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ARTICLE

Concurrent genetic alterations in DNA polymerase proofreading and mismatch repair in human colorectal cancer

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Genomic sequences encoding the 3′ exonuclease (proofreading) domains of both replicative DNA polymerases, pol delta and pol epsilon, were explored simultaneously in human colorectal carcinomas including six established cell lines. Three unequivocal sequence alterations, including one previously reported, were found, and all these were considered as dysfunctional mutations in light of the local amino-acid sequences. In particular, the F367S mutation found in the *POLE* gene encoding the pol epsilon catalytic subunit, which includes the proofreading domain, is the first found in human diseases. Surprisingly, the tumours carrying these proofreading domain mutations were all defective in DNA mismatch repair (MMR). In addition to the two cell lines with acknowledged MMR gene mutations, the third tumour was also demonstrated to harbour a distinct mutation in *MLH1*, and indeed exhibited a microsatellite-unstable phenotype. These findings suggest that, in concert with MMR deficiency, defective polymerase proofreading may also contribute to the mutator phenotype observed in human colorectal cancer. Our observations may suggest previously unrecognised complexities in the molecular abnormalities underlying the mutator phenotype in human neoplasms.
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INTRODUCTION

Mutation rates on the genome are invariably regulated and typically suppressed to an extremely low level, such as 10^{-10} per base replicated, in the organisms.[1] The high fidelity of DNA replication is achieved by several molecular mechanisms. The frequency of erroneous nucleotide incorporation is indeed extremely low compared with that expected from base-pairing energetics, and the vast reduction of the misincorporation frequency involves three steps of molecular events: (a) correct incorporation of complementary nucleotides by DNA polymerases, (b) removal of the newly added nucleotides, particularly incorrectly paired nucleotides, by the 3′ exonuclease activities associated with the DNA polymerases (proofreading) and (c) postreplicative scanning of mispaired bases by DNA mismatch repair (MMR).[2,3] Various *Escherichia coli* (*E. coli*) mutator strains have been used to study these molecular mechanisms by which organisms maintain their mutation rates at very low levels. The *dnaQ*$^+$ (*mutD*$^+$) gene of *E. coli* encodes the epsilon subunit of the DNA polymerase III holoenzyme that is involved in 3′ exonuclease proofreading activity,[4] and, therefore, the mutation frequencies in the *dnaQ* mutators are sometimes 1000–10 000 times higher than the wild-type levels.[4,5] In *E. coli*, MMR is chiefly directed by the proteins encoded by the three genes: *mutS*$^+$, *mutL*$^+$ and *mutH*$^+$.[6] The *mutS* or *mutL* mutators exhibit an approximately 100 times increase in the mutation frequency.[7] Thus, polymerase proofreading and MMR are the two major systems that counteract the replication errors caused by the DNA polymerases and, consequently, guarantee the high fidelity of DNA replication on the genome.

In 1993, mutations in the human homologues of the *E. coli* MMR genes were reported in the familial cancer-prone syndrome, hereditary non-polyposis colorectal cancer (HNPCC).[8,9] The cellular state with an elevated mutation rate, that is, the 'mutator phenotype',[10] has since then received attention as a cause for tumourigenesis. Indeed, tumours frequently arise in MMR gene-knockout mice[11,12] and the mutation rates in the MMR-defective mouse cells are known to be markedly increased.[11,13] Human cell lines with an acknowledged defect in the MMR genes also exhibit significantly elevated mutation rates.[14–17] On the other hand, defects in polymerase proofreading have not been documented in human populations. Various types of DNA polymerases have been identified in diverse species to date. Studies on the *in vitro* replication of SV40 DNA have elucidated that pol alpha and delta have essential roles in chromosomal DNA replication in higher eukaryotic cells.[18] Pol alpha functions at the replication folks and initiates DNA replication in the both leading- and lagging strands, synthesising the RNA primers and short stretches of DNA. Pol delta and epsilon elongate the DNA. As pol epsilon possesses a relatively high processivity, it is now widely accepted that in eukaryotic cells pol delta and epsilon are responsible for the lagging- and leading-strand synthesis, respectively,[19] although pol epsilon has been demonstrated not to be essential in yeast.[20] The catalytic subunits of pol delta and epsilon,

