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Frameshift Mutations of the *hMSH6* Gene in Human Leukemia Cell Lines

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Defects in DNA mismatch repair mechanisms, including frameshift mutations of the *hMSH6* and *hMSH3* genes at their $(C)_8$ and $(A)_8$ tracks, respectively, have been shown to be associated with human malignancies. To clarify the possible involvement of these mutations in hematopoietic malignancies, we screened a total of forty-four human leukemia and lymphoma cell lines for mutations in the *hMSH6* and *hMSH3* genes, as well as in other genes required for DNA replication or repair, by polymerase chain reaction single-strand conformation polymorphism analysis and sequencing analysis. Frameshift mutations at the $(C)_8$ track of the *hMSH6* gene were detected in two cell lines established from lymphoid leukemias. These two cell lines had no wild-type alleles, and both of them showed microsatellite instability. This is the first report that describes mutations and inactivation of the *hMSH6* gene in hematological malignancies, suggesting that defects of the *hMSH6* gene may be associated with development of hematological malignancies.

Key words: *hMSH6* gene — Mismatch repair gene — Mutations — Leukemia — Microsatellite instability

Increased genetic instability is now considered as one of the most important mechanisms for tumorigenesis, through which a number of genetic alterations in protooncogenes and tumor suppressor genes take place in series, contributing to cell transformation or immortalization, and finally leading to cancer formation. Genetic instability manifests itself in a variety of phenotypes, including chromosome instability, higher frequency of recombination, decreased fidelity in DNA replication and others. Microsatellite instability is one of the phenotypes of genetic instability, representing variability in the length of repetitive nucleotide sequences.[1)] First described in hereditary nonpolyposis colorectal cancer (HNPCC),[2, 3)] microsatellite instability has been shown to exist in a wide variety of sporadic human malignancies, including cancers of the colorectum, stomach, pancreas, endometrium, ovary, bladder, lung, and hematopoietic systems.[4–8)] HNPCC is an autosomal dominantly inherited disease with a marked increase in susceptibility to cancers, for example, of the colorectum, and recent studies have revealed that it is due to inheritance of germ line mutations in one of four genes required for DNA mismatch repair; *hMSH2*,[9–11)] *hMLH1*,[12–14)] *hPMS1*, and *hPMS2*.[15)] Moreover, non-HNPCC tumors with microsatellite instability have also been shown to carry mutations of these genes. These observations suggest that genetic instability, which appears as microsatellite instability or hypermutability of cancer-related genes, might be a result of prior mutations in "stability genes," such as DNA mismatch repair genes, which normally ensure the fidelity of DNA replication and repair.[16)]

The mechanism of DNA mismatch repair is best understood in *Escherichia coli* (*E. coli*), and is known as the MutHLS system. Although eukaryotes have a more complex system for mismatch repair, some of the components are evolutionally well conserved. The yeast *Saccharomyces cerevisiae* has six homologs of MutS, MSH 1–6, and at least two homologs of MutL, MLH1 and PMS1. The MSH2 is responsible for mismatch recognition, and binds to DNA at the site of a mispaired base. Recent studies in *Saccharomyces cerevisiae* showed that there are at least two MSH2-dependent pathways of mismatch recognition; single-base mispairs are recognized by MSH2-MSH6 (also called *GTBP* or *hMSH6* in humans) complex, and insertion/deletion mispairs are recognized by either MSH2-MSH6 complex or MSH2-MSH3 complex. In the following steps, MLH1-PMS1 (*hPMS2* in humans) complex interacts with MSH2. Finally, the mismatched nucleotide pairs are removed and a correct DNA strand is newly synthesized by DNA helicases and DNA polymerases. Human cells also contain homologs of MutS (*hMSH2*, *hMSH3*, *hMSH6*) and MutL (*hMLH1*, *hPMS1–8*), which are considered to play similar roles to their *E. coli* counterparts.

