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Original Paper

Mutations in *hMSH6* Alone are not Sufficient to Cause the Microsatellite Instability in Colorectal Cancer Cell Lines

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Microsatellite instability (MSI) at simple repeated sequences characterises a distinct mechanism of carcinogenesis in hereditary nonpolyposis colorectal cancer (HNPCC), as well as sporadic colorectal cancers displaying MSI. Such MSI is associated with mutations of *hMSH2* and *hMLH1*, and somatic frameshift mutations in *TGF-βRII*, *IGFIIR*, *BAX*, *hMSH3* and *hMSH6* at simple repeated sequences in coding regions. The aim of this study was to look for a correlation between mutations in mismatch repair genes and frameshift mutations in colorectal cancer cell lines with MSI. Using 22 colorectal cancer cell lines, we examined the MSI status at mononucleotide repeat microsatellite markers and mutations in *hMSH2* and *hMLH1*, *TGF-βRII*, *IGFIIR*, *BAX*, *hMSH3* and *hMSH6*. Thirteen of 22 lines (59%) displayed MSI. In these 13 lines showing MSI, 10 lines (77%) had mutations in *TGF-βRII*, nine lines (69%) in *BAX*, seven lines (54%) in *hMSH6*, and six lines (46%) in *hMSH3*, while mutations in the *IGFIIR* gene were identified in only two lines (15%). Of the 13 lines with MSI, six lines (46%) harboured mutations/deletions in *hMSH2* (two nonsense mutations, three deletions and one no expression of transcripts) and three of these cell lines (50%) had mutations both in the *hMSH2* and *hMSH3* genes. Two cell lines (15%) had mutations/deletions in *hMLH1* (one missense mutation and one deletion) and these two cell lines also had mutations in *hMSH3*. One line had a mutation in *hMSH3* only, although this line showed MSI and had mutations in *TGF-βRII*, *IGFIIR* and *BAX*. All lines with mutations in *hMLH1*, *hMSH2*, *TGF-βRII*, *IGFIIR*, *BAX* and *hMSH3* genes showed MSI. However, of the nine lines without MSI, two (22%) had homozygous mutations in *hMSH6*. In these two cell lines, no mutations were identified in *hMLH1*, *hMSH2*, *TGF-βRII*, *IGFIIR*, *BAX* and *hMSH3*. Our results indicate that mutations in *hMLH1*, *hMSH2* and *hMSH3* are associated with MSI, but mutations in *hMSH6* are not. We conclude that mutations in *hMSH6* alone are not sufficient to cause MSI, although protein functional effects of these mutations should still be examined. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: microsatellite instability, colorectal cancer cell lines, *hMSH2*, *hMLH1*, *TGF-βRII*, *IGFIIR*, *BAX*, *hMSH3*, *hMSH6*
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INTRODUCTION

MICROSATELLITE INSTABILITY (MSI) is manifested by frequent somatic variation in the size of microsatellite alleles within tumour DNA, as compared with matching normal DNA [reviewed in 1, 2]. The MSI phenotype has been found in up to 90% of HNPCC (hereditary nonpolyposis colorectal cancer)-associated colon cancers, as compared with only 16% of sporadic colon cancers [3]. The germline mutations within mismatch repair genes (*hMSH2*, *hMLH1*, *hPMS1*, *hPMS2* and *hMSH6*) and somatic mutations in the *hMSH3* gene have been found in HNPCC patients as well as some apparently non-familial tumours showing MSI [4–14].

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Although corresponding normal DNA is a prerequisite to determining the MSI status of tumours and cell lines, recently it was reported that the mononucleotide repeat microsatellite marker BAT-26 is an indicator which can be used to screen the MSI status of primary colorectal tumours and cell lines, even in the absence of matched normal DNA [15–17]. Two other mononucleotide repeat microsatellite markers, BAT-25 and BAT-40, are also unstable in HNPCC and sporadic colorectal cancers [16]. MSI has been demonstrated in multiple primary human tumour types, although most MSI has been described in noncoding DNA.

