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Enhanced Detection of Deleterious and Other Germline Mutations of hMSH2 and hMLH1 in Japanese Hereditary Nonpolyposis Colorectal Cancer Kindreds

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Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal, dominantly inherited cancer-prone syndrome. Here, we describe a novel and efficient approach for screening mutations of two major HNPCC susceptibility genes, hMSH2 and hMLH1. The system consists of RNA extraction from whole blood treated with the translation inhibitor, followed by long RT-PCR of the entire coding regions combined with direct sequencing. In analysis of 15 kindreds suspicious for HNPCC, 8 samples were subjected to analysis after puromycin treatment and 7 samples were analyzed without puromycin treatment. Three deleterious mutations were detected in the kindreds with puromycin treatment, while none were observed in those without puromycin. Signals from mutated alleles were enhanced after puromycin treatment and easily distinguished from the wild-type allele, achieved by suppression of nonsense-mediated mRNA decay. Furthermore, 12 other mutations were detected in 15 kindreds. The system is considered to be a reliable and useful approach for detecting germline mutations of hMSH2 and hMLH1 in HNPCC kindreds.

Key Words: **hereditary nonpolyposis colorectal cancer; mutation; long RT-PCR; translation inhibitor; nonsense-mediated mRNA decay.**

Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal, dominantly inherited syndrome that is considered to account for 0.5 to 5% of all colorectal cancer (CRC) cases in Western countries (1). Carcinomas originating from various organs, such as the colorectum, endometrium, ovary, stomach, small bowel, hepatobiliary tract, pancreas, ureter, and renal pelvis, usually develop in the affected individuals with an earlier onset than those in sporadic cases (1). Recently, germline mutations of several genes such as hMSH2, hMLH1, PMS1, PMS2 and MSH6 were reported in HNPCC kindreds (2–7). These genes are homologues to their counterparts involved in the mismatch repair systems of *E. coli* and/or yeast. Screening and identification of germline mutations of the susceptible genes in HNPCC kindreds would be important for presymptomatic detection of heterozygous carriers and secondary prevention of the cancer. Linkage analysis of the HNPCC kindreds fulfilling Amsterdam minimal criteria showed that hMSH2 and hMLH1, human homologues of the genes for bacterial mismatch repair proteins Mut S and Mut L, are assumed to account for 90% of the cases (8) and mutations either in hMSH2 or hMLH1 were observed in about 70% of kindreds (9). Various methods have been employed to detect mutations in hMSH2 and hMLH1, such as single-strand conformation polymorphism (SSCP) analysis (10), denaturing gradient gel electrophoresis (DGGE) (11), in vitro synthesized protein assay (IVSP) (12), yeast functional assay (13, 14) and direct sequencing of each exon amplified from genomic DNA (15) or RT-PCR (16). The regions for hMSH2 and hMLH1 span approximately 73 kb (15) and 58 kb (17) in genomic DNA, which comprise 16 exons for hMSH2 and 19 exons for hMLH1, respectively. In detecting mutations of these 2 genes, exon-by-exon analyses are laborious, and difficult for detecting mutations in cases of interstitial deletions missing multiple exons (18). Furthermore, if mutations are found in exon-intron boundaries, it is difficult to clarify whether these mutations result in splicing variants

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TABLE 1

Primer Sequences

Genes	Names	Primer sequences	Directions
hMSH2			
PCR primers	RT-Sf1	5′-GGCGGGAAACAGCTTAGTGGGTGTG-3′	(Sense)
	RT-Sr1	5′-CCCATGGGCACTGACAGTTAACACTATG-3′	(Antisense)
Sequencing primers	Sf1	5′-TCTTCAACCAGGAGGTGAGG-3′	(Sense)
	Sr1	5′-CCGGGTAAAACACATTCCTT-3′	(Antisense)
	Sf2	5′-GATAATGATCAGTTCTCCAA-3′	(Sense)
	Sr2	5′-GGCAAGTCGGTTAAGATCTG-3′	(Antisense)
	Sf3	5′-GCAGAATTGAGGCAGACTTT-3′	(Sense)
	Sr3	5′-GCATCCTGGGCTTCTTCATA-3′	(Antisense)
	Sf4	5′-AATTGACTTCTTTAAATGAAGA-3′	(Sense)
	Sr4	5′-CCCATGCTAACCCAAATCCA-3′	(Antisense)
	Sf5	5′-CTGCAACCAAAGATTCATTA-3′	(Sense)
	Sr5	5′-ATATTACCTTCATTCCATTA-3′	(Antisense)
hMLH1			
PCR primers	RT-Lf1	5′-CATCTAGACGTTTCCTTGGCTCTTC-3′	(Sense)
	RT-Lr1	5′-TAAAGGAATACTATCAGAAGGCAAGTATA-3′	(Antisense)
Sequencing primers	Lf1	5′-TTTCCTTGGCTCTTCTGGC-3′	(Sense)
	Lf1.2	5′-GATGATTGAGAACTGTTTAG-3′	(Sense)
	Lr1	5′-ATCAGCTACTGTCTCTCCTT-3′	(Antisense)
	Lf2	5′-GGAAAATTTTGGAAGTTGTT-3′	(Sense)
	Lf2.2	5′-TCTTCATCAACCATCGTCTG-3′	(Sense)
	Lr2	5′-GCATCAAGCTTCTGTTCCCG-3′	(Antisense)
	Lf3	5′-CCACAACAAGTCTGACCTCG-3′	(Sense)
	Lr3	5′-CGATAACCTGAGAACACCAA-3′	(Antisense)
	Lf4	5′-AACTGTTCTACCAGATACTC-3′	(Sense)
	Lr4	5′-GAGAAAGAAGAACACATCCC-3′	(Antisense)