The *hMSH6* and *hMSH3* genes have recently been reported to be mutated in several kinds of cancers, suggesting that these genes might also be associated with tumorigenesis.[17–20)] These mutations frequently occur at their $(C)_8$ or $(A)_8$ track, leading to frameshifts, in more than 30% of colon tumors with microsatellite instability.[20)] Human polymerase delta gene (*pol* δ) is another gene contributing to the high accuracy of DNA replication, and was found to be mutated in colorectal cancer cell lines with microsatellite instability.[21)] The mutations are located in the 3′–5′ exonuclease "proofreading" domain, raising the possibility that defects in "proofreading" function by the exonuclease domain of this polymerase could also make a contribution to microsatellite instability.[21)] Later, one colorectal cancer cell

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line was shown to contain a mutation in both the *pol δ* and *hMSH6* genes.[22]

In hematological malignancies, several lines of evidence suggested that genetic instability also exists in blastic crisis of chronic myelogenous leukemia (CML),[4] myelodysplastic syndrome,[5] chronic lymphocytic leukemia,[6] Burkitt lymphoma,[7] and human immunodeficiency-virus associated lymphoma.[8] As for DNA mismatch repair genes, we have reported mutations and inactivation of the *hMLH1* gene in human lymphoid leukemia cell lines.[23] To our knowledge, however, alterations of the other mismatch repair genes have not yet been demonstrated in hematological malignancies.

In this study, we screened forty-four human hematopoietic tumor cell lines for mutations in the *hMSH6*, *hMSH3*, *hMSH2* and human *pol δ* genes, and found frameshift mutations and inactivation of the *hMSH6* gene in two human leukemia cell lines. Possible involvement of defects in the DNA mismatch repair system in development of hematological malignancies is discussed.

MATERIALS AND METHODS

Cell lines and preparation of samples Forty-four human leukemia and lymphoma cell lines including 10 myelocytic/monocytic cell lines (HL60, SKH1, KG1, KU812, JOSK-I, JOSK-K, THP1, U937, J-111, and ML1) , 4 erythroid cell lines (F36E, K562, HEL, and TF1), 4 megakaryocytic cell lines (UT7, CMK, MegJ, and MOLM1), 18 B-lymphocytic cell lines (BALL-1, Daudi, Raji, IM9, HA, SCMCL-L3, SCMCL-L4, P30OHK, KCL22, BALM 1, Ri-1, RPMI 8226, NALM17, NALM6, ARH77, REH, SKT1B, and OND39), and 8 T-lymphocytic cell lines (Jurkat, A3/KAW, MOLT 4, MOLT16, SKW3, CEM, MT2, and HPB ALL) were included in this study. The cells were grown in suspension culture in RPMI medium 1640 supplemented with 10% fetal calf or bovine serum. TF1, UT7, CMK, and F36E are factor-dependent cell lines and were cultured in the presence of 5 ng/ml of recombinant human granulocyte-monocyte colony-stimulating factor (rhGM-CSF). Genomic DNAs and total RNAs of these cells were extracted as previously described.[24] Complementary DNAs (cDNAs) were synthesized from the total RNAs of forty cell lines (above-listed cell lines except CMK, SCMCL-L3, HPB ALL, and U937) according to the methodology described in the literature.[25]

(Reverse transcription-)polymerase chain reaction single-strand conformation polymorphism analysis ((RT-)PCR-SSCP analysis) We performed mutation analysis of four genes, the *hMSH6*, *hMSH3*, *hMSH2*, and human *pol δ* genes, using (RT-)PCR-SSCP analysis. The locations of the analyzed regions and the primers for (RT)-PCRs are listed in Table I. These are the regions where mutations have been reported at high frequencies,[20, 21, 26] or nucleotide sequences are highly conserved among human and other species. (RT-)PCR-SSCP analyses were performed according to the previously described methodology.[27] The conditions for the PCRs are listed in Table I. Genomic DNAs (50 ng)

