. MSI was rare in these panels of oesophageal carcinomas. The frequencies of MSI in the Japanese and Chinese subjects were 8 and 4%, respectively. All the alterations were mild (within 2 base pairs) and were observed in a limited number of markers. More drastic types of MSI, such as those typical in colorectal cancer, were not observed. On the other hand, the incidence of LOH was high, reaching 50% for the Japanese and 70% for the Chinese subjects. In many of these cases, LOH was observed in multiple microsatellite markers. The frequency of LOH in each marker was not apparently biased. Although in many cases MSI and LOH were clearly distinguished with use of the sensitive and quantitative fluorescent assay, theoretically indistinguishable patterns were noted in some cases. In conclusion, MSI is rare and LOH predominates in squamous cell carcinoma in the oesophagus.

Introduction

Cancer may arise from an imbalance between DNA damage derived from the internal or external environment and cellular activities to counteract it. In some cancers, specific environmental factors are regarded as risk factors, while in others, genetic factors are considered more important. In colorectal cancer, genetic defects which cause deficiency in DNA mismatch repair are known to be risk factors for multiple cancer and familial

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predisposition since in individuals with a familial cancer syndrome, hereditary non-polyposis colon cancer (HNPCC), mutations in genes encoding DNA mismatch repair proteins are inherited [1, 2]. Since DNA mismatch repair removes not only mispaired nucleotides but also ones misaligned on repetitive sequence contexts, the result of slippage of DNA polymerases, alterations in repeat sequences such as microsatellites, i.e. microsatellite instability (MSI), serve as a marker to detect mismatch repair deficiency. Indeed, MSI is frequently observed in tumours occurring in HNPCC individuals and, in addition, in sporadic cases of colon cancer [3–6]. Since MSI was reported in sporadic cases of other cancers, numerous studies have been performed concerning a wide variety of human malignancies. Oesophageal cancer is one of the most malignant tumours occurring in the digestive tract. It is known that this malignancy is prevalent in relatively limited geographical areas and populations [7], which implies that specific environmental or genetic factors are involved. Since smoking and drinking have been identified as risk factors, the contribution of environmental factors is considered important. MSI has also been examined in patients with oesophageal cancer. However, reported frequencies for MSI in oesophageal cancer differ widely in the literature [8–21], while relatively high frequencies have been uniformly reported in tumours arising in the other regions of the digestive tract [22–24]. This discrepancy may derive from the high incidence of loss of heterozygosity (LOH) in oesophageal cancer [25–31]. As discussed in the international workshop on MSI sponsored by the National Cancer Institute in 1997 [32], some patterns of MSI are indistinguishable from LOH. Particularly in some of the conventional MSI assays using radiolabelled polymerase chain reaction (PCR) and X-ray films, sensitive and accurate detection is limited and, consequently, LOH may be misinterpreted as MSI. Technologies to label nucleic acids with fluorescent dye compounds have evolved, and a combination of fluorescent labelling and laser scanning such as utilised in automated DNA sequencers is now used in a wide variety of nucleic acid analyses. In such systems, various improvements, including sensitive and quantitative detection, have been made. Indeed, use of an automated sequencer for microsatellite analyses is now on the increase. Such fluorescent systems have not thus far been used to analyse MSI in oesophageal cancer. Using a new fluorescent system that we have developed [33], we analysed microsatellite alterations in detail in 100 patients with oesophageal cancer.

Materials and Methods

Specimens and Extraction of Genomic DNA

Pairs of cancer tissues and corresponding normal mucosa were obtained from 100 patients with squamous cell carcinoma in the oesophagus, including 50 Japanese patients who underwent surgery in the Department of Surgery and Science, Kyushu University Hospital in Fukuoka, Japan, and 50 Chinese patients treated in the Department of Thoracic Surgery, Chinese Academy of Medical Sciences in Beijing, China. The specimens, taken immediately after resection, were placed in liquid nitrogen and used for analysis of genomic DNA. The remaining tissues were routinely processed for histopathological analyses. All the tumours were diagnosed by specialists in histopathology in each hospital.