until their transcripts are analyzed. RNA-based analyses are much more advantageous than those from genomic DNA, for they could detect exon skipping caused by interstitial deletions and mutations in exon-intron junctions concurrently. However, it has been reported that RNA-based analysis harbors a risk for

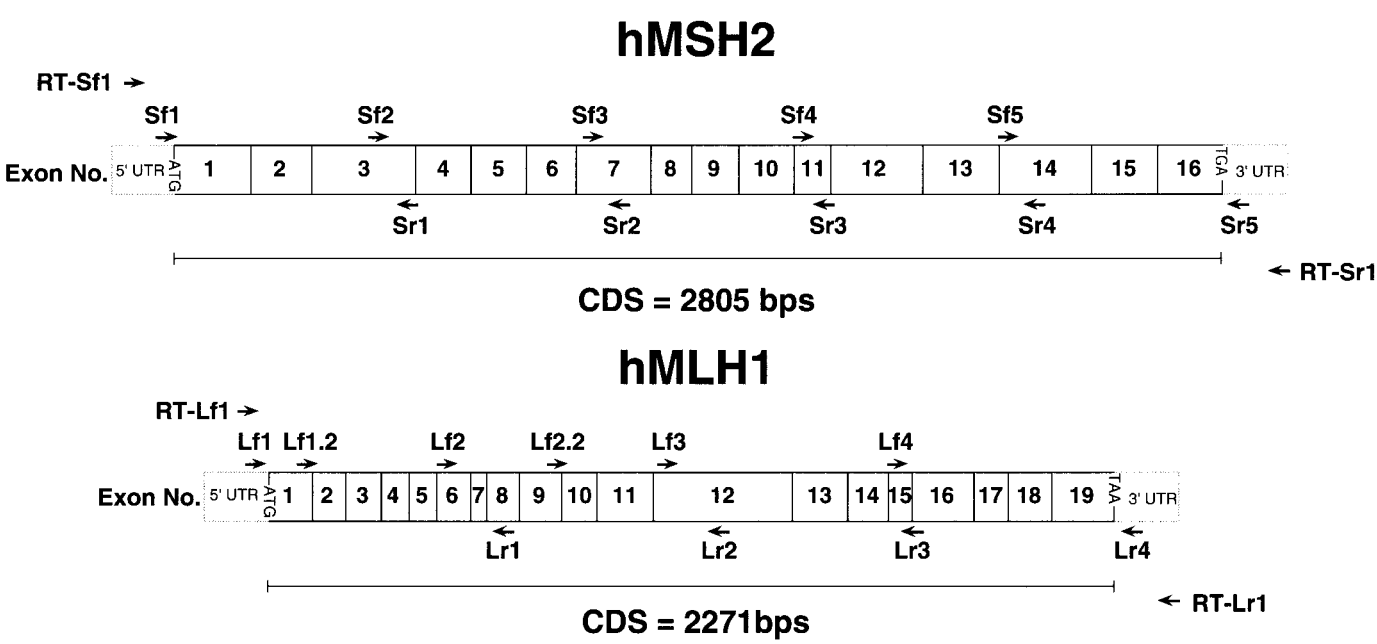

FIG. 1. Exon structures and primer settings for hMLH1 and hMSH2. Arrows indicate primer positions for RT-PCR and direct sequencing. Solid lines indicate coding regions of hMSH2 and hMLH1, separated by exons. Broken lines indicate 5′ and 3′ untranslated regions (UTRs).

