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LOSS OF EXPRESSION OF THE HUMAN MSH3 GENE IN HEMATOLOGICAL MALIGNANCIES

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SUMMARY : Human MSH3 (hMSH3), previously named human mismatch repair protein 1 (MRP1), is one of the human homologs of the bacterial DNA mismatch repair protein MutS. The hMSH3 gene is expressed at low level in most types of cells. Using the RT-PCR technique, we examined the expression of the hMSH3 gene in bone marrow cells from 40 patients with various hematological malignancies. The hMSH3 mRNA was not detectable in 7 cases including 3 of chronic myelogenous leukemia, 2 of acute myelogenous leukemia, and 1 of acute lymphocytic leukemia, and 1 of myelodysplastic syndrome. In addition, 17 cases showed significantly reduced expression of the hMSH3 gene. Southern blot analysis of genomic DNA demonstrated no remarkable changes in the structure and the copy number of the hMSH3 gene in all cases. These results suggest that inactivation of the hMSH3 gene may be involved in the development of hematological malignancies.

Genomic instability due to defects in DNA mismatch repair is now considered to be an important mechanism for carcinogenesis. Alterations in simple repeated sequences or microsatellites (RER+ phenotype) have been detected in some hereditary nonpolyposis colorectal cancers (HNPCC) (1, 2). In vitro repair assay has shown that RER+ cancer cells are defective in repair of base-base mutation (3, 4). The hMSH2 gene encoding a human homolog of the bacterial mismatch repair protein MutS has been cloned as a candidate for the HNPCC gene. Mutations of the hMSH2 gene have been identified in some HNPCC patients (5, 6). Therefore, it is postulated that hMSH2 plays an essential role in DNA mismatch repair in human cells. Inactivation of the hMSH2 gene increases the spontaneous mutation rate and therefore, results in

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both genetic instability of microsatellites and accumulation of the oncogenic mutations of critical transforming genes such as K-ras, p53, DCC and APC.

We have previously identified a gene encoding another MutS homolog (7) in the upstream of the human dihydrofolate reductase (DHFR) gene which is located on chromosome 5q11-13 (8). The amino acid sequence of this gene has 31% identity with that of MutS. In the most conserved region of 156 amino acids in the carboxy-terminal portion, there is more than 50% homology. A similar gene has been identified in the upstream of the mouse DHFR gene (9). These two genes were the first two mammalian genes with apparent sequence homologies to the gene for bacterial mismatch repair protein MutS and named the human mismatch repair protein 1 (MRP1) (7, 10-12) and the mouse repair gene 3 (Rep-3) (9, 13). However, the exact role of their protein products in the mismatch repair system remains unclear.

Recently, a number of MutS related proteins have been isolated in *S. cerevisiae* and are classified into at least four groups (14-16). Human MutS homolog 2 (hMSH2) was named on the basis of sequence similarity to *S. cerevisiae* MSH protein, MSH2 (5). Human MRP1 and mouse Rep-3 are most closely related to yeast MSH3 (15). Therefore, human MRP1 was renamed hMSH3. In this study, we found that expression of the hMSH3 gene was significantly reduced in a variety of hematological malignancies.

MATERIALS AND METHODS

Patients : Bone marrow samples were obtained from patients of hematological malignancies and hematologically normal volunteers after receiving informed consent. All leukemic cells examined showed no 5q chromosome aberrations. None of the patients had received therapy prior to study. The diagnosis and classification (based on the French-American-British (FAB) criteria (17, 18)) and the percentage of blast cells were presented in Table 1. All chronic myelogenous leukemia (CML) patients were Philadelphia (Ph1)-positive.