Targets of MSI within coding regions have been identified in the *TGF-βRII*, *IGFIIR*, *BAX*, *hMSH3* and *hMSH6* genes in colon cancer cells. These genes have a mutational hotspot comprising a 10 bp deoxyadenine (*TGF-RII*), or a 8 bp deoxyguanine (*IGFIIR* and *BAX*), or a 8 bp deoxyadenine (*hMSH3*) or a 8 bp deoxycytosine (*hMSH6*) repeat tracts in their coding regions. Frequent frameshift mutations in these tracts caused by small insertions or deletions and resulting in the synthesis of truncated proteins, have been reported in endometrial, gastric and colorectal cancers showing MSI [10–13, 15–29]. Of these, mutations of *BAX* in colon cancers with MSI provide a good example of the differences between the mutator and suppressor pathways of tumorigenesis and help to explain the observation that colon cancer with MSI exhibits a negative correlation with *TP53* mutations [18, reviewed in refs 13, 30].

For the accumulation of microsatellite mutations in MMP (microsatellite mutator phenotype) tumours, a 'mutator that mutates another mutator' model to describe the stepwise nature of the unfolding of the MMP has been proposed [10, 13, 27]. According to this model, the inactivation of the 'primary' mutators including *hMLH1* and *hMSH2* accelerates the mutational inactivation of 'secondary' mutators such as *hMSH3* and *hMSH6*. Moreover, it has been shown that HNPCC tumours tend to follow this model [13]. Especially, mutations in *hMSH6* have been observed in a small number of human gastro-intestinal tumour tissues, leukaemic, endometrial cancers and cell lines displaying MSI [8–10, 12, 13]. These mutations, however, have often been observed in conjunction with mutations in other mismatch repair genes [19, 20, 28]. Recently, it has been shown that *MSH6* mutation causes cancer susceptibility and does not cause microsatellite instability in mice carrying a null mutation in *MSH6* [31]. However, the precise role of *hMSH6* mutations in the onset and progression of cancer has not been determined in human tumours.

In this study, we determined the MSI status of the non-selected 22 colorectal cancer cell lines, and analysed mutations of *hMLH1* and *hMSH2* genes. In addition, we investigated frameshift mutations of *TGF-βRII*, *IGFIIR*, *BAX*, *hMSH3* and *hMSH6* genes containing simple repeated sequences in the coding region, and looked for a correlation between mutations in mismatch repair genes and frameshift mutations in colorectal cancer cell lines with MSI.

MATERIALS AND METHODS

Cell lines and nucleic acid isolation

A total of 22 non-selected colorectal cancer cell lines (Table 1) and two gastric cancer cell lines (SNU-1 and SNU-16) were obtained from the KCLB (Korean Cell Line Bank)

Table 1. *Microsatellite instability and mutational status of* TGF-βRII, IGFIIR, BAX, hMLH1, hMSH2, hMSH3 *and* hMSH6 *genes in colorectal cancer cell lines*

	MSI status			*TGF-βRII*	*IGFIIR*	*BAX*	*hMLH1*	*hMSH2*	*hMSH3*	*hMSH6*
BAT-26 [Ref.]	BAT-25	BAT-40	(A)10	(G)8 [Ref.]	(G)8	[Ref.]	(A)8 (C)8 [Ref.]	[Ref.]	[Ref.]	[Ref.]
SNU-61	–	–	–	–	–	–	wt	wt	–	–
SNU-81	–	–	–	–	–	–	wt	wt	–	– 1
SNU-175	+⋆	+	+	–	–	–	wt	wt	–	–
SNU-283	– †	–	–	–	–	–	wt	wt	–	–
SNU-407	+	+	+	+ 1/ – 1‡	–	+ 1/ – 1	wt	Mt‖	–	–
SNU-503	–	–	–	–	–	–	wt	wt	–	– 1
SNU-769A	+	+	+	– 2	–	+ 1/ – 1	wt	Del¶	– 1	– 1
SNU-769B	+	+	+	– 1/ – 2	–	+ 1/ – 1	wt	Del¶	– 1/wt	–
SNU-1033	–	–	–	–	–	–	wt	wt	–	–
SNU-1040	+	+	+	–	–	– 1/wt	Mt§	wt	– 1/wt	–
SNU-1047	+	+	+	– 1	– 1/wt	– 1/wt	wt	wt	– 1/wt	–
SNU-1197	–	–	–	–	–	–	wt	wt	–	–
C2A	+	+	+	–	– 1/wt	–	wt	NE⋆⋆	–	+ 1
C4	+	+	+	– 1	–	– 1	wt	Mt⋆⋆	– 1	– 1
HCT-116 [30]⋆	+	+	+	– 1 [16]	–	– 1/wt [27]	Del [33]	wt	– 1 [34]	wt [35]
LoVo [30]⋆	+	+	+	– 2 [16]	–	+ 1/ – 1 [27]	wt	Del [6]	– [34]	– [35]
LS174T [30]⋆	+	+	+	– 1 [16]	–	– 1 [27]	wt	wt	–	– 1 [35]
HCT – 8	+	+	+	– 1/wt	–	–	wt	wt	–	–
Colo205	–	–	–	–	–	–	wt	wt	–	–
SW480	–	–	–	–	–	–	wt	wt	–	–
DLD-1 [17]⋆	+	+	+	– 1/wt [16]	–	– [27]	wt	wt	– [34]	Mt [9]
WiDr	–	–	–	–	–	–	wt	wt	–	–