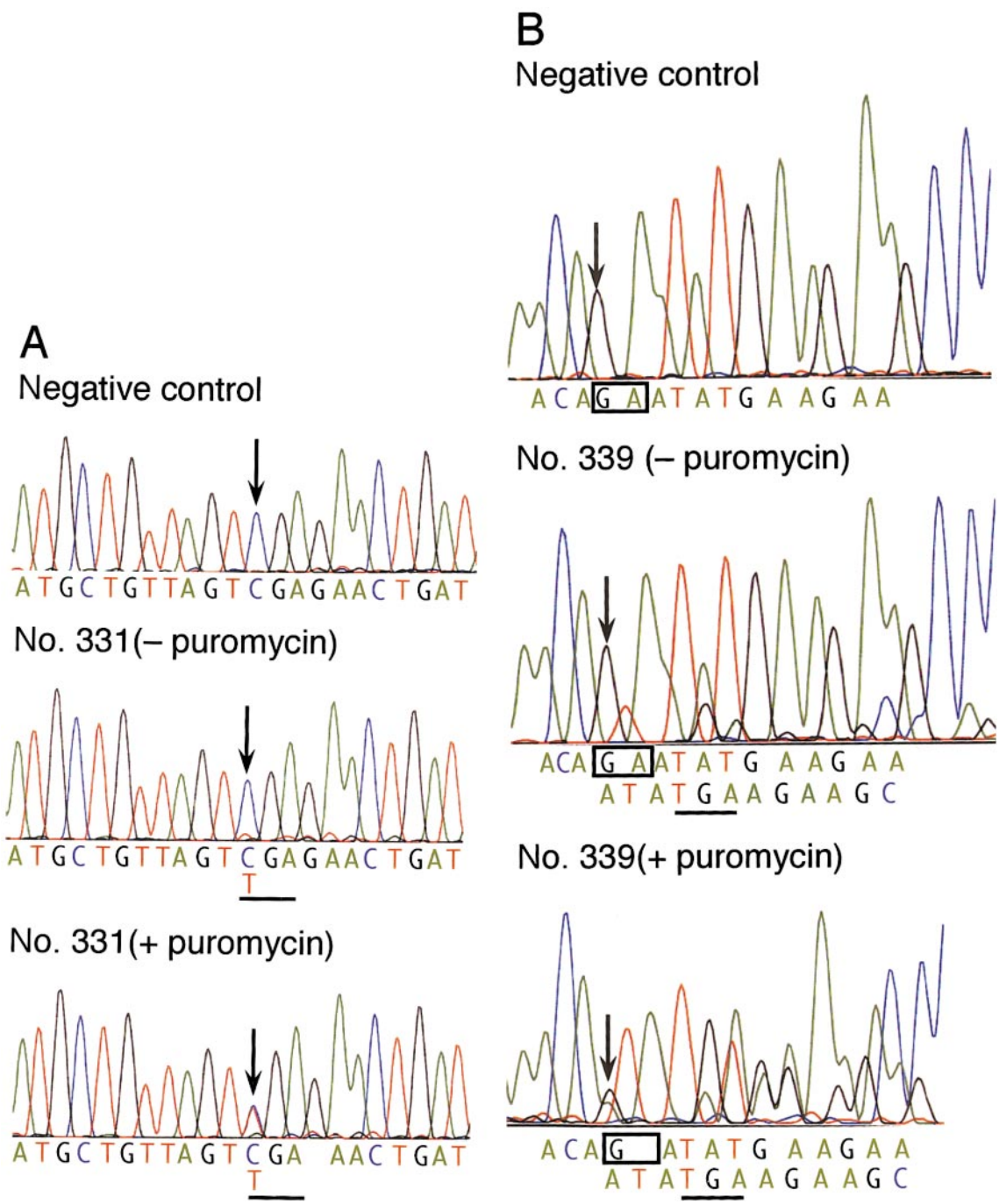

FIG. 2. Effects of puromycin treatment on the sequencing profiles for detecting germline mutations. Top panels indicate the sequencing profile of the negative control (wild type), middle panels are the sample without puromycin treatment, and lowermost panels are the sample with puromycin treatment. Arrows indicate the positions for the point mutation (A) or, 2 bps deletions (B and C) and the exon skipping causing in-frame deletion (D). Boxes indicate sequences of 2 bps deletions. Underline indicates premature termination codon appearing in the mutated allele. (A) Nonsense mutation of R226X in exon 8 of hMLH1 (patient 331); (B) frameshift mutation, 1705delGA in exon 11 of hMSH2 (patient 339); (C) frameshift mutation of 223delCT in exon 2 of hMSH2 (patient 352); (D) skipping of hMSH2 exon 5 resulting in in-frame deletion (patient 354).

overlooking mutations resulting in premature termination of protein synthesis, e.g., nonsense or frameshift mutations, due to the unstable and degradable property of the RNA transcript carrying premature termination codons (19). This fragile property of mRNA coding deleterious mutations were designated as nonsense-mediated mRNA decay (NMD) (24). Treating cells with puromycin, an antibiotic inhibiting transla-