Cell preparation and extraction of DNA and RNA : Mononuclear cells were obtained by Ficoll-Hypaque centrifugation (Lymphoprep, Neegard, Norway). DNA of high molecular weight was extracted from the mononuclear cells by digesting nuclei with protease K followed by phenol/ chloroform extraction and ethanol precipitation as described previously (19). Extraction of total RNA was performed using a RNAzol kit (Biotex Laboratories, Inc., Houston, TX), which is based on the technique described by Chomczynski and Sacci (20).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of hMSH3 gene expression : RT-PCR analysis of hMSH3 gene expression was performed according to the previously described protocol (21) with a slight modification. First, complementary DNA was synthesized with 500 ng of total RNA using 200 ng of MSH-A primer (5'-AGGCAATGGCAATTCCATCA-3', antisense strand sequence in exon 21 corresponding to cDNA residues of 2971-2952), and 2.5 units of Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer/Cetus Corp, GB) in 10 μL of solution containing 1 mmol/L each of four dNTPs, 1 unit of RNase inhibitor, 10 mmol/ L Tris-HCl (pH 8.3), 50 mmol/L KCl, and 5 mmol/L $MgCl_2$. The reaction was allowed to proceed for 15 min at 42°C and the reaction solution was heated at 95°C for 15 s. To the cDNA reaction solution (10 μL), 40 μL of solution containing 200 ng of MSH-S primer (5'-GAGAGTAATGATAATTACCG-3', sense strand sequence in exon 20 corresponding to cDNA residues of 2667-2686), 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.3 units of Taq DNA polymerase (Perkin-Elmer/Cetus Corp, GB) was added. Twenty-five cycles of PCR were

performed with 30 s at 94°C for denaturation, 30 s at 55°C for annealing, and 1 min at 72°C for extension using a DNA Thermal Cycler (Perkin-Elmer/Cetus Corp, GB). To show the intactness of RNA, we examined the expression of ß$_2$-microglobulin (ß$_2$m) mRNA as a control using oligonucleotide primers (ß$_2$m-1 primer : 5'-ACCCCCACTGAAAAAGATGA-3', ß$_2$m-2 primer : 5'-ATCTTCAAACCTCCATGATG-3') (19) by the same RT-PCR assay.

The amplified products of the RT-PCR analysis were fractionated by electrophoresis on 2.5% agarose gels and visualized by staining with ethidium bromide. After staining, they were transferred onto a nylon membrane filter (Gene Screen Plus, New England Nuclear, Boston, MA) and hybridized with ^{32}P-labeled 3.5 kb cDNA of the hMSH3 gene (7).

RT-PCR analysis of DHFR gene expression : Expression of DHFR was analysed by the same RT-PCR assay described above. DHFR-A primer (5'-GTTTAAGATGGCCTGGGTGA-3', antisense strand sequence in exon 5) and DHFR-S primer (5'-ATGGTTGGTTCGCTAAACTG-3', sense strand sequence in exon 1) were used. Hybridization was performed using ^{32}P-labeled 1.8 kb genomic DNA containing exons 1 and 2 of the DHFR gene (22).

RESULTS

Using the RT-PCR technique, we examined the expression of the hMSH3 gene in bone marrow cells from 7 normal volunteers and 40 patients with hematological malignancies including 18 of acute myelogenous leukemia (AML), 10 of chronic myelogenous leukemia (CML), 2 of acute lymphocytic leukemia (ALL), 1 of multiple myeloma (MM) and 9 of myelodysplastic syndrome (MDS). Quality and quantity of RNA samples were monitored by RT-PCR analysis of the ß$_2$-microglobulin mRNA (Fig 1-A and C). Comparable amounts of RT-PCR products of the hMSH3 mRNA were detected in all seven normal volunteers. However, the expression levels of hMSH3 were highly variable in hematological malignancies (Fig 1-C and Table 1). No hMSH3 signals were detected in 3 CML, 2 AML, 1 ALL and 1 MDS cases even in a longer exposed autoradiogram of the blotted filter. In addition, 17 cases showed a significant reduction of the hMSH3 mRNA. Therefore, 24 of 40 cases (60 %) showed inhibition of the hMSH3 expression. Although the abnormal expression of the hMSH3 gene was detected in all types of hematological malignancies examined, its frequency seemed to vary dependent upon diseases. Reduced or absence of the hMSH3 gene was observed in 9 of 10 CML cases (90%) and 7 of 18 AML cases (38%).