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for example, p125[21–23] and p261[24,25] in mammals, include the 3′ exonuclease, that is, proofreading, domains. Indeed, in the yeast mutants in which pol delta or epsilon proofreading is selectively inactivated, the mutations rates are 10–100 times higher than the wild-type strains.[26–28] Recently, mice carrying artificial alleles with substitutions at essential amino-acid residues in the proofreading domains of pol delta[29,30] and epsilon[31] have been reported. The observed mutation rates were indeed significantly elevated over the wild-type levels.[31] Intriguingly, tumours arose in various organs of the animals.[31] Defective polymerase proofreading warrants particular attention in human cells. However, the genomic sequences that encode proofreading domains of the both replicative DNA polymerases, pol delta and epsilon, have not previously been explored simultaneously in human diseases, particularly in human neoplasms, although the *POLD1* gene encoding the p125 subunit of pol delta has been sequenced in several human colorectal tumours.[32] In this present study, we first sequenced both the genomic sequences encoding the pol delta and epsilon proofreading domains in a large panel of human colorectal carcinomas, including six established human colorectal cancer cell lines, and found novel mutations in both the genes. In particular, the mutation in the *POLE* gene that encodes p261 catalytic subunit of pol epsilon is the first found in human cells. Furthermore, we found common and unique molecular characteristics in the proofreading-mutant tumours. Our findings may suggest previously unrecognised complexities in the molecular abnormalities underlying the mutator phenotype in human tumours.

MATERIALS AND METHODS

Patients and tissue specimens

Cancer tissues and the corresponding normal tissues were collected from 76 consecutive patients who underwent surgery in the Department of Surgery and Science, Kyushu University Hospital from 1994 to 1999. Ethical approval for this study was obtained from the IRB of Kyushu University. Specimens were taken immediately after resection and placed in liquid nitrogen. High-molecular weight chromosomal DNA was extracted and subjected to the analyses.