⋆MSI, microsatellite instability. †MSS, microsatellite stable. ‡wt, if sample is wild-type at both alleles, or the number (e.g. – 1/ – 2) indicates the number of bases lost at each allele. §CGC→TGC (Arg to Cys) at codon 725 [32]. ‖TCA→TGA (Ser to Stop) at codon 251 [32]. ¶Deletion from exons 7 to 16 [32]. ⋆⋆NE, no expression by RT-PCR, Mt, mutant (data not shown). Del, deletion; Mt, mutant.

(Seoul, Korea) or ATCC (American Type Culture Collection) (Rockville, Maryland, U.S.A.). Twelve SNU-colorectal cancer cell lines except SNU-C2A and SNU-C4 were newly established and reported from our laboratory [32]. These cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS). Cultures were maintained in humidified incubators at 37°C in an atmosphere of 5% CO_2 and 95% air. Genomic DNA and total RNA were isolated from washed-cell pellets. Total genomic DNA was extracted according to the standard sodium dodecyl sulphate (SDS) proteinase K procedure; total RNA was isolated using TRI reagent according to the manufacturer's instructions (Molecular Research Center, Cincinnati, Ohio, U.S.A.). For cDNA synthesis, 2 μg of total RNA was subjected to reverse transcription with random oligo (dT) primer, dNTPs, and 1 μl (200 units) of the Superscript™ II reverse transcriptase (Life Technologies, Gaithersburg, Maryland, U.S.A.).

Analysis of microsatellite instability

Because of a lack of corresponding normal DNA in colorectal carcinoma cell lines in this study, MSI status was assessed using the mononucleotide repeat microsatellite markers, *BAT-26*, *BAT-25* and *BAT-40* [15–17]. The sense primer of each marker was labelled with [γ-^{32}P]dATP (Amersham, Aylesbury, U.K.) using T4 polynucleotide kinase (New England Biolabs, Massachusetts, U.S.A.). Polymerase chain reaction (PCR) conditions consisted of 35 cycles of 95°C for 30 sec, 45°C for 1 min and 70°C for 1 min, and final elongation was 70°C for 10 min. PCR products were denatured and separated on 6 M urea/32% formamide/7% polyacrylamide gels at 60 W constant. After electrophoresis, the gel was transferred to 3MM Whatman paper, dried and subjected to autoradiography. Cell lines with additional bands without normal mobility bands in all three markers were classified as microsatellite unstable.

Mutation screening in the hMSH2 *and* hMLH1 *genes*

Reverse transcriptase (RT)-PCR and PCR-SSCP analyses were employed to investigate the genetic status of *hMSH2* and *hMLH1* genes. Oligonucleotide primers for RT-PCR and genomic PCR and procedures for PCR-SSCP have been previously described [36].

