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Microsatellite instability is associated with genetic alteration but not with low levels of expression of the human mismatch repair proteins hMSH2 and hMLH1

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Abstract

Mutational inactivation of *hMSH2* or *hMLH1* has been known to be responsible for microsatellite instability and cellular resistance to DNA-damaging alkylating agents. However, the effects of altered expression of hMSH2 or hMLH1 on microsatellite stability and cellular response to alkylating agents has not been well investigated. Previously, we have reported that downregulation of the hMLH1 protein was a frequent event and was closely associated with cellular resistance to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) in human gastric carcinoma cell lines. Therefore, to investigate the relationship between microsatellite instability and quantitative changes in hMSH2 and hMLH1, we compared the genetic status and expression levels of hMSH2 and hMLH1 with microsatellite instability in 11 human gastric carcinoma cell lines. Five cell lines contained wild-type *hMSH2* and *hMLH1* and expressed adequate levels of hMSH2 and hMLH1 proteins. In three cell lines, genetic alterations such as mutation in the *hMLH1* gene (SNU-1) or the *hMSH2* gene (SNU-638), or hypermethylation in the promoter region of the *hMLH1* gene (SNU-520) were observed. Microsatellite instability assays revealed that only these three cell lines exhibited microsatellite instability. Three cell lines (SNU-216, -484, and -668) containing wild-type *hMSH2* and *hMLH1* genes produced significantly downregulated hMSH2 and/or hMLH1 proteins. In spite of the substantial decrease in the protein levels, these cell lines did not show microsatellite instability. Together with our previous report, this study suggests that: microsatellite instability of cells is associated only with genetic alteration of the mismatch repair genes; relatively low levels of the hMSH2 and hMLH1 proteins may be sufficient to retain the microsatellite stable phenotype; and the cellular response to alkylating agents is associated with genetic alteration and decreased expression of the mismatch repair genes in human gastric carcinoma cell lines.

Keywords: Human gastric carcinoma cell lines; *hMLH1*; *hMSH2*; Microsatellite instability; MNNG tolerance

1. Introduction

A significant proportion of human cancers are associated with defects in DNA mismatch repair (MMR) genes and germ line mutations in the MMR genes, namely *hMSH2*, *hMLH1*, *hPMS1*, *hPMS2* and *hMSH6* have been identified in patients with hereditary non-polyposis colorectal cancer [1–6]. The majority of germ line mutations have been found in *hMSH2* and *hMLH1*, whereas germ line mutations in *hPMS1*, *hPMS2* and *hMSH6* appear to be rare. Based upon studies from the distribution of mutations in the MMR genes as well as the bacteria and yeast mismatch repair system, it has been suggested that the hMSH2 and hMLH1 proteins may play a key role in the MMR pathway [7–9].

Inactivation of MMR genes causes destabilisation of microsatellite sequences in both non-coding and coding portions of the genome, resulting in the microsatellite instability (MSI) phenotype. Such sequences are found in the coding regions of the TGF-β type II receptor (*TGF-βRII*), *hMSH6*, *hMSH3*, *BAX* and insulin-like growth factor II receptor (*IGFIIR*) genes, indicating that they may be target genes of genomic instability in MSI tumorigenesis [10–14]. Therefore, tumours and cell lines with defective MMR genes exhibit MSI and contain altered lengths of repeat sequences in the genes [15–19].

Defective MMR is also implicated in acquired cellular resistance to DNA-damaging alkylating agents, such as

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N-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), *N*-methyl-*N*-nitrosourea (MNU), procarbazine and temozolomide [20–25]. The implication of MMR defects in the cellular resistance to alkylating agents is based on several observations. Cells with a defective MMR system were resistant to the cytotoxic effect of alkylation agents [26]. Mammalian cell lines containing genetic defects in MMR genes such as *hMLH1* or *hMSH2* have a high level of resistance to the cytotoxic effects of DNA alkylating agents [20,22]. Furthermore, we previously demonstrated that the introduction of wild-type *hMLH1* cDNA into human cancer cell lines with inactivated *hMLH1* increased cellular sensitivity to MNNG [21].

Many studies have shown that human cancer cells containing mutant *hMSH2* or *hMLH1* show MSI and increased cellular resistance to DNA-damaging alkylating agents. Although we demonstrated that down-regulation of the hMLH1 protein was closely associated with cellular resistance to MNNG in human gastric carcinoma cell lines [21], the effects of altered expression of hMSH2 or hMLH1 on microsatellite stability and the cellular response to alkylating agents have not been well investigated. Therefore, to investigate the relationship between quantitative changes in mismatch repair proteins (hMSH2, hMLH1) and microsatellite instability, we compared the genetic status and expression levels of hMSH2 and hMLH1 with microsatellite instability in 11 human gastric carcinoma cell lines.