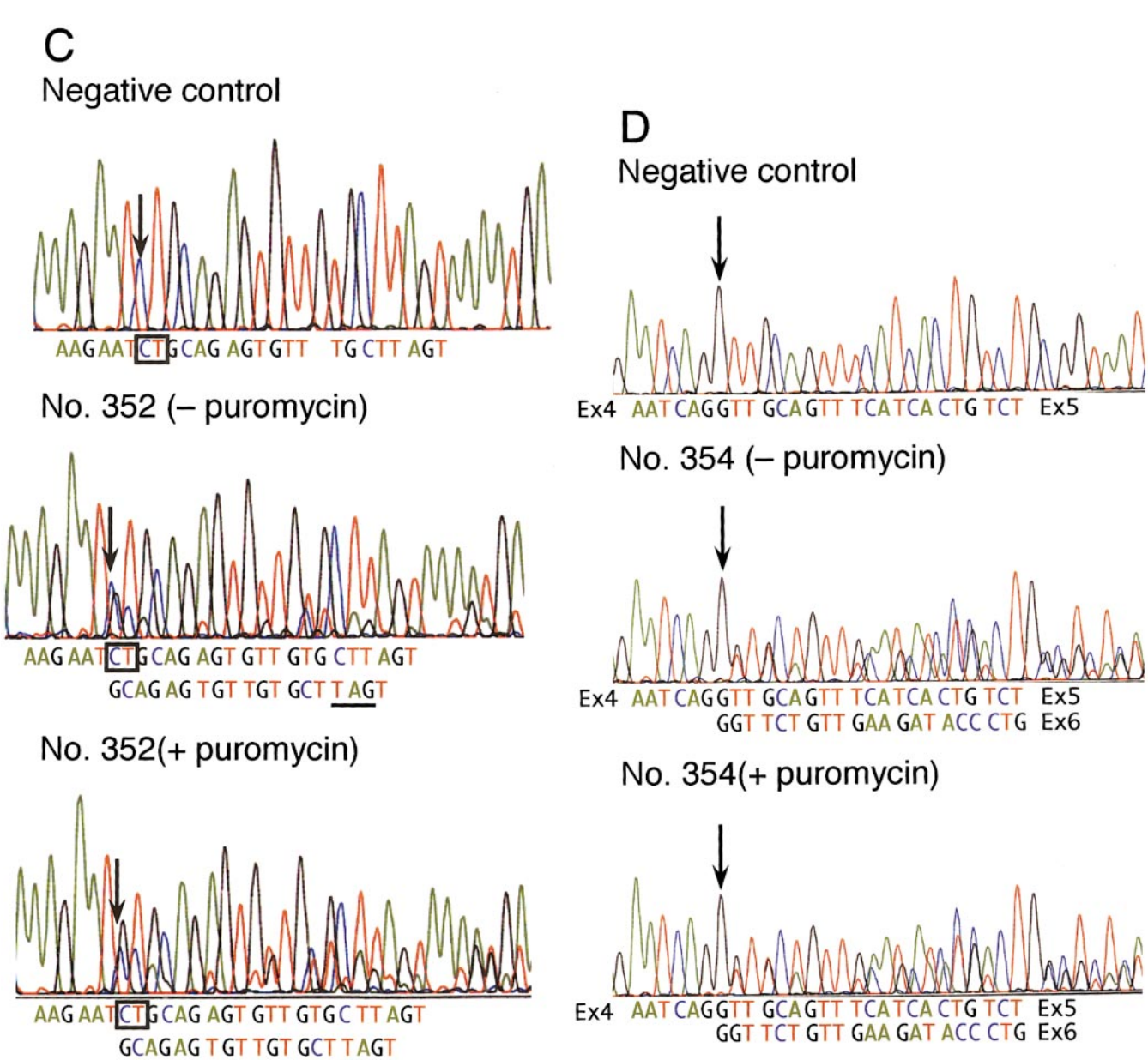

FIG. 2—*Continued*

tion, was reported to be useful for reducing the decay of the transcripts carrying premature termination codons in the yeast functional assay (13). We applied long RT-PCR to amplify whole coding exons from RNA samples extracted after puromycin treatment and subjected the PCR product to direct sequencing using a fluorescence-based automated DNA sequencer. This mutation screening system could be available as a suitable and reliable method for detecting germline mutations of hMSH2 and hMLH1.

MATERIALS AND METHODS

The HNPCC criteria and pedigree profiles. In 1990, the International Collaborative Group on HNPCC (ICG-HNPCC) defined HNPCC by the following minimum diagnostic criteria (Amsterdam criteria) (20). (1) At least three relatives should have histologically verified colorectal cancer; one of them should be a first degree relative to the other two. Familial adenomatous polyposis should be excluded. (2) At least two successive generations should be affected. (3) In one of the relatives, colorectal cancer should be diagnosed under 50 years of age. In Japan, other clinical diagnostic criteria were proposed at the 34th Annual Meeting of the JSCCR in 1991, Tokushima, Japan (21). Japanese criteria to define HNPCC are as follows: group A (JA), a family with three or more colorectal cancer patients within first degree relatives; group B (JB), a family with two or more colorectal cancer patients within first degree relatives and with any of the following—(a) age at onset of colorectal cancer is less than 50 years of age, (b) right colon involvement, (c) synchronous or metachronous multiple colorectal cancers, and/or (d) association with extracolorectal cancers. In this study, 15 kindreds were analyzed for germline mutations of hMSH2 and hMLH1. Of these, 4

kindreds were eligible for the Amsterdam criteria, 1 for JA and 10 for JB, respectively.