Mutation screening of TGF-βRII, IGFIIR, BAX, hMSH3 *and* hMSH6 *genes*

For mutational analysis of *TGF-βRII*, *IGFIIR*, *BAX*, *hMSH3* and *hMSH6* genes, the repeated deoxyadenine, deoxyguanine or deoxycytosine regions of each gene were amplified from 50–100 ng of genomic DNA using the sense primer of each gene labelled with [γ-^{32}P]dATP using T4 polynucleotide kinase and the unlabelled antisense primer of each gene. PCR conditions consisted of 35 cycles of 95°C for 30 sec, 45°C for 1 min, and 70°C for 1 min in *hMSH3*, *hMSH6* and *BAX*, and PCR conditions for *TGF-βRII* and *IGFIIR* were as previously described [22, 24]. PCR products were electrophoresed in 6 M urea/32% formamide/7% polyacrylamide gels at 60 W constant, and the gel was transferred to 3 MM Whatman paper, dried and visualised by autoradiography. Mutation screening of the full seven exons of the *TGF-βRII* gene was performed by PCR-SSCP (single strand conformation polymorphism) analysis. The primers for genomic PCR and PCR conditions were described by Lu and colleagues [29]. PCR products were electrophoresed in a non-denaturing SSCP gel, and separated for 12–16 h in a cool room (16°C) at a constant 300 volts. After electrophoresis the gel was transferred to 3 MM Whatman paper, dried and subjected to autoradiography.

Cloning and sequencing

Samples showing abnormal bands were subjected to DNA sequencing analysis. Fresh PCR products were ligated into PCR™II vectors and subcloned using the TA cloning system (Invitrogen, San Diego, California, U.S.A.). A minimum of 10 individual clones was then pooled and used for DNA isolation. Bi-directional DNA sequencing analysis was performed by using the dideoxy chain termination method with a T7 DNA polymerase sequencing kit (Pharmacia Biotech Inc., Piscataway, New Jersey, U.S.A.), or directly sequenced using a Taq dideoxy terminator cycle sequencing kit on an ABI 377 DNA sequencer (Perkin-Elmer, Foster City, California, U.S.A.).

RESULTS

MSI was detected in 13 of 22 (59%) cell lines using three markers. For the BAT-40, five cell lines without MSI in BAT-25 and BAT-26 had both the normal mobility bands and additional bands (Figure 1 and Table 1).

After screening of mutations in *hMLH1* and *hMSH2*, we found that four lines of 12 (33%) newly established colorectal cancer cell lines had mutations in *hMSH2* or *hMLH1*. In addition, we detected alterations of *hMSH2* in two previously reported cell lines (SNU-C2A and SNU-C4). It has been reported earlier that HCT-116 had a homozygous nonsense

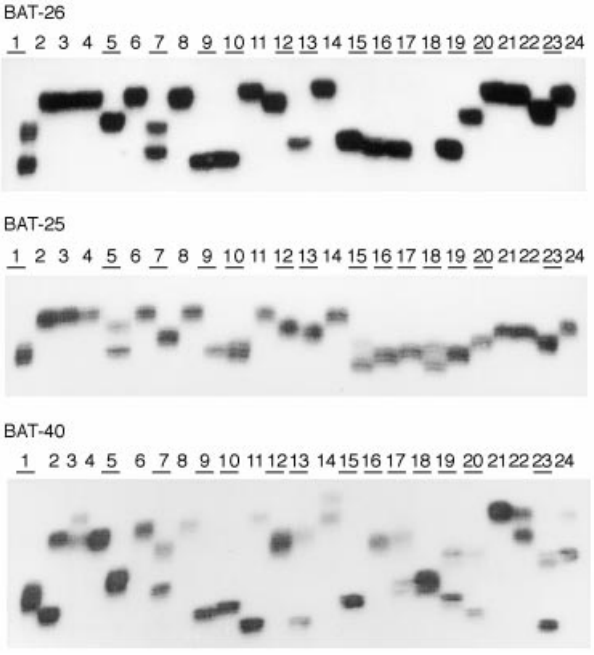

Figure 1. Analysis of microsatellite instability in colorectal cancer cell lines by BAT-26, BAT-25, and BAT-40. Each underlined number indicates MSI. Lane numbers (1–24): 1, SNU-1; 2, SNU-16; 3, SNU-61; 4, SNU-81; 5, SNU-175; 6, SNU-283; 7, SNU-407; 8, SNU-503; 9, SNU-769A; 10, SNU-769B; 11, SNU-1033; 12, SNU-1040; 13, SNU-1047; 14, SNU-1197; 15, SNU-C2A; 16, SNU-C4; 17, HCT-116; 18, LoVo; 19, LS174T; 20, HCT-8; 21, Colo205; 22, SW-480; 23, DLD-1; and 24, WiDr) indicate each cell line. Gastric cancer cell lines, SNU-1 and SNU-16, were used as positive and negative control, respectively, for microsatellite instability [22].

mutation in *hMLH1* and LoVo was deleted in *hMSH2* (reviewed in [30]) (Table 1).