Table I. Primer Pairs and Conditions for PCR

Genes	Locations	Primers	Conditions for PCR			Cycles	Method of analysis	Templates
hMSH6	Codons 1069–1100 (94 bp)	5′-GGGTGATGGTCCTATGTGTC-3′ 5′-CGTAATGCAAGGATGGCGT-3′	94°C 1 min	62°C 1 min	72°C 2 min	35	PCR-SSCP	Genome DNA
hMSH3	Codons 342–393 (153 bp)	5′-AGATGTGAATCCCCTAATCAAGC-3′ 5′-ACTCCCACAATGCCAATAAAAAT-3′	94°C 1 min	62°C 1 min	72°C 2 min	35	PCR-SSCP	Genome DNA
hMSH3	Codons 888–1006 (354 bp)	5′-GAGAGAGTAATGATAATTACCG-3′ 5′-CAGGGTTAAGGATTTCAC-3′	94°C 1 min	58°C 1 min	72°C 2 min	30	RT-PCR-SSCP	cDNA
hMSH3	Codons 516–612 (285 bp)	5′-AGATGCTCTCCAAACCTGAG-3′ 5′-CTATCTGACCAAACACACTAG-3′	94°C 1 min	62°C 1 min	72°C 2 min	30	RT-PCR-SSCP	cDNA
hMSH3	Codons 748–891 (431 bp)	5′-CAGGACAGGAGTTTATGATAG-3′ 5′-TACTCTCTCTGAGTCCTCTG-3′	94°C 1 min	62°C 1 min	72°C 2 min	30	RT-PCR-SSCP	cDNA
hMSH2	Exon 5 (311 bp)	5′-CCAGTGGTATAGAAATCTTCG-3′ 5′-GTCTAATATTGATGTCGAACTC-3′	94°C 30 s	55°C 30 s	72°C 1 min	35	PCR-SSCP	Genome DNA
hMSH2	Exon 7 (207 bp)	5′-TTCAGATTGAATTTAGTGGAAGC-3′ 5′-ACCTTCATGTTTTTCCAGAGC-3′	94°C 30 s	55°C 30 s	72°C 1 min	35	PCR-SSCP	Genome DNA
hMSH2	Exon 12 (326 bp)	5′-ATTATTCAGTATTCCTGTGTAC-3′ 5′-ACCCCCACAAAAGCCCAAA-3′	94°C 30 s	55°C 30 s	72°C 1 min	35	PCR-SSCP	Genome DNA
human *pol δ*	Codons 226–327 (303 bp)	5′-CCTGGAACAGGGCATCCG-3′ 5′-AGGGAAGATGCCTTTGCG-3′	94°C 1 min	55°C 1 min	72°C 2 min	30	RT-PCR-SSCP	cDNA
human *pol δ*	Codons 325–459 (404 bp)	5′-ATCTTCCCTGAGCCTGAG-3′ 5′-ATGTCCATCTGCACGCG-3′	94°C 1 min	55°C 1 min	72°C 2 min	30	RT-PCR-SSCP	cDNA
human *pol δ*	Codons 447–588 (424 bp)	5′-AAGGTTGTCAGCATGGTGGGC-3′ 5′-CGATGACAGTGGCTCCCG-3′	94°C 1 min	60°C 1 min	72°C 2 min	30	RT-PCR-SSCP	cDNA

Table II. *hMSH6*, *hMSH3*, and Human Polymerase Delta Gene Mutations in Human Leukemia/Lymphoma Cell Lines

Genes	Cell lines	Codon	Nucleotides	Amino acid	Genotype
hMSH6	Jurkat	Codons 1085–1087	$(C)_8 \rightarrow (C)_7$	Frameshift (Stop codon at codon 1089)	
hMSH6	KCL22	Codons 1085–1087	$(C)_8 \rightarrow (C)_7$	Frameshift (Stop codon at codon 1089)	
hMSH6	KCL22	Codons 1085–1087	$(C)_8 \rightarrow (C)_9$	Frameshift (Stop codon at codon 1092)	
hMSH6	Josk-I, SKW 3, U937	Codon 1082	CCG→CCT	No change	Heterozygous
hMSH3	ARH77, JIII, HEL, CEM	Codon 949	CGG→CAG	Arg → Gln	Heterozygous
pol δ	Daudi, JIII, A3KAU, Raji, MOLT16	Codon 270	GCT→GCC	No change	Heterozygous
pol δ	OND39, NALM6, HL60, KG1, REN	Codon 495	ACC→ACT	No change	Heterozygous
pol δ	Daudi, JIII, Raji, MOLT16	Codon 513	CTG→CTA	No change	Heterozygous

or reverse-transcribed cDNAs (100 ng) were used as templates.