Blood samples and RNA extraction. Heparinized peripheral blood lymphocytes (PBL) were obtained from 15 kindreds who visited the National Cancer Center Hospital for genetic counseling, and provided informed consent for the study. Fresh blood samples were available in 8 kindreds and they were incubated with the translation inhibitor, puromycin (SIGMA CHEMICAL CO., MO) prior to RNA extraction at a concentration of 200 μg/ml for 2 to 6 h at 37°C. In the patient No. 331 carrying germline mutation of hMLH1 at codon 226 substituting stop codon (TGA) for arginine (CGA), the sample was incubated with puromycin at the same concentration but for 0, 5, 90, 360 and 1440 minutes to examine the time course effect of puromycin treatment. Other translation inhibitors, cycloheximide and anisomycin were used to treat the peripheral blood for 6 h at 2 μg/ml and 40 μg/ml, respectively. After incubation, leukocytes were isolated from peripheral blood using VACUTAINER CPT tubes (Becton Dickinson, NJ). The cells were collected by centrifuge at 1500*g* for 15 min at room temperature and washed with 15 ml of phosphate-buffered saline (PBS). Total RNAs were extracted from the leukocytes using the acid guanidine phenol chloroform method (22). In all experiments, RNAs extracted from fresh blood samples without puromycin treatment were also analyzed as puromycin-negative controls. In the remaining 7 kindreds, PBL were separated and stored at −80°C immediately after blood sampling so that RNA extraction was carried out without puromycin treatment.

Long RT-PCR. Reverse transcription was carried out with 200 units of MMLV reverse transcriptase SuperScript II (Life Technologies, Inc., MD), 0.5 mM oligo(dT) primer and 1 μg of the total RNA in 20 μl of 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM of dNTPs and 10 mM dithiothreitol and incubated for 50 min at 42°C. Primers used for long RT-PCR were RT-Sf1 and RT-Sr1 for hMSH2 and RT-Lf1 and RT-Lr1 for hMLH1 (Table 1, Fig. 1). Long PCR for cDNA was carried out using the Advantage cDNA PCR kit (CLONTECH Laboratories, Inc., CA), according to the manufacturer's recommendations with a minor modification, i.e., addition of 10% glycerol in the PCR mixture. The mixture was heat denatured at 94°C for 1 min, followed by 40 cycles of shuttle PCR at 94°C for 15 s and 65°C for 4 min with final extension at 65°C for 10 min. The yield and quality of the PCR product was ascertained by electrophoresis in 0.8% agarose gel.

Sequencing reaction. Prior to sequencing, PCR products were treated with shrimp alkaline phosphatase and exonuclease I using a PCR product pre-sequencing kit (Amersham Pharmacia Biotech, Sweden) to allow cleavage of oligonucleotide primers and dNTPs. Sequencing reaction was performed using Bigdye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Inc., CA). Electrophoresis was carried out using a Genetic Analyzer 310 (PE Applied Biosystems, Inc., CA) equipped with long-read sequencing capillary and POP-6 sequencing polymer (PE Applied Bio Systems, Inc., CA). Primers used for direct sequencing reactions are shown in Table 1 and Fig. 1. Sequencing reactions were usually carried out unidirectionally using 5 sequencing primers (Sf1, Sf2, Sf3, Sf4, and Sf5) for hMSH2 and 6 sequencing primers (Lf1, Lf1.2, Lf2, Lf2.2, Lf3, and Lf4) for hMLH1. If the results were ambiguous, the opposite strand was sequenced using reverse primers (Sr1, Sr2, Sr3, Sr4, and Sr5 for hMSH2 and Lr1, Lr2, Lr3, and Lr4 for hMLH1). In analysis of hMLH1, Lf1.2 and Lf2.2 were designed to anneal sequences overlapping 2 exons, to reduce background signals of normal splicing isomers skipping exon 2 or exon 10 that were frequently observed in healthy individuals. All mutations detected by long RT-PCR/direct sequencing were confirmed by PCR-based sequencing of the corresponding regions of genomic DNA. Mutations or polymorphisms were designated according to the nomenclature proposed by Antonarakis (23) and nucleotide positions of mutations were counted from the first letter of the initiation codon.

Allelic frequencies of the DNA variants. Allelic frequencies of the DNA variants observed in this study were examined by PCR-RFLP and/or PCR/SSCP analysis of the genomic DNAs obtained from normal Japanese donors. PCR conditions and primer sequences are accessible via e-mail to the corresponding author of this article.

RESULTS

Effect of Puromycin Treatment for Detecting Nonsense and Frameshift Mutations

Three mutations (R226X in hMLH1, 1705delGA and 223delCT in hMSH2), resulting in premature termination codons, were expected to NMD (24) and their sequencing profiles were compared between the RNA samples extracted with or without puromycin treatment. In analysis of blood samples treated with puromycin, signals from the mutated alleles were strong enough to discriminate all of these mutations from the wild type sequence. In analysis of samples without puromycin treatment, the mutant signal derived from the nonsense mutation at R226X in patient 331 was barely detectable by direct sequencing, while it was enhanced similar to the intensity of the wild-type allele after puromycin treatment (Fig. 2A). In analysis of the samples causing frameshift mutations due to 2 bps deletion (patients 339 and 352), signals from mutated alleles became more intense and easier to detect in samples treated with puromycin than those without puromycin treatment (Figs. 2B and 2C). In contrast, an in-frame deletion caused by the skipping of exon 5 in the hMSH2, derived from the point mutation at the splicing junction in the 3′ end of the exon 5 (patient 354), showed that the signal intensity from the mutated allele was almost identical between samples with or without puromycin treatment (Fig. 2D).