Frozen tissues were broken up in liquid nitrogen and lysed in digestion buffer (10 m*M* Tris-Cl, pH 8.0, 0.1 *M* EDTA, pH 8.0, 0.5% SDS, 20 μg/ml pancreatic RNase). After treatment with proteinase K and extraction with phenol, DNA was precipitated with ethanol, then dissolved in 1 × TE (10 m*M* Tris-Cl, pH 7.5, 1 m*M* EDTA).

High-Resolution Fluorescent Microsatellite Analysis

Five human dinucleotide microsatellites, D2S123, D5S107, D10S197, D11S904 and D13S175, were used as markers for MSI. Using genomic DNA derived from the tissue specimens, the five microsatellite sequences were amplified by PCR. Oligonucleotide primers corresponding to the microsatellite sequences [33] were synthesised and purified by HPLC, and forward primers were labelled with fluorescent compounds, 6-carboxy-x-rhodamine (ROX) or 6-carboxy-2',4',7',4,7-hexachloro-fluorescein (HEX). PCR reactions were performed using *TaKaRa Taq* Reagent Kits (TaKaRa Co. Ltd., Tokyo, Japan) and Applied Biosystems GeneAmp PCR system 9600 or 9700 (Applied Biosystems, Foster City, Calif., USA). A 50-μl reaction mixture contained 1 × reaction buffer, 350 μ*M* of each dNTP, 10 pmol of each primer, 2.5 units of *Taq* polymerase and 25 ng of genomic DNA. The thermal conditions of the system were as follows: 1 cycle at 95 °C for 4 min, 35 cycles at 95 °C for 0.5 min, 55 °C for 0.5 min and 72 °C for 0.5 min, and 1 cycle at 72 °C for 10 min. Then, 0.5 units of T4 DNA polymerase was added to the mixture, followed by incubation at 37 °C for 10 min. 1.5 μl of the product was mixed with 0.5 μl of loading buffer (blue dextran, 25 m*M* EDTA), 2.5 μl of formamide and 0.5 μl of dH_2O. To compare electrophoretic profiles between two samples, 1.2 μl of ROX-labelled product and 0.3 μl of HEX-labelled product were mixed, denatured and loaded onto an ABI 373A sequencer (Applied Biosystems). The running conditions were 1,500 V, 20 mA and 30 W for 5.5 h. Data were processed using ABI software, GeneScan version 1.2.2 (Applied Biosystems).

Results

Fifty tumours were examined in each panel of Japanese and Chinese patients with oesophageal cancer, using high-resolution fluorescent microsatellite analysis (HRFMA) [33]. In all cases, the histological type was squamous cell carcinoma, and no case of adenocarcinoma was included. In HRFMA, five dinucleotide micro-