Sequencing analysis Mutations in the (RT-)PCR products that showed abnormally migrating bands in SSCP analysis were confirmed by sequencing analysis. The RT-PCR products of the *hMSH3* or human *pol δ* gene were directly sequenced using a Sequenase PCR Product Sequencing Kit (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. The PCR products of the *hMSH6* and *hMSH3* genes were subcloned into the pCR 2.1 Vector (Invitrogen, San Diego, CA) and at least four independent clones were sequenced with an "ABI PRISM" Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, CA) or a Sequenase Version 2.0, 7-deaza-dGTP Kit (Amersham).

Isolation of single cell clones Single cell clones of Jurkat were isolated by limiting dilution in 96-well microtiter plates. Genomic DNAs were extracted from each single cell clone as previously described.[24]

Assay for microsatellite instability Microsatellite instability in twenty-five single cell clones of Jurkat was tested by PCR-based analysis. We analyzed the status of four microsatellite regions containing repetitive sequences; D18S58, D2S123, BAT25, and BAT26. The primers used for these microsatellite markers and conditions for the PCRs were taken from the literature.[28] The PCR products were denatured and separated on a 6% polyacrylamide denaturing gel containing 8.3 mol/liter urea.

Accession to the GenBank database The codon numbers of *hMSH3*, *hMSH6*, and human *pol δ* in Table I and Table II have been submitted to the GenBank database under the accession numbers U28946, J04810, and M80397, respectively.

RESULTS

The positive findings of mutation analysis in the four genes (the *hMSH6*, *hMSH3*, *hMSH2*, and human *pol δ* genes) are summarized in Table II.

Mutations in the *hMSH6* gene We screened forty-four human hematopoietic tumor cell lines for mutations in PCR-amplified regions encompassing the $(C)_8$ track of the *hMSH6* gene (codons 1,085–1,087), which was recently reported to be a hotspot for frameshift mutations in colorectal tumors and other sporadic tumors.[20] In SSCP analysis, abnormally migrating bands were detected in five cell lines; Josk-I, SKW3, Jurkat, KCL22, and U937 (Fig. 1). Jurkat and KCL22 showed only altered bands and no normal bands. Josk-K, SKW3, and U937 gave both normal and abnormal bands showing similar SSCP patterns. The sequencing analysis of these *hMSH6* fragments revealed frameshift mutations in two cell lines; Jurkat and KCL22 (Fig. 2). All 4 sequenced clones from Jurkat had one base deletion at the $(C)_8$ track (Fig. 2B) . On the other hand, one base deletion (Fig. 2C) and one base insertion (Fig. 2D) were detected at the $(C)_8$ track in 6 clones and in 5 clones, respectively, when 11 clones from KCL22 were sequenced. No wild-type sequences were obtained in the analysis on both cell lines. As for the other three cell lines, Josk-I, SKW3, and U937, 10 clones of each were sequenced. As expected from their similar SSCP patterns, the same silent mutation at codon 1082 (CCG to CCT), as well as the wild-type *hMSH6* sequence, was detected for each cell line (data not shown), indicating that these cell lines were heterozygous for this mutation.

Mutations in the *hMSH3* gene Because the $(A)_8$ track of the *hMSH3* gene (codons 381–383) has also been reported to be a hotspot for frameshift mutations in colorectal tumors and other sporadic tumors,[20] we analyzed mutations of the same region in forty-four cell lines by PCR-SSCP analysis. In addition, we also analyzed three coding regions which are highly conserved among human and other species by RT-PCR-SSCP analysis. In the SSCP analysis of the region encompassing codons 888–1006, we detected altered bands in four cell lines; ARH77, JIII, HEL, and CEM (data not shown). All of them showed the same shift pattern. Sequencing analysis revealed that all these four cell lines were heterozygous for the same missense mutation at

codon 949 (CGG to CAG) (Table II), suggesting that it is most likely to be a polymorphism. SSCP analysis of the other three regions, including that with the $(A)_8$ track, disclosed no altered bands.

Mutations in the *hMSH2* and human *pol* δ genes We analyzed three exons (exon 5, 7, and 12) of the *hMSH2* gene in which mutations had been reported at a high frequency.[26] SSCP analysis of thirty-eight cell lines (the above-listed cell lines except ML1, BALM 1, Ri-1, RPMI 8226, A3/KAU, and OND 39) revealed no altered bands. We also analyzed three coding regions of the human *pol δ* gene, containing three highly conserved exonuclease sites, termed Exo I, Exo II, and Exo III. RT-PCR-SSCP and sequencing analyses revealed only silent mutations in some of the cell lines, and all of them were heterozygous (Table II).