Time Course of Puromycin Treatment for Inhibition of NMD

The time course of the puromycin effect for inhibiting NMD was examined by incubating the blood sample from patient 331, which was carrying the R226X germline mutation of hMLH1, for 0, 5, 90, 360 and 1440 min with puromycin. Signal ratios of mutant (T) to wild-type (C) alleles were plotted against the incubation periods (Fig. 3A). The ratio of mutant to wild-type alleles was increased at 90 min and reached maximum at 360 min.

Effect of Cycloheximide and Anisomycin for NMD

The effect of other translation inhibitors, cycloheximide and anisomycin, for inhibiting NMD was examined in the same sample carrying R226X mutation of the hMLH1. At the incubation periods for 360 min, these 2 agents were also effective in suppressing the NMD as well as puromycin (Fig. 3B).

Mutations Observed in the Study

In analysis of 15 kindreds, 15 mutations were detected in hMSH2 and/or hMLH1. Ten mutations were detected in hMSH2, including 3 deletions (1 in-frame and 2 frameshift mutations), 1 splice mutation and 3 missense mutations. In analysis of hMLH1, 5 mutations were detected, including 1 nonsense and 4 missense mutations. Three patients (patients 92, 331, and 350) had 2 mutations in hMSH2 and/or hMLH1. T8M mutation of the hMSH2 were independently detected in 2 kindreds (patients 344 and 350). Except for 2 silent mutations, 12 mutational types were detected in this study, and 4 mutations, i.e. I219V, R226X and V384D of the hMLH1 and 1705delGA of hMSH2 were reported previously in HNPCC kindreds, either as pathogenic mutations or DNA variants (25–27). However, there have been no previous reports of the remaining 8 mutational types. In analysis of 8 kindreds, blood samples were treated with puromycin and 10 mutations were detected. All 3 deleterious mutations were detected in the puromycin treated samples. In analysis of 7 kindreds without puromycin treatment, 5 mutations were detected, including 3 missense mutations, 1 silent mutation, 1 in-frame mutation and no deleterious mutations.

Allelic Frequencies of the DNA Variants among Japanese

All single-base substitutions, except for the nonsense mutations and splice mutations, were examined for their allelic frequencies among normal Japanese donors. Four variant alleles (hMSH2 at codon 8, 157, hMLH1 at codon 219 and 384) were observed in normal Japanese donors, of which the allelic frequencies were 1.0% (2/200 alleles), 2.8% (5/180 alleles), 3.8% (5/132 alleles) and 2.8% (5/180 allele), respectively (Table 2). The other base substitutions at codons 324, 688 and 845 of hMSH2 and at codons 111 and 588 of hMLH1 were not observed in analysis of the control DNAs.

DISCUSSION

The need for detecting germline mutations of responsible genes for cancer predisposition is increasing in the fields of cancer genetics. The numbers of causative genes reported are increasing annually, rendering the genetic analysis laborious. This prompted us to perform an RNA-based approach for screening germline mutations. The RNA-based approach was suggested to be disadvantageous due to the appearance of normal splicing variants (28, 29) and NMD (30). Several splicing variants were reported in analysis of hMSH2 and hMLH1 in normal cells using RT-PCR and/or the protein truncation test (28–30). In the present analysis, 2 splicing variants were commonly observed in hMLH1, one of which was 5 bps skipping of 5′ end of exon 2 and another of exons 10 and 11. The signal from aberrantly transcribed RNA isomers were reduced using sequencing primers Lf1.2 and Lf2.2 that were designed to anneal exon–exon boundaries between exons 1 and 2 and exons 9 and 10 for Lf1.2 and Lf2.2, respectively. No splicing isomer was observed to interfere with the analysis of hMSH2. NMD has been thought to be associated with the translation process (19). Recently, treating cells with the translation inhibitor was reported to suppress the decrease of mRNA with premature termination codon in analysis of hMSH2 (13) and bullous pemphigoid antigen 2/type XVII collagen (BPAG2) (31). In the present study, NMD was confirmed in the hMLH1 gene as well as in hMSH2. The translation inhibitor, puromycin, showed improvement of NMD caused by nonsense and frameshift mutated hMLH1 transcripts in the present study. In contrast, enhancement of signals derived from mutated allele was not observed in the case of in-frame deletions such as skipping of exon 5 (Fig. 3D) and 1235del33 of hMSH2 (data not shown), suggesting this effect was specific for deleterious mutations. Other translation inhibitors, such as cycloheximide and anisomycin, also appeared to be effective in suppression of NMD.