We also examined the expression of the DHFR gene which is located immediately upstream from the hMSH3 gene and transcribed from a common bidirectional promoter (10-12). Normal levels of the DHFR mRNA were detected in all cases (Fig 2).

To evaluate the sensitivity of our RT-PCR assay, we analyzed serial dilutions of hMSH3 positive RNA from normal bone marrow cells with hMSH3 negative RNA from CML bone marrow cells (Fig 3). The relationship between the concentration of the hMSH3 mRNA and that of the PCR product was roughly linear under the conditions used. The hMSH3 signals were detectable in the 1,000-fold diluted sample.

A patient with multiple myeloma (MM) had colon cancer (adenocarcinoma) at the same time. The RNA samples extracted from bone marrow cells, peripheral mononuclear cells, and

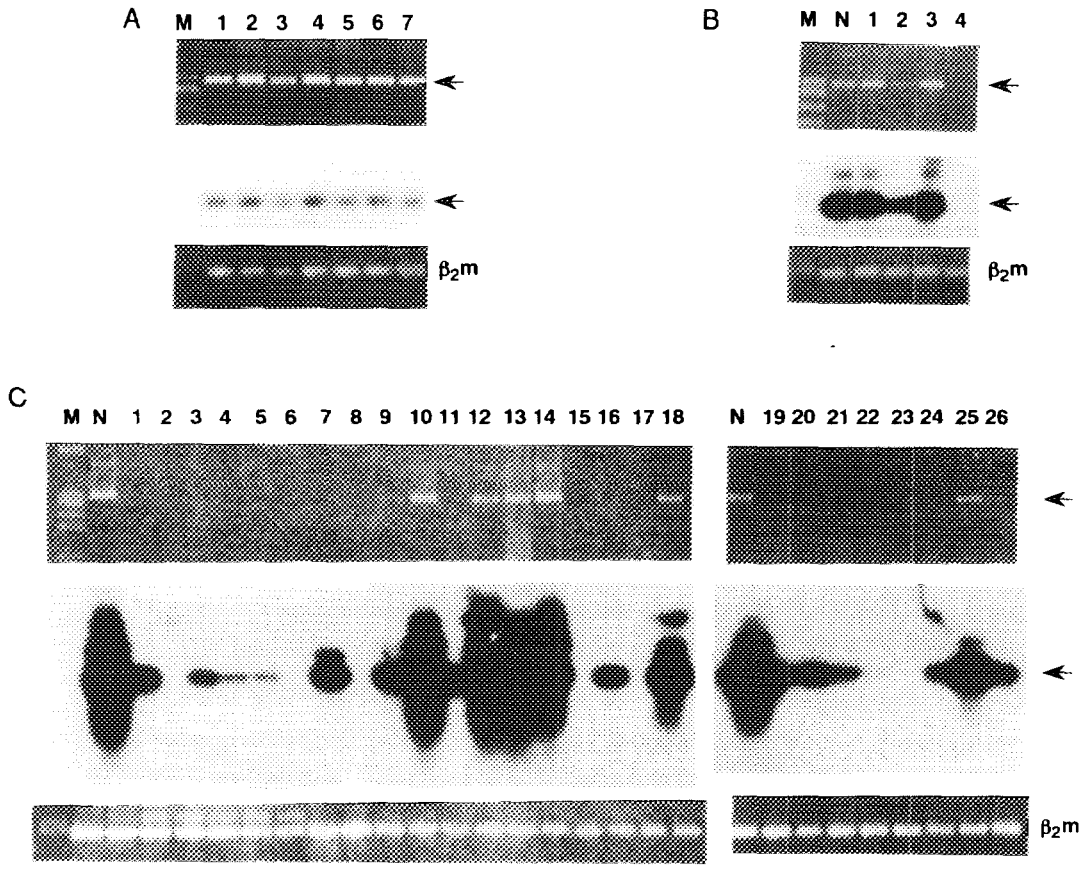

Fig. 1. Expression of the hMSH3 gene. RT-PCR products of hMSH3 (305 bp) were detected by ethidium bromide staining (upper panel) and the Southern blotting (middle panel) after electrophoresis. The blotted membranes were exposed on X-ray films overnight (A and B) or for 4 days (C). The RT-PCR products of β_2m (115 bp) as an internal control were also detected by ethidium bromide staining (lower panel). (A) RNA from bone marrow cells of 7 normal volunteers. Lane M represents HaeIII digested ϕx174 DNA as a size marker. (B) RNA from a patient with multiple myeloma and colon cancer at the same time. Lane N, bone marrow cells from a normal volunteer (shown in lane 1 of panel A); Lane 1, peripheral blood mononuclear cells from the patient; Lane 2, bone marrow cells from the patient; Lane 3, colon cancer cells from the patient; Lane 4, bone marrow cells from a CML patient (shown in lane 6 of panel C). (C) RNA from bone marrow cells of 24 patients with various hematological malignancies including CML (lanes 1-9, 18, 22), MDS (lanes 12, 13, 17, 19, 20, 24), AML (lanes 10, 11, 14-16, 21, 25, 26), and ALL (lane 23). Lanes 5 and 22 (CML) and lanes 16 and 26 (AML) are the same patients.