Ten of 22 cell lines (45%) showed frameshift mutations at the poly $(A)_{10}$ tract in the *TGF-βRII* gene. Sequencing analysis from isolated single clones of each cell line confirmed these results (Figure 2, Table 1). A representative example is shown in Figure 2 where the SNU-16 cell line has a homozygous wild-type in the poly $(A)_{10}$ tract and the SNU-407 cell line has a -1 bp/+1 bp mutation in the poly $(A)_{10}$ tracts. In PCR-SSCP analysis, three of 12 lines (25%) (SNU-175, SNU-283 and SNU-1033) showed band shifts in exon 4. However, sequencing analysis revealed that all three alterations were the silent mutation from AAC to AAT at codon 389 (Asn to Asn) (data not shown).

Only two of 22 cell lines (9%) (SNU-1047 and SNU-C2A) had length changes in a poly $(G)_8$ tract of *IGFIIR* and they have a one base truncated band and a wild-type band (2 out of 13 with positive MSI status, 15%). Sequencing analysis from the isolated single clones confirmed that the SNU-1047 and SNU-C2A lines had a one base deletion and an intact eight base guanine in the poly deoxyguanine tract of this gene (Figure 3, Table 1).

In the poly $(G)_8$ tract in *BAX*, nine of 22 cell lines (41%) tested harboured length changes resulting in a one base deletion or addition in the poly deoxyguanine tracts in *BAX* and all of these showed MSI (Figure 3, Table 1).

In the poly $(A)_8$ tracts in *hMSH3*, six of 22 cell lines (27%) showed a one base truncated band resulting in a premature stop codon at the poly deoxyadenine tract and all six displayed MSI (46%) (Figure 3, Table 1). In the poly $(C)_8$ tract in *hMSH6*, six of 22 cell lines (27%) showed the shortening poly deoxycytosine tracts of the *hMSH6* gene (Figure 3, Table 1). One base truncated bands in poly $(C)_8$ tract of this gene were noted in five lines and one base addition band in one line (SNU-C2A). Table 1 summarises these mutational analyses.

DISCUSSION

Five cell lines having normal mobility bands and additional bands by BAT-40 did not show any MSI in BAT-25 and BAT-26. This result may represent polymorphisms in these lines because BAT-40 is polymorphic with a larger number of different alleles and behaved like most of the dinucleotide microsatellites. The frequency of MSI in non-selected colorectal carcinoma cell lines of our study is relatively high 13/22 (59%) even allowing for the fact that one cell line was derived from an HNPCC patient. Other reports of sporadic colon cancer have demonstrated a frequency of MSI of approximately 10–15% (reviewed in [13]).

The extent of involvement of *hMLH1* and *hMSH2* genes in sporadic colorectal tumours is not clear; however, a 65% incidence of mutations in mismatch repair (MMR) genes has been reported in sporadic cases with enhanced MSI (reviewed in [37]). In the present study, we found that eight of 22 cell lines (36%) had alterations in *hMLH1* or *hMSH2* genes. Of those with mutations in MMR genes, two lines (15%) had mutations in *hMLH1*, whereas six cell lines (46%) had alterations of *hMSH2*. Moreover, all lines with defects in these genes displayed MSI and the mutation frequency of MMR genes with enhanced MSI was 62% (8/13).

It has been known that colorectal cancers and gastric cancers displaying MSI contain somatic slippage-related frameshift mutations in *TGF-βRII*, *IGFIIR*, *BAX*, *hMSH3* and *hMSH6* genes containing mononucleotide repeat sequences in their coding regions [10–13, 14–29]. Of these, microsatellite mutations in the *TGF-βRII* gene are common in HNPCC, as well as sporadic gastro-intestinal cancers displaying MSI [10, 13–17, 21, 22, 26, 29] and uncommon in sporadic pancreatic, hepatic and breast cancers [38]. In our study, 10 of 13 lines (77%) with MSI displayed frameshift mutations at the mononucleotide repeat sequence of this gene, moreover, frameshift mutations were not detected in other cell lines without MSI.