As to the missense mutations of hMSH2 and hMLH1, their allelic frequencies among normal donors were examined. Reportedly, I219V and V384D of hMLH1 were DNA polymorphisms and their allelic frequencies among Swedish and Japanese were 34% (25) and 2.5% (32), respectively. We examined the allelic frequencies of these DNA variants among Japanese subjects (Table 2). The T8M of the hMSH2 was a novel DNA variant found in this study. This polymorphism was observed at a relatively low frequency in Japanese donors, but observed in 2 unrelated pedigrees fulfilling the Japanese criteria group B (patients 344 and 350). Its role in colon carcinogenesis remains to be elucidated through segregational analysis of the pedigree. Other missense mutations such as M688V in exon 13 and K845E in exon 15 of the hMSH2, A111V in exon 4 and L588P in exon 16 of the hMLH1 were not detected among other Japanese donors in this study. These missense changes may have a causative effect in cancer predisposition and further studies are in progress to examine their co-segregation with the phenotype.

Reportedly, in-frame deletion of exon 5 in hMSH2 was frequently observed in HNPCC kindreds. In all cases, this splicing variant was caused by A → T transversion at the 3 bps downstream of exon 5 (12, 16, 26). However, in patient 354, skipping of exon 5 in hMSH2 was caused by the A → T transversion at the 3′ end of the exon 5. This mutation of hMSH2 could be regarded as a silent mutation if the analysis was performed using only genomic DNA. In RT-PCR analysis, adenine at nucleotide position 942 was not detected, suggesting

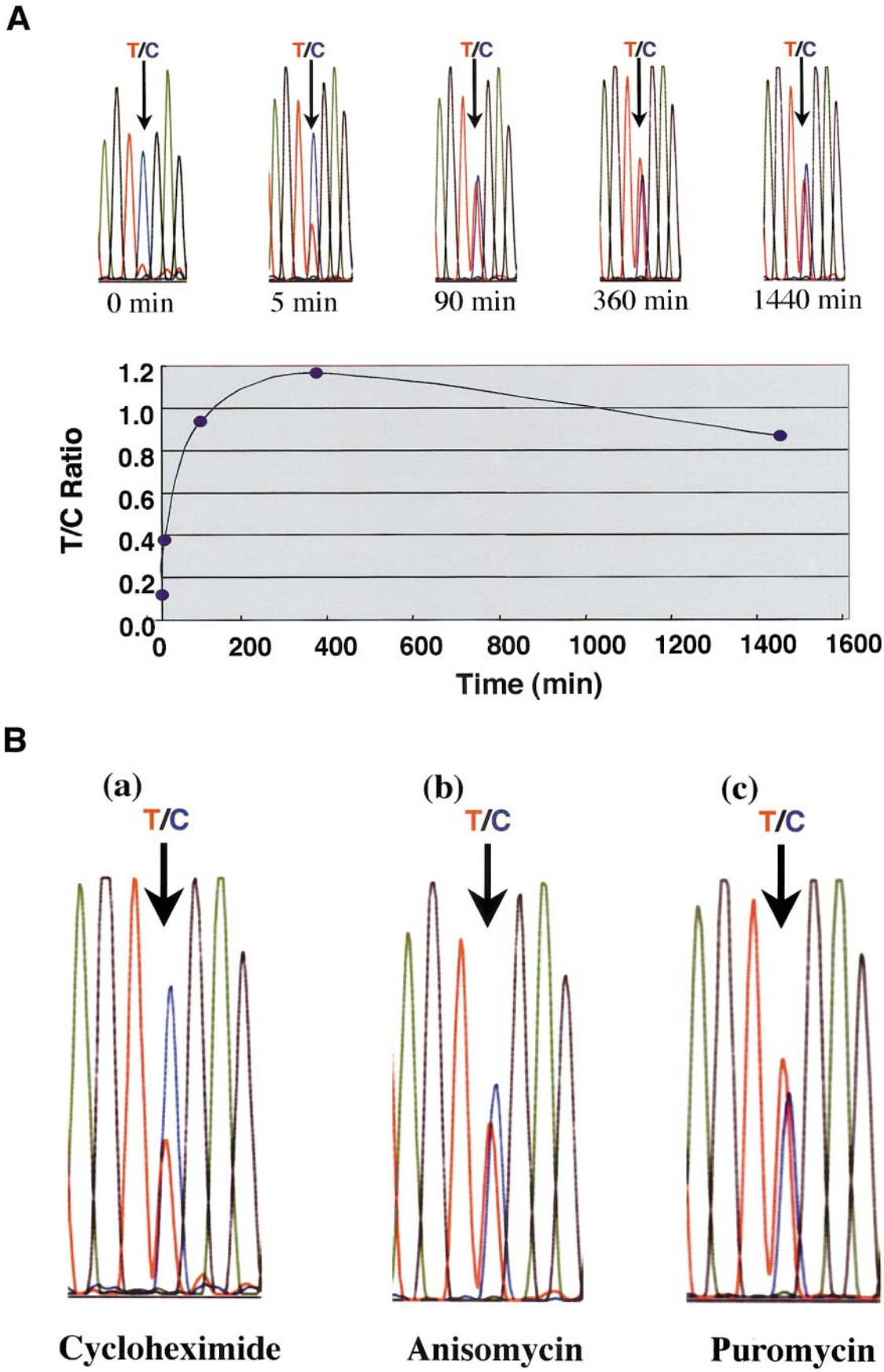

FIG. 3. Effect of protein translation inhibitors for NMD. Arrows indicate signals of mutation R226X. C (blue) indicates wild-type allele and T (red) indicates mutant allele. (A) Time course of puromycin effect for NMD. (B) Comparison of several translation inhibitors for suppressing NMD effect.