2. Materials and methods

2.1. Cell lines

Eleven human cell lines established from gastric carcinomas of individual patients were used in this study. The characteristics of the cell lines have been previously described [27,28] and used in various studies [21,29,30]. The cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. Cultures were maintained in a humidified incubator at 37°C in an atmosphere containing 5% CO_2.

2.2. Nucleic acid isolation

High molecular weight cellular DNA was extracted from the cells with phenol/chloroform/isoamyl alcohol (25:24:1) and ethanol precipitation [31]. Total RNA was extracted from cells with TRI REAGENT (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions.

2.3. DNA sequencing analysis

Randomly primed cDNAs were reverse-transcribed from 5 μg of total RNA by Superscriptase (GIBCO/BRL, Grand Island, NY, USA) in 40 μl of mixture. Two microlitres of the cDNA mixture was used to amplify the *hMLH1* and the *hMSH2* transcript. PCR products were cloned into pCRII using a TA™ Cloning Kit (Invitrogen, San Diego, CA, USA) under the conditions recommended by the manufacturer. The nucleotide sequence of the cloned DNA was determined by the primer extension method as previously described [32] or with a Taq dideoxy terminator cyclic sequencing kit on an ABI 377 automatic DNA sequencer (Perkin-Elmer, Foster City, CA, USA).

2.4. Northern blot hybridisation analysis

20 or 40 μg of total RNA were denatured and loaded onto a 1.2% formaldehyde agarose gel and separated by electrophoresis. The RNAs were transferred to a nylon filter (Amersham Corp., Arlington Heights, IL, USA) and crosslinked with ultraviolet light. The filter was hybridised to [^{32}P]-labelled *hMLH1* cDNA, *hMSH2* cDNA (from B. Vogelstein, Howard Hughes Medical Institute, Baltimore, MD, USA) or β-actin cDNA at 42°C for 24 h in 50% formamide/10% dextran sulphate/5×SSPE (0.15 M NaCl, 0.01 M Na_2HPO_4, and 0.001 M EDTA)/5× Denhardt's solution/denatured salmon sperm DNA (100 μg/ml). After hybridisation, the filter was washed twice in 5× SSPE for 15 min at 42°C, in 1× SSPE/0.1% SDS for 30 min at 42°C and in 0.1× SSPE/0.1% SDS for 30 min at room temperature. The filter was then autoradiographed on SB-5 X-ray film (Eastman Kodak, Rochester, NY, USA) for 12 h at −70°C.

2.5. Western blot analysis

Nuclear proteins were prepared as described in [33]. Briefly, exponentially growing cells in 100-mm dishes were washed three times with tris-buffered saline solution and 2.5 ml of lysis buffer (20 mM HEPES, pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM MgCl, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulphonyl fluoride (PMSF) was added. Cells were dislodged by scraping and pelleted by centrifugation. Nuclei were resuspended in nuclear extraction buffer (lysis buffer containing 500 mM NaCl). Nuclei were gently rocked for 1 h at 4°C and centrifuged at 12 000*g* for 10 min, the supernatants were saved and the amount of protein was quantitated. Fifty micrograms of nuclear proteins were subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (Novex, San Diego, CA, USA), and probed with mouse monoclonal ab-1 antibody specific for human MLH1, human MSH2 (Calbiochem, Cambridge, MA, USA) or α-tubulin. Quantification of Northern and Western analysis was done by phosphor screen autoradiography with the

Storm 860 phosphoimager and ImageQuant software (Molecular Dynamics, CA, USA) quantification.

2.6. PCR-methylation assay

To address the methylation status of the *hMLH1* promoter, 5 μg of genomic DNA from the cell lines were digested with 20 units of methylation sensitive enzyme (*Hpa*II) or methylation-insensitive enzyme (*Msp*I) for 16 h. After digestion, 33 ng of DNA from each digest were analysed by PCR using the Taq PCR Core Kit (Qiagen, Inc., Chatsworth, CA, USA). Multiplex PCR was performed with primer pairs for the *hMLH1* promoter region [34] and exon 15 of the *hMLH1* gene devoid of *Hpa*II and *Msp*I sites as a control for amplification.