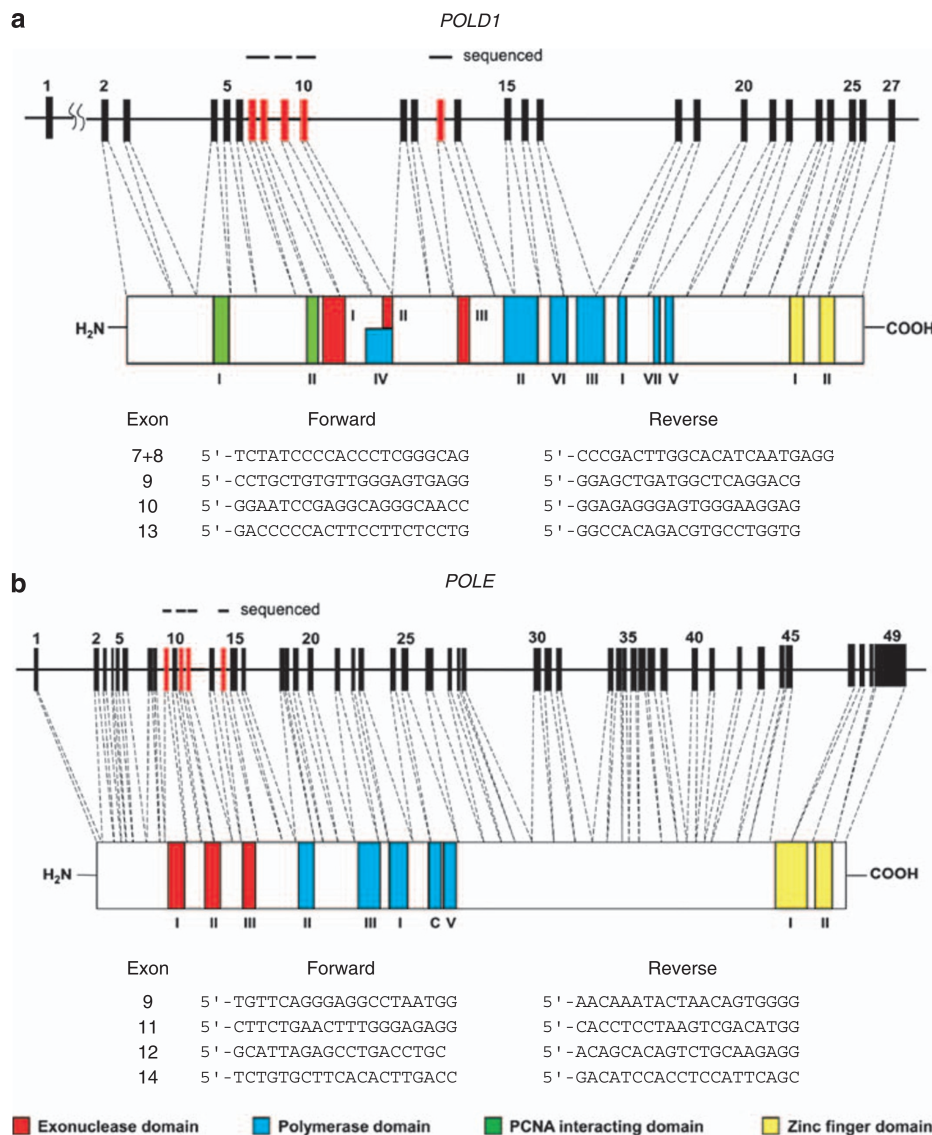

Figure 1 The genomic structures of the *POLD1* (**a**) and *POLE* (**b**) genes and the functional domains in their protein products. The exons corresponding to the 3′ exonuclease, that is, proofreading, domains are indicated in red and the genomic regions sequenced in this study by bar. The oligonucleotide primers used are also shown.

Cell culture

Human colorectal adenocarcinoma cell lines, HCT116, SW620, SW48 and HT29, were distributed by the American Type Cell Culture (Rockville, MD, USA). LoVo and DLD-1 were obtained from the Japanese Cancer Research Bank (Tokyo, Japan). HCT116 and HT29 were maintained in MacCoy's 5a medium. For SW620 and SW48, L15 medium was used. DLD-1 and LoVo cells were maintained in RPMI 1640 and F12, respectively. All the media were purchased from Life Technologies, Inc. (Rockville, MD, USA) and supplemented with 10% foetal calf serum.

DNA sequencing

The 2.5-kbp genomic sequences in the *POLD1* and *POLE* genes that encompass the regions encoding the proofreading domains of pol delta and epsilon (Figure 1) were amplified by PCR using *Taq* polymerase with the 3′ exonuclease activity, *EX Taq* (TaKaRa Bio Inc., Otsu, Japan), and the oligonucleotide primers shown in Figure 1. PCR products were directly used as a template for cycle sequencing reactions using BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA), and the reaction products were loaded onto ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Mutations found in one PCR product were verified by reverse sequencing and finally confirmed in three independently amplified PCR products. Primer sequences for sequencing analyses of the *MLH1* gene were the same as reported by Kolodner *et al*,[33] except that the additional sequence complementary for M13 universal primer was deleted. The *KRAS* sequence was analysed as described in our previous report.[34] Sequencing analyses of *TP53* (exons 5–9) were performed using *p53* primers (Nippon Gene, Tokyo, Japan). Mutations found were similarly confirmed in three independently amplified PCR products.

Table 1 Clinicopathological features of 76 colorectal cancer patients

Variables	*Number*
Sex	
Male	42
Female	34
Age (years)	62.4
Location	
Ascending colon	23
Transverse colon	6
Descending colon	7
Sigmoid colon	18
Rectum	22
Stage	
0/I	11
II	22
IIIa/IIIb	30
IV	13
Histology	
Well differentiated adenocarcinoma	44
Moderately differentiated adenocarcinoma	22
Poorly differentiated adenocarcinoma	7
Other	3
Depth	
Mucosa	1
Submucosa	3
Musculiris propia	13
Subserosa	31
Serosa	20
Invasion to surrounding tissues	8
Lymph node metastasis	
Positive	38
Negative	38
Lymphatic invasion	
Positive	28
Negative	48
Venous invasion	
Positive	20
Negative	56

Microsatellite instability

Microsatellite analysis using fluorescence-labelled primers and an automated DNA sequencer has been described in detail.[35] Five human dinucleotide microsatellites, D2S123, D5S107, D10S197, D11S904 and D13S175, were used as markers. PCR products of the microsatellite sequences were analysed using ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Data were processed by ABI software, GeneScan ver. 3.1.2. (Applied Biosystems).