Mutational frequency of the mutational hot spot of *BAX* was relatively higher 9/22 (41%) than other reports [13, 27]. Recently, it was reported that colorectal cancer of the MSI

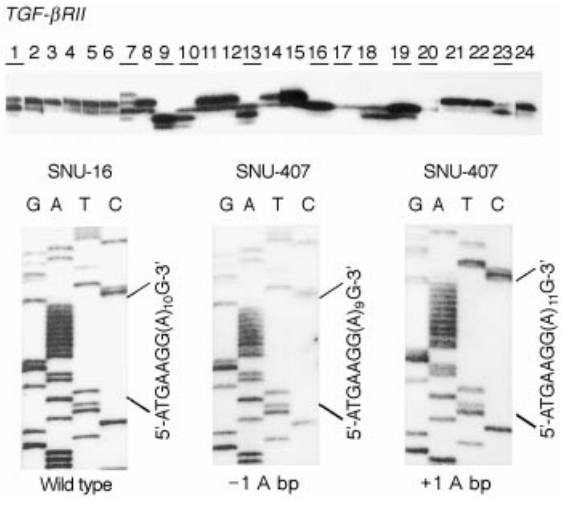

Figure 2. (a) PCR detection of mutations at the poly $(A)_{10}$ tract in *TGF-βRII* gene. Each underlined number indicates frameshift mutations. Lane numbers (1–24) match numbers from Figure 1. (b) Sequencing analysis of the *TGF-βRII* gene.

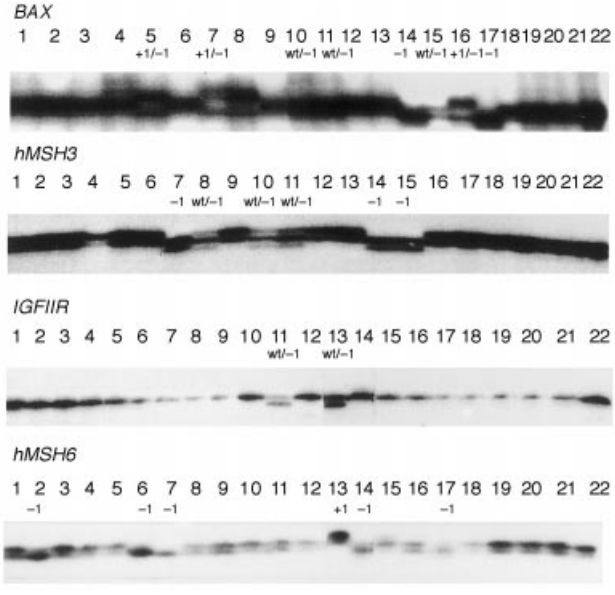

Figure 3. Mutations in the mononucleotide repeat tract of *BAX*, *hMSH3*, *IGFIIR* and *hMSH6* genes. Lane numbers (1–22) indicate the following cell lines: 1, SNU-61; 2, SNU-81; 3, SNU-175; 4, SNU-283; 5, SNU-407; 6, SNU-503; 7, SNU-769A; 8, SNU-769B; 9, SNU-1033; 10, SNU-1040; 11, SNU-1047; 12, SNU-1197; 13, SNU-C2A; 14, SNU-C4; 15, HCT-116; 16, LoVo; 17, LS174T; 18, HCT-8; 19, Colo205; 20, SW-480; 21, DLD-1; and 22, WiDr.

phenotype exhibits a negative correlation with *TP53* mutations and the proapoptotic gene *BAX* is frequently inactivated by frameshift mutations in a $(G)_8$ tract in gastrointestinal cancer of the MSI phenotype, and this provides a good example of the differences between mutator and suppression pathways for colon cancer [13]. In a previous study using the newly established 12 cell lines included in this study, we found that six lines (50%) (SNU-61, SNU-503, SNU-1033, SNU-1040, SNU-1047, and SNU-1197) harbour *TP53* mutations. Of these six lines, two lines (33%) (SNU-1040 and SNU-1047) displayed MSI and had mutations in *BAX*, and four lines (67%) did not. Yamamoto and colleagues [13] suggested that *BAX* mutations in MSI tumours may occur sooner than mutations in the *TP53* gene, which lack such repeats. Therefore, our results support this concept because mutations in *BAX* were exclusively found in MSI tumours and most of the mutations in *TP53* were found in tumours without displaying MSI 4/6 (67%).