2.7. Analysis of microsatellite instability

The MSI status was determined by screening a polyadenine sequence, BAT-26, which showed 99.4% efficiency to detect MSI-associated cancer [35]. The repeat sequence was amplified using one ^{32}P labelled primer and one unlabelled primer. The amplified products were separated on urea–formamide polyacrylamide gels and exposed to film. Simple repeat sequences within the open reading frames of *TGF-βRII*, *hMSH6* and the *MSH3* genes were also examined. The primer sequences and the detailed conditions for amplification have been described elsewhere [6,10,14].

3. Results

3.1. Mutational status of the hMLH1 and hMSH2 genes in human gastric cancer cell lines

The genetic integrity of the *hMLH1* and *hMSH2* genes in 11 human gastric cancer cell lines was examined. With the exception of the SNU-1 cell line, which contains a nonsense mutation at codon 226 in the *hMLH1* gene [21], the cDNAs containing *hMLH1* and *hMSH2* from the other 10 cell lines were amplified and sequenced. Sequencing analysis demonstrated that the SNU-638 cell line contained two missense mutations of GCA (Ala) to CCA (Pro) at codon 700 and GAA (Glu) to CAA (Gln) at codon 701 in exon 13 of the *hMSH2* gene (Fig. 1). No mutation was identified in the other cell lines.

3.2. Expression of hMLH1 and hMSH2 in human gastric cancer cell lines

Expression of hMLH1 and hMSH2 in the 11 cell lines were examined by Northern and Western blot analysis (Fig. 2). Northern blot analysis showed a loss of *hMLH1* mRNA in SNU-1 and -520 and low level in SNU-638 (Fig. 2a). These observations were consistent with results from Western blot analysis (Fig. 2a). However, SNU-216, -484 and -668 lysates contained negligible amounts of the hMLH1 protein yet expressed an adequate amount of the *hMLH1* mRNA (Figs. 2a and 3). With a longer exposure of the Western blot, SNU-216 and -484 showed a faint hMLH1-specific band. This result therefore suggests that the low levels

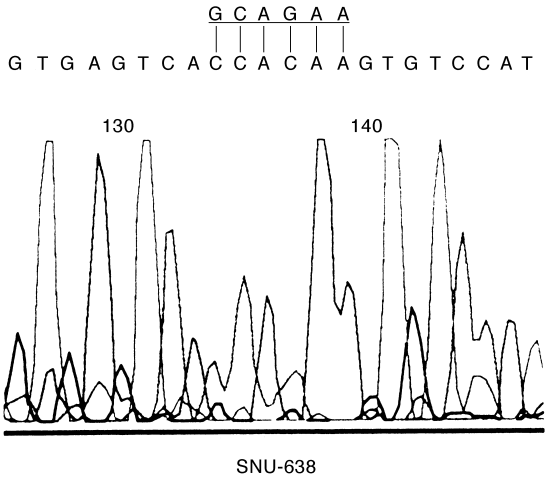

Fig. 1. Nucleotide sequencing analysis of *hMSH2* cDNA of the SNU-638 cell line.

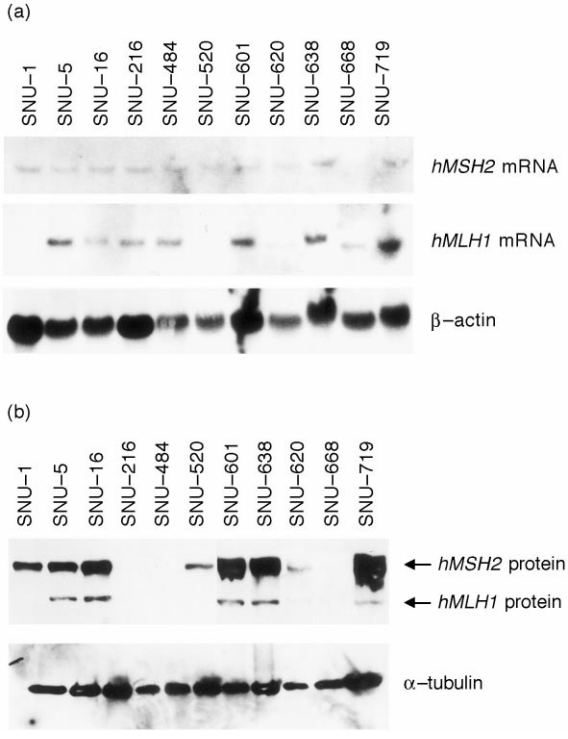

Fig. 2. Expression of hMLH1 and hMSH2 in human gastric cancer cell lines. (a) Northern blot analysis showing the level of transcripts of *hMLH1*, *hMSH2* and β-actin. (b) Western blot analysis showing the level of proteins of hMLH1, hMSH2 and α-tubulin. β-actin and α-tubulin were used as controls.