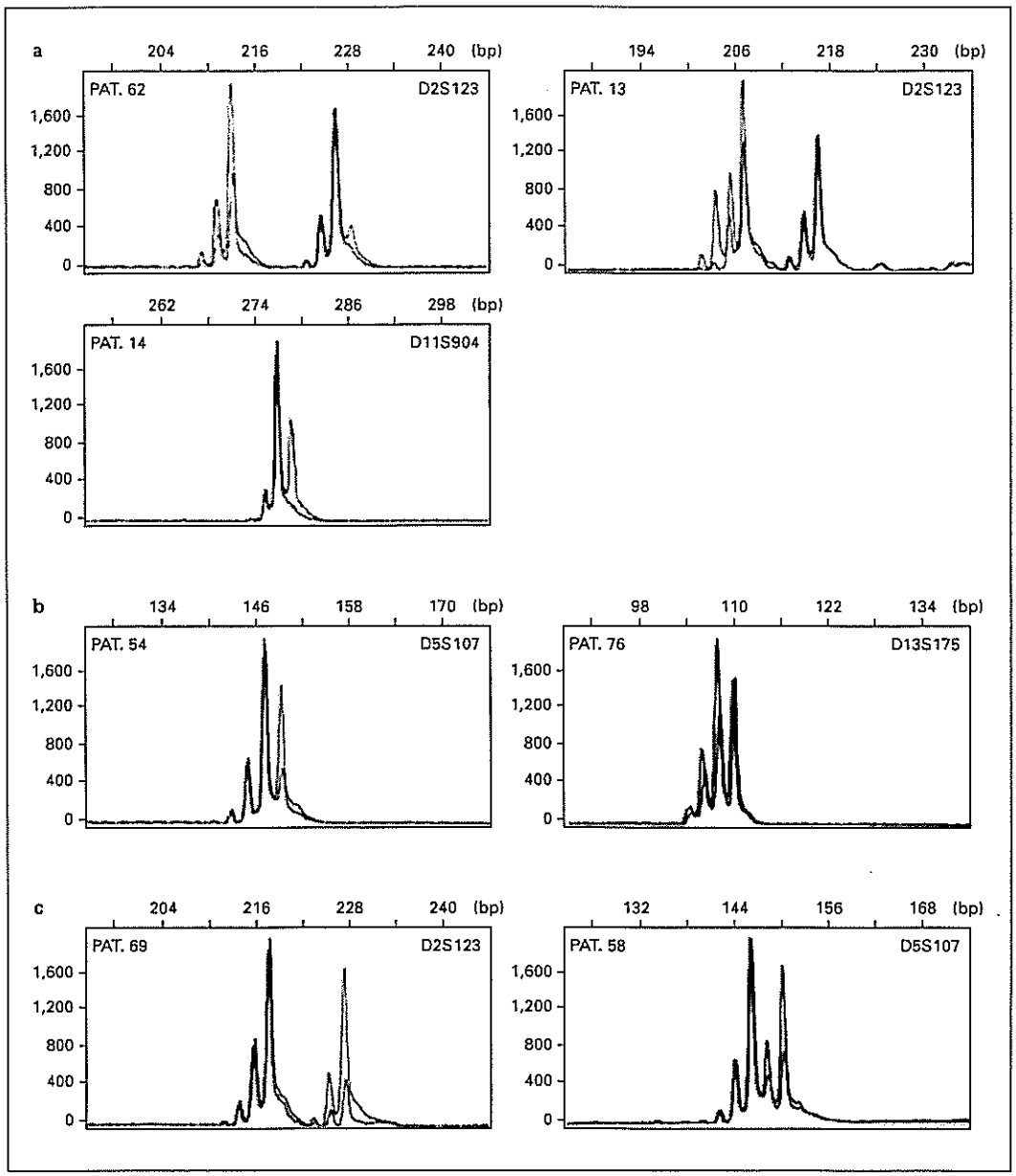

Fig. 1. Microsatellite alterations observed in oesophageal cancer. Using genomic DNA samples derived from cancer and corresponding normal mucosa, five dinucleotide microsatellite sequences were independently amplified by PCR, using two differentially labelled fluorescent primers. PCR products were then mixed and electrophoresed in the same lane in an automated DNA sequencer. All alterations observed were classified as MSI (**a**), alterations ambiguous for MSI and LOH (**b**) and LOH (**c**). Representative results for each category are shown. Red lines: cancer; green lines: normal mucosa. The patient codes correspond to those used in table 1. PAT. = Patient.