RESULTS

Mutations found in the genomic sequences encoding the proofreading domains of pol delta and epsilon in human colorectal cancer

High-molecular weight genomic DNA was extracted from 6 established human colorectal cancer cell lines and clinically obtained carcinoma tissue specimens derived from a panel of 76 colorectal cancer patients that is representative of the average patient population with this malignancy (Table 1). Five and four exons of human *POLD1* and *POLE* genes, respectively, that encompass the genomic regions encoding the 3′ exonuclease, that is, proofreading, domains of pol delta and epsilon were amplified by PCR and sequenced by direct cycle sequencing (Figure 1a and b). Sequence alterations causing amino-acid substitutions were identified in two cell lines and one tissue sample (Figure 2, Table 2),[36–43] and these sequence alterations were confirmed in three independently amplified PCR products by more than three cycle sequencing reactions. Other types of sequence alterations such as insertions/deletions were not found. Among these sequence alterations, *POLD1* R506H in DLD-1 cells has been previously reported[32,44] and therefore is not novel. On the other hand, *POLD1* V312M in LoVo cells has not thus far been reported in the literature. In particular, *POLE* F367S in the IC678 tumour is the first identified sequence alteration of the *POLE* gene in human diseases.

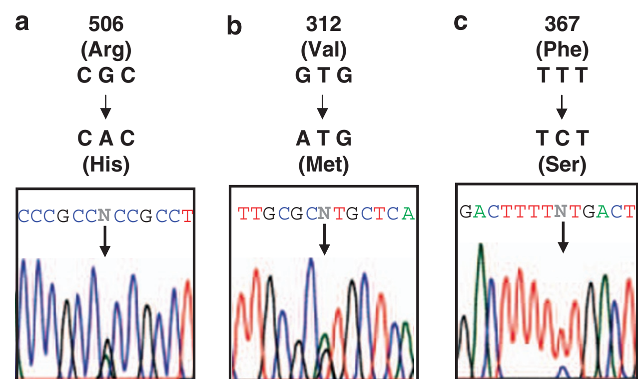

Figure 2 *POLD1* and *POLE* mutations in human colorectal tumours. Sequence alterations found at codon 506 (DLD-1) (**a**) and 312 (LoVo) (**b**) of the *POLD1* gene and codon 367 (IC678) (**c**) of the *POLE* gene are shown. Arrows indicate base changes.

Table 2 Mutations found in proofreading-mutant colorectal carcinomas

Subject	Gene	Exon	Nucleotide position	Codon change	MSI	MMR gene	TP53	KRAS	APC
Cell line									
DLD-1	*POLD1*	13	1517	(CGC)His506Arg(CAC) (G:C→A:T ts)	+	*MSH6*: Leu222stop[36,37] (C del) *MSH6*: Asp1103Cys-1106stop[36,37] (GATAGA→T)	Ser241Phe[38,39] (G:C→A:T ts)	Gly013Asp[38] (G:C→A:T ts)	Arg2166stop[42,43] (G:C→A:T ts) Ile1417Leu-1418stop[42,43] (C del)
LoVo	*POLD1*	8	934	(GTG)Val312Met(ATG) (G:C→A:T ts)	+	*MSH2*: exons 3–8 del[37]	wt[39,40]	Gly013Asp[40,41] (G:C→A:T ts)	Arg1114stop[43] (G:C→A:T ts) Thr1431Thr-1472stop[43] (C del)
Tissue									
IC 678	*POLE*	11	1110	(TTT)Phe367Ser(TCT) (A:T→G:C ts)	+	*MLH1*: Leu521Ile (G:C→T:A tv)	wt	wt	wt

Abbreviations: del, deletion; MSI, microsatellite instability; ts, transition; tv, transversion; wt, wild type.

POLD1 has previously been explored in a limited number of human colorectal carcinoma tissue specimens, and no sequence alterations causing amino-acid substitutions were found in the genomic sequences encoding the proofreading domain.[32] Similarly, we identified no *POLD1* alterations in a relatively large panel of colorectal cancer patients.