colon cancer cells were analyzed for the expression of the hMSH3 gene (Fig 1-B). Reduced hMSH3 expression was observed in the bone marrow sample, whereas the peripheral blood and colon cancer samples showed normal expression of the hMSH3 gene. The hMSH3 mRNA was also examined in surgical specimens of the cancer and the adjacent noncancerous tissues from

Table 1 Decreased hMSH3 Expression in Hematological Malignancies

Diagnosis	FAB-Classification[1]	Myeloblasts in MNC Preparation (%)[2]	No. of Patients Examined	No.of Patients with Reduced or Absent Expression	
Normal			7	0 (0%)	
Hematological Malignancies			40[3]	24 (60%)	
Myelodysplastic syndrome (MDS)			9	6 (67%)	
	RA[4]	5-20	5		2
	RAEB	28	1		1
	RAEB-t	32-45	3		3
Acute myelogenous leukemia (AML)			18	7 (38%)	
	M1	95-97	3		2
	M2	80-96	7		2
	M3	98, 99	2		2
	M4	91-98	6		1
Acute lymphocytic leukemia (ALL)	L2	96, 97	2	1 (50%)	
Chronic myelogenous leukemia (CML)[5]		5-22	10	9 (90%)	
Multiple myeloma (MM)		94[6]	1	1 (100%)	

[1]MDS, AML and ALL patients were diagnosed in accordance with FAB criteria.
[2]Myeloblasts were counted by a light microscope after Wright-Giemsa staining of mononuclear cell (MNC).
[3]Data of RT-PCR analysis of 25 cases were shown in Fig. 1. Results of additional cases were included in this table.
[4]In MDS, there were 5 cases of refractory anemia (RA), 1 case of RA with excess of blasts (RAEB) and 3 cases of RAEB in transformation (RAEB-t).
[5]All CML patients were Philadelphia (Ph1)-positive.
[6]Bone marrow examination showed a hypercellular marrow (82% myeloma cells), and there was 94% myeloma cells in MNC preparation.

five esophageal cancer patients and five renal cell carcinoma patients. RT-PCR products of the hMSH3 mRNA were detected in all these samples (data not shown). Therefore, reduced or absent expression of the hMSH3 gene was observed only in bone marrow cells from hematological malignancies.

Genomic DNAs from the patients lacking hMSH3 expression were subjected to Southern blotting analysis using the hMSH3 cDNA probe (Fig 4). No remarkable changes in the structure and the copy number of the genomic hMSH3 gene in all cases.