TABLE 2

Colorectal Cancer Patients with Gene Alterations of hMSH2 and/or hMLH1

Patient No.	Gene	Name[a]	Nucleotide change[b]	Effect on coding sequence	Location	Puromycin	Allele frequency[e]		Criteria
							Variant	Wild type	
43	hMSH2	1235del33	Deletion of 33 bp from 1235	In-frame deletion	EX7	ND[d]	ND		JB
92	hMSH2	M688I	G → A at 2064	Met → Ile at 688	EX13	ND	0 (0.0)	158 (100)	JB
175	hMSH2	471C → A	C → A at 471	No change Gly 157	EX3	ND	5 (2.8)	175 (97.2)	JB
331	hMSH2	972G → A	G → A at 972	No change Gln 324	EX6	+	0 (0.0)	142 (100)	JB
339	hMSH2	1705delGA[c]	Deletion of GA at 1705–1706	Frameshift	EX11	+	ND		AM
344	hMSH2	T8M	C → T at 23	Thr → Met at 8	EX1	+	2 (1.0)	198 (99.0)	JB
350	hMSH2	T8M	C → T at 23	Thr → Met at 8	EX1	+	same as No. 344		JB
350	hMSH2	K845E	A → G at 2533	Lys → Glu at 845	EX15	+	0 (0.0)	130 (100)	JB
352	hMSH2	223delCT	Deletion of CT at 223–224	Frameshift	EX2	+	ND		JB
354	hMSH2	942G → A	G → A at 942	Splice mutation	Ex5	+	ND		JB
45	hMLH1	V384D[c]	T → A at 1151	Val → Asp at 384	EX12	ND	5 (2.8)	175 (97.2)	JB
92	hMLH1	I219V[c]	A → G at 655	Ile → Val at 219	EX8	ND	5 (3.8)	127 (96.2)	JB
319	hMLH1	L588P	T → C at 1763	Leu → Phe at 588	EX16	+	0 (0.0)	200 (100)	AM
331	hMLH1	R226X[c]	C → T at 676	Arg → Stop at 266	EX8	+	ND		JB
355	hMLH1	A111V	C → T at 332	Arg → Val at 111	Ex4	+	0 (0.0)	140 (100)	AM

[a] Nomenclatures are defined according to the guideline proposed by Antonarakis (23).
[b] Position 1 is the first letter of the initiation codon.
[c] Previously reported.
[d] ND, not done.
[e] Figures in parentheses indicate the percentage of the examined allele.

that the mRNA species derived from the variant allele lacked exon 5, and that the 942G → A mutation caused for this exon skipping.

In pedigrees that did not qualify for the Amsterdam criteria, the frequencies of the germline mutations were still controversial. In the present study, 4 kindreds met the Amsterdam criteria; 3 (75%) showed germline mutations for hMSH2 or hMLH1 and at least 1 (25%) was considered to be pathogenic causing a truncated protein. Of the 11 kindreds satisfying the Japanese clinical criteria, 10 mutations were found in 10 kindreds constituting Group B, while there was no mutation in 1 kindred in Group A. Except for 4 polymorphic DNA variants, 6 kindreds showed germline mutations (55%) and at least 4 (36%) caused a truncated protein; one nonsense mutation, one frameshift mutation and two in-frame deletions (Table 2). Wijnen *et al.* analyzed a total of 125 HNPCC families using DGGE. In their analysis, 49% (n = 86) of the families satisfying the Amsterdam criteria had germline mutations of either hMSH2 or hMLH1, while only 8% (n = 39) of those incompatible with the Amsterdam criteria showed germline mutations (33). Other studies reported that the frequencies of germline mutations in hMSH2 or hMLH1 in incomplete HNPCC families were 44% (n = 9) (27) and 60% (n = 10), respectively (34). Nakahara *et al.* examined the frequencies of hMSH2 and hMLH1 in Japanese kindreds fulfilling the Japanese criteria and reported that germline mutations were detected in 4 of 7 (67%) Japanese HNPCC kindreds not fulfilling the Amsterdam criteria, although 3 of 4 (75%) were missense mutations (35). Frequencies of germline mutations in this study were high, considering that only 4 of 15 kindreds were eligible for the Amsterdam criteria and the analysis of hMSH2 and hMLH1 was indicated in diagnosis of HNPCC for kindreds not fulfilling the Amsterdam criteria.

Mutation screening based on long RT-PCR is a high-throughput approach, which enables direct sequencing of the entire coding regions of these 2 genes from small amounts of the template obtained in a single PCR in a few days. All 3 deleterious mutations were found in analysis of the 8 kindreds undergoing puromycin treatment, and the number of the other mutations found in this study indicates the usefulness of long RT-PCR/direct sequencing analysis combined with puromycin treatment. Further studies are in progress to elucidate the pathogenecity of the missense mutations detected in the HNPCC kindreds through segregation analysis of the pedigrees.

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