In seven cell lines with *hMSH6* mutations, five lines displayed MSI 5/13 (38%), however, two lines (SNU-81 and SNU-503) did not show microsatellite instability by all mononucleotide repeat microsatellite markers (Figure 1, Table 1). Frequencies of frameshift mutations in *hMSH3* and *hMSH6* of MSI tumours are consistent with a previous report [13]. Although the DLD-1 cell line showing MSI did not show frameshift mutation in the poly $(C)_8$ tract in *hMSH6*, this line has been found to be mutated in both codon 222 and 1103 of the different allele resulting in a termination codon [9].

According to the model of the 'mutator that mutates another mutator', the first step is inactivation of a gene which generates a first level of genomic instability and this accelerates the mutational inactivation of *hMSH3* and/or *hMSH6* secondary mutators (reviewed in [13]). Biochemical studies based on yeast revealed that MSH2 is capable of forming a complex with MSH3 or MSH6, MSH3 and MSH6 may at least partially substitute for each other. The genetic studies support the view that the MSH2–MSH6 complex functions in the repair of single base–base mispairs and smaller insertion/deletion mispairs. In contrast, the MSH2–MSH3 complex does not function in the repair of single base–base mispairs, but rather functions in the repair of insertion/deletion mispairs and is predominantly responsible for the repair of the larger insertion/deletion mispairs (reviewed in [31]). In our study, eight cell lines with mutations in the *hMSH2* or *hMLH1* genes all displayed MSI phenotype (8/13, 62%) (Table 1). Five and four of these lines also have mutations in *hMSH3* and *hMSH6*, respectively. Two lines with mutations in *hMSH2* did not harbour any mutations in *hMSH3* or *hMSH6*. However, these two lines have mutations in *TGF-βRII* and *BAX*. In contrast, two of the cell lines which had mutations in only the *hMSH6* did not show MSI, and had no mutations in the *hMLH1*, *hMSH2*, *hMSH3*, *TGF-βRII*, *IGFIIR* and *BAX* genes. It has been shown that the *hMSH6* mutant cell lines had weak MSI at mononucleotide repeats and little if any dinucleotide repeat instability, in contrast to the strong broad spectrum repeat instability observed in *hMSH2*, *hMLH1* and *hPMS2* mutant cell lines (reviewed in [31]). Moreover, recently Edelmann and colleagues [31] showed that *MSH6* null mutations, like those in some other members of the family of MMR genes, lead to cancer susceptibility. They also showed that the lack of either mononucleotide repeat or dinucleotide repair instability found in the tumours from *MSH6* mutant mice and repair of insertion/deletion mispairs were observed in extracts from *MSH6*-/- cells. They concluded that germline mutations in this gene are associated with a cancer predisposition syndrome that does not show MSI.

Mutations of the *hMSH6* gene have been previously reported in colorectal cancer cell lines and colorectal cancer tissues as well as gastric cancer, leukaemic and endometrial cancer cell lines displaying widespread MSI, and in the germline of HNPCC patients [8–10, 12–14]. However, since most of the cell lines with mutations in *hMSH6* had mutations in other mismatch repair genes, the precise role of *hMSH6* mutations in the onset and progression of cancer had not been determined (reviewed in [31]).

Therefore, our findings of two cell lines with mutations in *hMSH6* only without displaying MSI indicate that mutations in *hMSH6* alone are not sufficient to cause MSI and do not fit the model of 'mutator that mutates another mutator', at least in certain sporadic colorectal cancers. Nevertheless, the possible existence of redundancy between *hMSH6* and other MMR components means that this hypothesis is not disproved: it may simply be the case that the *hMSH6* mutations detected are unrelated to MMR and/or that they have no effect in the absence of *hMSH3* or alternative mutations.

In summary, we show that all cell lines with mutations in the *hMLH1* and *hMSH2* displayed MSI. Cell lines with mutations in the *hMLH1* and *hMSH2* genes harboured mutations in at least one of genes (*TGF-βRII*, *IGFIIR*, *hMSH3*, *hMSH6* and *BAX*) with mononucleotide repeats in the coding regions. Moreover, mutations in *hMSH6* alone were not sufficient to cause MSI because we found mutations in mononucleotide repeat sequences of the *hMSH6* gene in two cell lines without displaying MSI and no mutations in MMR and other genes containing mononucleotide repeat sequences.

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