of hMLH1 protein observed in SNU-216, -484 and -668 are not because of downregulation of *hMLH1* mRNA, indicating that post-transcriptional mechanisms are involved in the regulation of hMLH1 expression. Western blot analysis with anti-hMSH2 antibody significantly reduced the hMSH2 protein in SNU-216, -484, -638, and -668 (Fig. 2b). With longer exposure of Western blot SNU-216, -484, and -668 showed a faint hMSH2-specific band (data not shown). The level of *hMSH2* mRNA in the SNU-668 cell line was consistent with the level of hMSH2 protein observed from Western analysis (Figs. 2b and 3). However, similar to hMLH1 expression, post-transcriptional mechanisms involved in regulation of hMSH2 expression were noted in SNU-216 and -484 (Figs. 2b and 3). The results of expression analysis are summarised in Fig. 3.

3.3. *Methylation of the* hMLH1 *promoter in a cell line that lacks hMLH1 expression*

To examine methylation of the promoter region of *hMLH1*, we employed a PCR-based restriction enzyme assay [34]. Genomic DNAs from the cell lines were digested with *Hpa*II (methylation-sensitive enzyme) or *Msp*I (methylation-insensitive enzyme). Following endonuclease digestion, previously reported primers flanking the *Hpa*II sites were used for PCR amplification [34]. A PCR product is detected only when the promoter region of the *hMLH1* gene is methylated. The assay revealed that the *hMLH1* promoter region of only SNU-520 was resistant to digestion by *Hpa*II and was sensitive to digestion by the *Msp*I enzyme (Fig. 4), indicating that the *hMLH1* promoter region of the SNU-520 cell line is methylated. These data are consistent with expression analyses showing that the SNU-520 cell line does not express *hMLH1* mRNA and protein (Figs. 2 and 3).

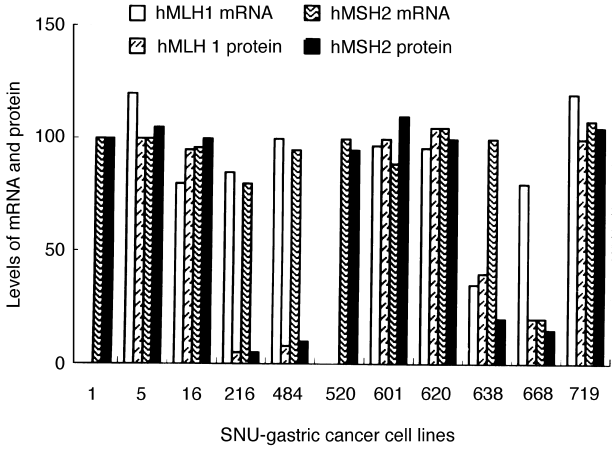

Fig. 3. Relative levels of hMLH1 and hMSH2 shown in bar graph form. The levels of mRNA and protein were depicted after normalisation to the levels of β-actin mRNA and α-tubulin protein, respectively.

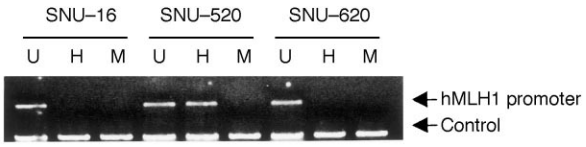

Fig. 4. Analysis for methylation of the *hMLH1* promoter region in the human gastric cancer cell lines. The *hMLH1* promoter regions of the indicated cell line DNAs were amplified before (U, undigested) or after digestion with *Hpa*II (H) or *Msp*I (M). Among the 11 cell lines, the *hMLH1* promoter region was amplified from *Hpa*II-digested DNA of SNU-520, indicating hypermethylation of the promoter region. Exon 15 of *hMLH1*, which is devoid of *Hpa*II and *Msp*I sites was amplified as a control.