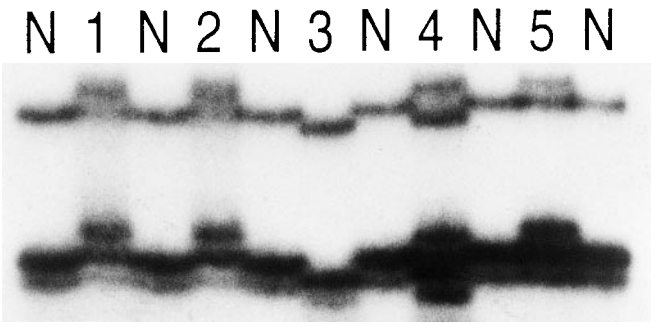

Fig. 1. SSCP analysis of the coding region encompassing the $(C)_8$ track of the *hMSH6* gene. Lanes: N, human placenta cells (normal control); 1, Josk-I; 2, SKW 3; 3, Jurkat; 4, KCL 22; 5, U937.

Microsatellite instability in Jurkat and KCL22 Because defects in the mismatch repair system have been considered to lead to a genetically unstable state, Jurkat cell clones were also analyzed for microsatellite instability at four different polymorphic loci. Single cell clones from Jurkat were found to exhibit microsatellite instability (Fig. 3) at all four loci. Clones from KCL22 have also been reported to display microsatellite instability.[23]

DISCUSSION

We detected frameshift mutations in a track of eight deoxycytosines present in the coding region of the *hMSH6* gene (codons 1085–1087) in two of the forty-four human leukemia/lymphoma cell lines, Jurkat and KCL22. Both cell lines were established from leukemias of lymphoid origin; Jurkat from T-lineage acute lymphocytic leukemia (T-ALL) and KCL22 from B-lymphoid crisis of CML. No wild-type sequences were obtained in sequencing analysis of these two cell lines. Because frameshift mutations cause the generation of truncated proteins, the function of *hMSH6* should be lost in both cell lines. This is the first report that describes mutations and inactivation of *hMSH6* in hematological malignancies. We also demonstrated that microsatellite instability exists in the two cell lines with inactivation of the *hMSH6* gene.

The frameshift mutations of the *hMSH6* gene in the two leukemia cell lines occur at the same hotspot that has recently been reported in colorectal and other tumors.[20] The mechanisms of these frameshift mutations are unknown. One possibility is that the frameshift mutations occur at the $(C)_8$ track, a hotspot for slippage-generated

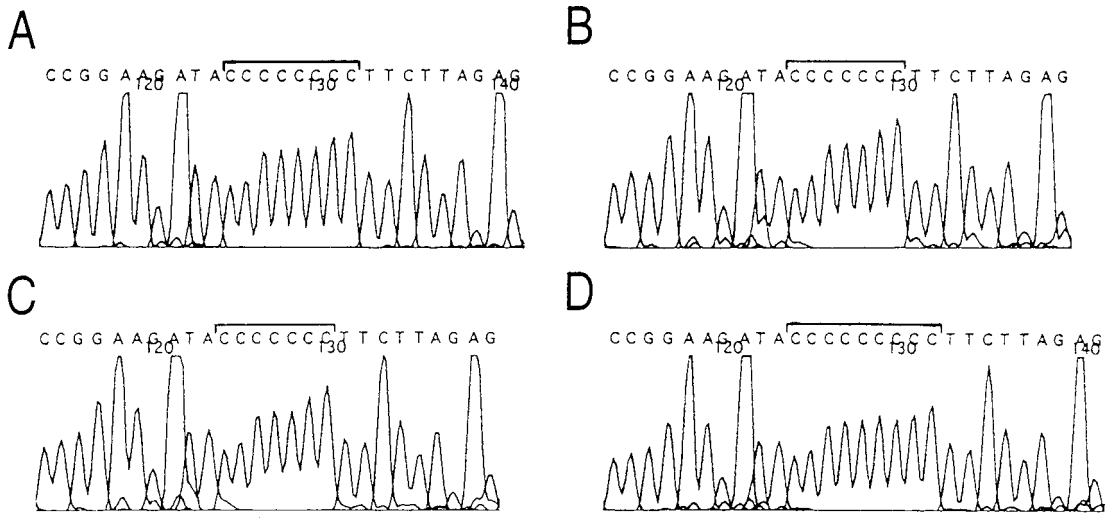

Fig. 2. Frameshift mutations at the $(C)_8$ track of the *hMSH6* gene. The sequences corresponding to the $(C)_8$ track and its surrounding regions are shown. Panels: A, human placenta (control); B, Jurkat; and C and D, KCL22.