DISCUSSION

In this study, we found that the expression of the hMSH3 gene was inhibited in bone marrow cells from the patients with various hematological malignancies. Of 40 cases examined, complete loss of expression was observed in 7 cases, and reduced expression was in 17 cases. Because the hMSH3 gene is constitutively expressed in all tissues (Watanabe, A., Ikejima, M., Suzuki, N., and Shimada, T., submitted) including normal bone marrow (Fig. 1A), inactivation of the hMSH3 gene may be involved in the development of these malignant diseases.

The decrease of hMSH3 expression was observed in all types of hematological malignancies examined in this study, but its frequency appeared to vary dependent upon the types of diseases.

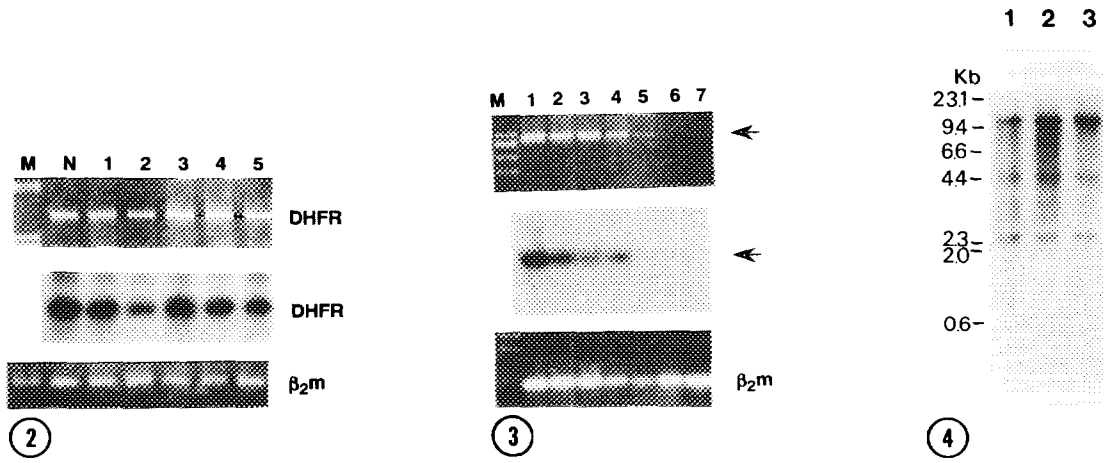

Fig.2. Expression of the DHFR gene. RT-PCR products of DHFR (400 bp) were detected by ethidium bromide staining (upper panel) and the Southern blotting (middle panel) after electrophoresis. The lower panel shows the ethidium bromide stained gel of the RT-PCR products of β_2m. Lane N, bone marrow cells from a normal volunteer (shown in lane 1 of Fig 1-A); Lanes 1-5, bone marrow cells from patients shown in lanes 17, 20, 21, 23 and 10 of Fig 1-C, respectively.

Fig.3. The sensitivity of the RT-PCR assay. hMSH3(+) RNA from a normal volunteer (shown in lane 1 of Fig 1-A) was mixed with hMSH3(-) RNA from a CML case (shown in lane 6 of Fig 1-C). The ratios of normal:CML are (lane 1) normal alone, (2) 1:20, (3) 1:50, (4) 1:100, (5) 1:1000, (6) 1:10000, and (7) CML alone. The total amounts of RNA were 500 nanograms. The upper panel shows the ethidium bromide-stained gel of the RT-PCR products of hMSH3, and the middle panel shows Southern blot analysis of the same gel. The RT-PCR products of β_2m are shown in the lower panel.

Fig.4. Southern blot analysis of the genomic hMSH3 gene. Genomic DNAs extracted from bone marrow cells were digested with EcoRI, electrophoresed and the blot was hybridized with ^{32}P-labeled cDNA of hMSH3. Lane 1, a normal volunteer (shown in lane 5 of Fig 1-A); Lane 2, a CML patient (lane 6 of Fig 1-C); Lane 3, a MM patient (Fig 1-B).