3.4. *Microsatellite instability in human gastric cancer cell lines*

To determine the MSI status of the 11 cell lines, the polyadenine sequence BAT-26 was analysed. The cell lines were divided into two groups. Cell lines that showed alteration in the BAT-26 sequence were considered to have MSI, and those that did not were classified as microsatellite stable (MSS). As shown in Fig. 5, SNU-1, -520, and -638 showed abnormal band patterns in the tested repeat sequence. However, no alteration in the repeat sequence was seen in the other cell lines. Therefore, we concluded that SNU-1, -520 and -638 exhibited MSI whereas the other cell lines exhibited MSS. Furthermore, *TGF-βRII*, *hMSH3* and *hMSH6*, which contain simple repeat sequences within the open reading frame, were also examined (data not shown). The results are summarised in Table 1. Frameshift mutations in the repeat sequence within the coding

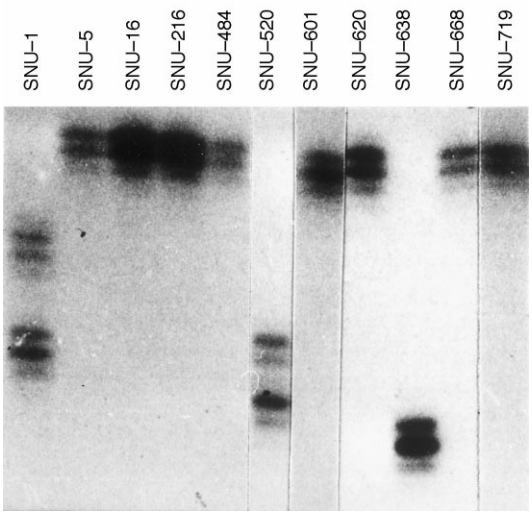

Fig. 5. Analysis of microsatellite instability (MSI) status from human gastric cancer cell lines. The polyadenine repeat sequence BAT-26 of the cell lines was amplified and separated in a denaturing polyacrylamide gel. Alteration in the BAT-26 repeat sequence was found in SNU-1, -520 and -638.

region of the *TGF-βRII* gene were identified in the all MSI cell lines (SNU-1, -520 and -638). Frameshift mutations in a repeat sequence of the *hMSH3* gene were detected in the MSI SNU-1 and -520. The MSI SNU-520 and -638 contained one base pair deletions in the repeat sequence of the *hMSH6* gene. Such alterations were not found in any of the MSS cell lines (Table 1).

In addition, the relationship between drug resistance and MMR status was investigated using the MTT assay. The effects of doxorubicin, cisplatin and etoposide in these gastric carcinoma cell lines was studied and no definite conclusions could be drawn from the results (data not shown). However, the cell lines (SNU-216, SNU-484, -520 and -668) with either no or low MMR protein expression were more resistant to the three anticancer drugs compared with the other cell lines.

4. Discussion

Although there have been several studies showing the involvement of mutational inactivation of the MMR genes in MSI and alkylation tolerance in human tumours and cancer cell lines, the effects of low levels of MMR proteins on such aspects have not been well investigated in human cancer cells. Recently, we reported that mutational inactivation as well as a low level of hMLH1 protein caused cellular resistance to alkylating agents [21]. Therefore, the aim of this study was to understand the relationship between quantitative changes in the MMR proteins (hMSH2, hMLH1) and microsatellite stability. The present study shows that MSI is associated only with genetic alteration of the MMR genes, such as mutation in the MMR genes and hypermethylation of the promoter region of the *hMLH1* gene. However, in spite of a substantial decrease in the proteins, the majority of cell lines containing low levels of wild type hMSH2 or hMLH1 proteins did not show MSI. As summarised in Table 2, this study yields several important observations.

1. Relatively low levels of the hMSH2 and hMLH1 proteins may be sufficient to retain the microsatellite stable phenotype.
2. Together with our previous observation [21], the cellular response to alkylating agents is associated with qualitative and quantitative changes of the MMR genes.
3. MSI is associated with the loss of wild-type MMR proteins but not with reduction of MMR proteins.
4. The roles of MMR genes in cellular responses to alkylating agents and in maintaining microsatellite stability may be mediated through different mechanisms.

There have been many studies showing that human cancer tissues and cell lines with defective MMR genes exhibit increased MSI. Major mechanisms of MMR gene inactivation are genetic alterations such as mutation of the coding region and hypermethylation of the promoter region, resulting in loss of activity [34,36]. Such genetic alterations in MMR genes are associated with MSI. In the present study, we found three cell lines containing genetically altered *hMLH1* or *hMSH2* and three cell lines expressing low levels of wild-type hMLH1 and hMSH2 proteins amongst 11 tested human gastric cancer cell lines. The MSI analysis showed that the three cell lines with genetically altered MMR genes