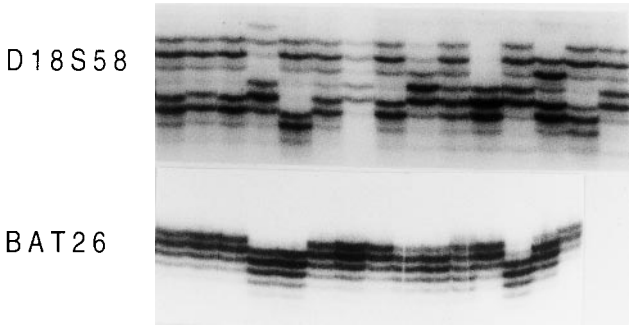

Fig. 3. Microsatellite instability observed in Jurkat. Each lane represents a single cell clone from Jurkat. Analyses performed for two polymorphic loci, D18S58 and BAT26, are indicated.

mutations,[29] and that both leukemia cell lines in fact showed microsatellite instability. It is possible that these mutations are a result of primary defects of other components in the mismatch repair systems, generating microsatellite instability. If this is the case, genetic alterations including slippage-generated mutations would occur in consecutive steps, where secondary mutator genes are inactivated one after another. This would increase the genetic instability of the tumor cells. However, a recent study by Malkhosyan *et al*. seems intriguing.[20] They showed that colorectal tumors having frameshift mutations of *hMSH6* at this $(C)_8$ track had no slippage-generated mutations in repetitive sequences in other genes, including the *hPMS2* mismatch repair gene, the DNA polymerase alpha gene, the retinoblastoma gene, and the fibronectin type III gene. Their observation suggested that the frameshift mutations at the *hMSH6* $(C)_8$ track were the primary abnormality selected for during tumorigenesis, generating genetic instability.[20]

We can not rule out the possibility that the mutations in the *hMSH6* gene developed *ex vivo*, since the original tumor samples from which the two cell lines, Jurkat and KCL22, were generated are not available. However, the fact that similar frameshift mutations are found in many other tumors, including tumor cell lines of colon and endometrium, colorectal tumors, and gastric tumors,[20] strongly suggests that the mutations at the *hMSH6* $(C)_8$ track might be common abnormalities during tumorigenesis. We have already screened 14 patients with ALL for mutations in the *hMSH6* genes. Although we found no mutations in these 14 patients (data not shown), it would be of great interest to screen a larger number of primary human hematological malignancies for frameshift mutations of the *hMSH6* gene.

In this study, silent mutations of the *hMSH6* and human *pol* δ genes were observed in some of the cell lines. All of them were heterozygous. These may represent polymorphisms with no biological effect. Four cell lines carried the same heterozygous missense mutation that causes an amino acid change (Arg→Gln) at codon 949 of the *hMSH3* gene. Although these mutations have not been reported in healthy individuals, it is unclear whether they are related to the diseases.

Responsible genes for defects in mismatch repair systems have not yet been fully studied in hematological malignancies. Robledo *et al*. analyzed one of the conserved regions of the *hMSH2* gene in leukemia/lymphoma cases showing microsatellite instability, and did not find any mutations.[7] Inokuchi *et al*. reported loss of expression of the *hMSH3* gene in patients with hematological malignancies.[30] However, it remains unclear whether these patients showed microsatellite instability. We describe here the frameshift mutations and inactivation of the *hMSH6* gene in two cell lines from lymphoid malignancies displaying microsatellite instability. This is the first report showing a defect of the *hMSH6* gene in cell lines of hematological malignancies. The fact that similar mutations of the *hMSH6* genes are found in colorectal tumors suggests that defects of the *hMSH6* gene may be involved in development of hematological malignancies as well as colorectal tumors. Further analyses would be of interest.

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