In CML, 90% of the patients showed decreased expression of the hMSH3, while 38% AML patients showed abnormal expression. This difference may be due to the purity of the cell preparations. CML is a typical clonal disease, and all bone marrow cells share genetic characteristics of the original transformed cell which derived from multipotential stem cells (23). Therefore, biochemical and genetic properties of the bone marrow cells should be homogeneous, although the cells are morphologically heterogenous. On the contrary, because AML cells are originated from the committed myeloid stem cell, it is likely that normal mononuclear cells are contaminated in the bone marrow cell preparation from the patients. The RT-PCR assay used in this study was able to detect the hMSH3 mRNA, when at least 0.1% of the total cells expressed the gene. Therefore, the rate of the patients with decreased hMSH3 expression may be underestimated in AML cases.

Although our data demonstrated a close correlation between reduced expression of the hMSH3 gene and hematological malignancies, the role of hMSH3 in leukemogenesis is not

known. Both hMSH3 and hMSH2 have significant sequence homologies to a bacterial mismatch repair protein MutS, suggesting that hMSH3, like hMSH2 (5, 6), may play a role in human DNA mismatch repair. However, there is no evidence supporting the repair function of hMSH3 at this moment. It has been reported that expression of hMSH2 cause a dominant mutator phenotype in *E. coli* (5). Expression vectors containing various parts of the hMSH3 cDNA were constructed and introduced into *E. coli* JM109. Although the protein products were detected by Western blot analysis, the frequency of spontaneous mutation of the RNA polymerase gene was not changed (unpublished). Therefore, the function of hMSH3 may be different from that of hMSH2. The MutS dependent mismatch repair system in bacteria and yeast has been shown to have multiple functions for maintaining the genetic stability of DNA (14-16, 24). One of them is to prevent genetic recombination between nonidentical sequences (antirecombination) (15). Therefore, hMSH3 may play a role in this antirecombination rather than correction of a single mismatch base pair.

In this context, it is interesting that the reduced expression of the hMSH3 gene was detected only in hematological malignancies, but not in other solid tumors. Chromosomal translocations are consistently associated with various types of hematological malignancies (25, 26). However, no specific cytogenetic abnormalities are found in solid tumors. One possibility is that inactivation of the hMSH3 gene may increase the rate of recombination and result in chromosomal translocation which is involved in the development of hematological malignancies.

We have previously reported that the hMSH3 mRNA are detected in established cell lines such as K562, HL-60 and CEM cells (7). Because these cells are hematological malignant cells, the previous results appear to be inconsistent with the findings in this study. A possible explanation is that the expression of the hMSH3 gene is transiently inhibited during malignant transformation, and the inhibition is released after long time culture. Recently, it has been also suggested that the genetic changes found in established cell lines are not always reflect those occurred in primary tumor cells. The gene for cyclin-dependent-kinase (CDK) inhibitor p16 is homozygously deleted in a striking proportion of tumor cell lines (27). However, the mutation frequency of the p16 gene in primary cells is much less than that in cultured cells (28).

Molecular mechanism of inhibition of the hMSH3 expression remains to be elucidated. Southern blot analysis of genomic DNA showed that there were no gross changes in the structure and the copy number of the hMSH3 gene. The hMSH3 and DHFR genes are organized in a head-to-head configuration separated by only 90 base pairs, and the expression of these two genes appears to be regulated by a bidirectional promoter containing common regulatory elements (10-12). RT-PCR analysis showed the promoter of the DHFR gene was intact, even though expression of the hMSH3 gene was totally reduced. Therefore, one possibility is that transacting factors specifically required for hMSH3 expression are absent or inactive in the cells from the patients. Alternatively, small deletions or point mutations in the

hMSH3 gene including the promoter region may inhibit transcription or decrease the stability of the mRNA. Such examples were known in specific mutations of the human RB1 gene (29) or the human ß-globin gene (30). We have recently succeeded in isolation of the genomic clones containing all exons of the hMSH3 normal gene (Watanabe, A., Ikejima, M., Suzuki, N., and Shimada, T., submitted). The gene consists of 24 exons, and the total length appears to be at least 160 kb. The complete characterization of the genomic clone will allow us to study the detailed structure and regulation of the hMSH3 gene.

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