Table 1
Summary of the MSI status in the human gastric carcinoma cell lines

SNU lines	MSI status[a]	*TGF-βRII*[b]	*hMSH3*[c]	*hMSH6*[d]
1	MSI	−1/wt[e]	−1/wt	wt/wt
5	MSS	wt/wt	wt/wt	wt/wt
16	MSS	wt/wt	wt/wt	wt/wt
216	MSS	wt/wt	wt/wt	wt/wt
484	MSS	wt/wt	wt/wt	wt/wt
520	MSI	−1/−1	−1/−1	−1/wt
601	MSS	wt/wt	wt/wt	wt/wt
620	MSS	wt/wt	wt/wt	wt/wt
638	MSI	−1/−2	wt/wt	−1/wt
668	MSS	wt/wt	wt/wt	wt/wt
719	MSS	wt/wt	wt/wt	wt/wt

[a] The MSI status was examined by screening the BAT-26 repeat sequence. MSI, microsatellite unstable phenotype; MSS, microsatellite stable phenotype.
[b] A 10 bp poly(A) tract at nucleotides 709–718 was examined.
[c] An 8 bp poly(A) tract comprising codons 381–383 was examined.
[d] An 8 bp poly(C) tract comprising codons 1085–1087 was examined.
[e] wt, if the sample has a wild-type allele, or the number (e.g. −1/−2) indicates the number of bases lost in each allele.

Table 2
Differential effects of MMR status on *N*-methyl-*N*′nitro-*N*-nitrosoguanidine (MNNG) responsiveness and microsatellite instability in SNU-gastric cancer cell line[a]

SNU line	MNNG response [21]	MSI status	Alteration in MMR gene [Ref.]	hMLH1 protein/ mRNA	hMSH2 protein/ mRNA
1	R	MSI	*hMLH1* [21]	No/No	AE/AE
5	S	MSS		AE/AE	AE/AE
16	S	MSS		AE/AE	AE/AE
216	R	MSS		Low/AE	Low/AE
484	R	MSS		Low/AE	Low/AE
520	R	MSI	*hMLH1*[b]	No/No	AE/AE
601	R	MSS		AE/AE	AE/AE
620	S	MSS		AE/AE	AE/AE
638	R	MSI	*hMSH2*	Low/Low	Low/AE
668	R	MSS		Low/AE	Low/Low
719	S	MSS		AE/AE	AE/AE

[a] MSI, microsatellite unstable phenotype; MSS, microsatellite stable phenotype; AE; adequate expression; R; resistant; S; sensitive.
[b] Hypermethylation of the *hMLH1* promoter region.

exhibited MSI, whereas the three cell lines with low wild-type MMR proteins showed a microsatellite stable phenotype. These results were consistent with the previous report that the downregulation of hMLH1 protein by antisense hMLH1 transfection into HCT116 + chromosome 3 cells did not affect mismatch repair activity [37]. Together, our findings indicate that low levels of expression of the human mismatch repair proteins hMSH2 and hMLH1 are sufficient to retain microsatellite stability and, therefore, broaden our knowledge of the effects of MMR in MSI.

In addition to cellular alkylation tolerance, defective MMR function has been shown to be associated with cellular resistance to a variety of clinically important drugs [38]. Therefore, we also examined the relationship between the resistance to drugs doxorubicin, cisplatin and etoposide, and the MMR status in the human gastric carcinoma cell lines (data not shown). Results from this MTT assay did not support the conclusion that both mutation and low levels of expression of MMR proteins were closely associated with the drug resistance.

It is worth noting that the levels of MMR proteins were not correlated with those of the MMR mRNAs in several of the human gastric cancer cell lines (Fig. 3). In the SNU-216 and -484 cell lines, the diminished hMLH1 and hMSH2 protein expression cannot be attributed to downregulated mRNA, because the mRNA levels were not decreased. This finding is consistent with another study [39] and raises the possibility that an abnormality in translational regulation or stability might be involved in the inactivating mechanisms of hMSH2 and hMLH1. Therefore, further research to understand how hMLH1 and hMSH2 are regulated by post-translational mechanisms is warranted.

In summary, we report that microsatellite instability of cells is associated only with genetic alteration of the mismatch repair genes and that relatively low protein levels of hMSH2 and hMLH1 may be sufficient to retain the microsatellite stable phenotype. According to our knowledge, this is the first report to show different effects of the qualitative and quantitative status of MMR genes on microsatellite stability in human cancer cells. Our data also suggest the possibility that an abnormality in translational regulation or stability might be involved in the inactivating mechanisms of hMLH1 and hMSH2.

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