satellites which have a wide variety of lengths and are located in an independent chromosome were analysed in HRFMA. Using two genomic DNA samples derived from cancer and corresponding normal tissues, each microsatellite sequence was independently amplified by PCR using primers labelled differentially with fluorescent dye compounds, then mixed and electrophoresed in the same lane in an automated DNA sequencer. All the alterations observed were classified into three categories: MSI, LOH and alterations ambiguous for both (fig. 1). Through analyses of various types of tumours, we have found that MSI observed in human cancers can be classified into at least two distinct subtypes, i.e. 'type A', showing relatively small changes within 6 base pairs, and 'type B', exhibiting drastic changes over 8 base pairs [34–36]. Similarly, in one of the earliest reports of the MSI phenomenon, Thibodeau et al. [6] divided MSI into two categories, i.e. type I and II mutations. The former was defined as a 'significant increase (expansion) or decrease (deletion) in the apparent fragment size' and the latter as a 'single 2-bp change'. Intriguingly, in oesophageal cancer, drastic changes over 8 base pairs, i.e. type B/type I mutation, were not observed. All the cases with MSI showed a limited extent of alterations within 2 bases, i.e. type A/type II mutation (fig. 1a). It seems to be generally accepted that MSI can also be classified in terms of the frequency of changes in a given set of microsatellite markers. The National Cancer Institute workshop mentioned above recommended classifying MSI into two different grades, i.e. MSI-H and MSI-L [32]. The first is defined as MSI showing microsatellite alterations in 'the majority of markers' (e.g. $\geq 40\%$) and the second as MSI exhibiting changes only in 'a minority of markers' (e.g. $<40\%$). These two categories may correspond to the type I and type II mutations of Thibodeau et al. [6], since those authors described that all tumours with type I mutations showed changes in multiple loci and that type II mutation was observed mainly at a single locus. Indeed, MSI noted in this study were all type II mutations and observed in only one locus, suggesting that MSI-L is dominant in oesophageal cancer. Some patterns of MSI were not distinguishable from LOH (fig. 1b). This type of alteration was often observed in this study. On the other hand, some patterns of microsatellite alterations were clearly judged to be LOH (fig. 1c). We considered given alterations to be LOH when fragments derived from both parental alleles were clearly separated in an electrophoretogram and the signal magnitude of either allele was more than 30% lower than that of the other. To verify the sensitivity and reproducibility in this system, we examined the quantitative relationship between PCR products of the parental alleles in various allelotypes, and concluded that over 10% relative change in the signal magnitude between the peaks in question is significant [unpubl. data].

Frequencies of these microsatellite alterations are summarised in tables 1 and 2. MSI was rare in both Japanese and Chinese subjects, the frequency being 8 and 4%, respectively. On the other hand, LOH was frequent in these panels. The incidence of LOH in the Japanese and Chinese patients reached 50 and 70%, respectively. Among these LOH cases, 13 Japanese (50%) and 18 Chinese patients (49%) had LOH in more than one locus. Intriguingly, LOH coexisted even in cases with MSI (tables 1, 2). The incidence of LOH in each microsatellite marker was not evidently biased. Ambiguous alterations were also noted. These changes are theoretically indistinguishable between MSI and LOH [36]. If these observed changes were all MSI, the frequency of MSI would increase to 48 and 38% in the Japanese and Chinese panels, respectively. In addition, cases in which both ambiguous alterations and LOH were simultaneously noted in the set of five microsatellites were not rare: 12/25 (48%) for the Japanese panels and 11/35 (31%) for the Chinese panels. It would appear rather likely that many of these ambiguous alterations are, in fact, LOH. This may be compatible with the knowledge that type II mutations rarely occur in multiple loci. Thus, in our panels of 100 patients with oesophageal cancer, MSI was rare and all the alterations were type A/type II mutation/MSI-L. On the other hand, LOH was frequent.

Discussion

Using HRFMA, we obtained details of microsatellite alterations in oesophageal cancer. All the alterations were separable into MSI, LOH and alterations ambiguous for both. MSI was rare and characterised by mild alterations, i.e. type A or type II mutations of Thibodeau et al. [6], in a limited number of markers, which implies that the type of MSI is MSI-L. LOH was frequent, but no hot spot was found in the set of five microsatellite markers. Cases showing LOH in multiple loci were also frequent.

Data on the frequency of MSI in oesophageal cancer differ widely in the literature. Some groups reported relatively high frequencies [8, 9, 14, 16–19], while others found low ones [11, 13, 15, 20, 21]. This discrepancy may result from the high incidence of LOH in oesophageal cancer [25–31]. As mentioned above, in some patterns of MSI, the possibility of LOH cannot be excluded. The con-

Table 1. Microsatellite alterations noted in 50 Japanese and 50 Chinese patients with oesophageal cancer

Patient No.	D2S123	D5S107	D10S197	D11S904	D13S175
Japanese patients					
1	MSI	–	A	–	–
2	–	L	–	–	L
3	–	A	–	–	–
4	–	–	–	A	–
5	–	–	–	–	–
6	–	–	–	–	–
7	L	L	–	–	L
8	L	–	–	–	A
9	–	L	–	–	A
10	L	–	L	–	A
11	–	L	A	L	–
12	–	–	A	–	–
13	MSI	–	–	–	–
14	–	L	–	MSI	–
15	–	–	L	L	A
16	–	A	–	–	–
17	–	L	–	–	L
18	–	–	–	L	–
19	L	–	L	–	A
20	L	–	–	L	A
21	–	–	–	–	–
22	–	–	–	–	–
23	–	–	–	–	–
24	–	L	–	–	–
25	L	–	–	–	L
26	L	A	–	–	A
27	–	A	–	–	L
28	–	–	–	–	A
29	–	–	–	–	–
30	–	–	–	–	–
31	L	–	–	–	A
32	–	–	–	–	–
33	–	–	–	–	A
34	L	–	–	–	A
35	–	–	–	–	–
36	–	–	–	–	L
37	–	–	–	L	–
38	–	A	L	L	–
39	–	MSI	–	–	–
40	–	A	–	–	A
41	–	–	–	L	L
42	–	A	–	–	A
43	–	–	–	–	–
44	L	L	–	L	–
45	–	–	–	–	–
46	L	–	–	L	L
47	–	–	–	–	–
48	–	–	–	–	–
49	–	–	–	L	–
50	–	L	–	–	–

Patient No.	D2S123	D5S107	D10S197	D11S904	D13S175
Chinese patients					
51	–	–	–	–	L
52	A	L	L	L	L
53	–	–	–	A	–
54	L	A	–	A	L
55	–	–	–	–	–
56	–	L	–	L	–
57	L	L	A	–	–
58	–	L	–	L	A
59	–	–	L	–	L
60	–	–	–	L	–
61	–	A	–	L	MSI
62	MSI	MSI	L	–	–
63	–	–	A	–	–
64	–	L	L	–	L
65	–	–	–	–	–
66	–	–	–	–	A
67	–	–	A	–	–
68	–	–	–	L	–
69	L	L	A	L	L
70	L	–	–	–	–
71	–	–	A	–	A
72	–	–	–	L	–
73	–	–	–	L	–
74	A	–	L	–	L
75	–	–	–	L	–
76	–	L	–	–	A
77	–	–	–	L	–
78	L	–	–	–	–
79	–	–	–	–	–
80	–	–	L	L	A
81	L	–	–	–	–
82	L	–	L	–	–
83	–	–	–	–	A
84	–	–	–	–	–
85	–	L	–	–	A
86	L	–	L	L	L
87	–	L	–	L	L
88	–	L	–	L	–
89	–	–	–	–	L
90	–	–	–	L	L
91	–	A	–	A	–
92	–	L	–	–	–
93	–	–	–	–	–
94	–	–	L	L	–
95	–	–	–	–	L
96	L	–	–	L	L
97	L	–	–	–	–
98	–	–	–	–	–
99	L	L	–	L	A
100	–	–	–	L	–

L = LOH (≥ 30% reduction of signal in either peak cluster); A = ambiguous for MSI and LOH.