To discuss the significance of these sequence alterations, we compared the local amino-acid sequences among several different organisms (Figure 3). Arginine residues at codon 506 of the *POLD1* gene are highly conserved from yeast to mammals[21,44] and, therefore, R506H in DLD-1 cells is regarded as impairing the protein function and, consequently, as a dysfunctional mutation.[44] Valine residues at codon 312 are also conserved between mouse and human, although differing in yeast.[21,45,46] Intriguingly, phenylalanine residues of the first identified sequence alteration in *POLE*, F367S, is highly conserved in these four organisms,[47] suggesting that F367S is dysfunctional and possibly pathogenic. We thus found two novel and one known mutations in the genomic sequences encoding the proofreading domains of pol delta and epsilon in two human colorectal cancer cell lines and one colorectal carcinoma tissue. These mutations have not been confirmed using genomic DNA extracted from the corresponding normal tissues. The IC678 tumour carrying the *POLE* mutation was derived from a male rectal cancer patient without any remarkable clinicopathological features.

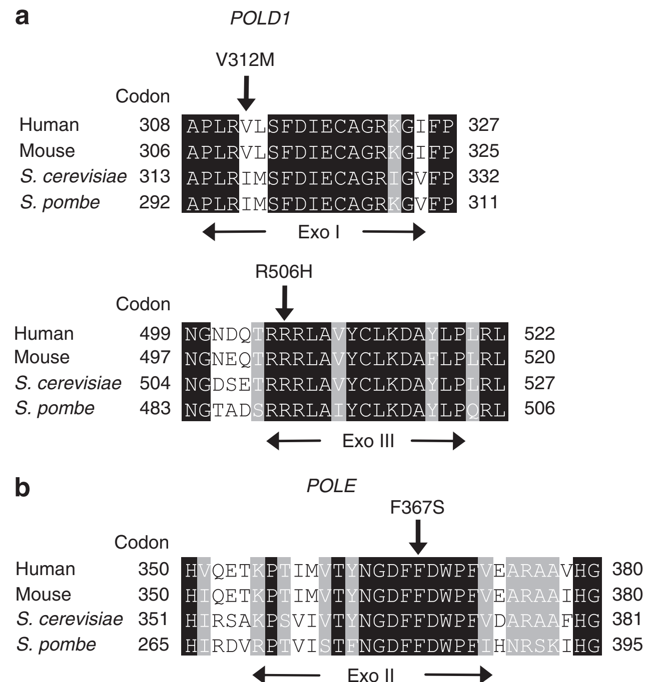

Figure 3 Comparison of the local amino-acid sequences encoded by *POLD1* (**a**) and *POLE* (**b**) among the different species. Amino-acid substitutions found in human colorectal tumours are indicated by vertical arrows.

MMR deficiency in proofreading-mutant colorectal carcinomas

One important common characteristic of DLD-1 and LoVo cells is that both cell lines are defective in MMR. A large deletion involving from exons 3–8 of one of the essential human MMR genes, *MSH2*, has been reported in LoVo cells (Table 2) and, therefore, LoVo cells have been regarded as MMR-deficient. Indeed, LoVo cells exhibit a remarkable phenotype with microsatellite instability (MSI),[40] a hallmark genomic change observed in MMR-defective cells. On the other hand, DLD-1 cells are known to harbour a 1-bp deletion mutation at codon 222 of one allele of another important MMR gene, *MSH6*, and a sequence deletion/insertion involving 5-bp in the other allele (Table 2). As a consequence, MSH6 protein is not expressed in DLD-1 cells.[36,48] Intriguingly, the IC678 tumour also exhibits a MMR-defective phenotype.[49] As shown in Figure 4a, alterations in a human dinucleotide microsatellite, D13S175, were readily detected in the genomic DNA extracted from this tumour. Indeed, a sequence alteration resulting in an amino-acid substitution was found at codon 521 of another essential MMR gene, *MLH1* (Figure 4b).[49] As normal leucine residues in this codon are conserved from yeast to human,[50] this sequence alteration, L521I, may be regarded as a dysfunctional mutation. Thus, all the three subjects that carry *POLD1* or *POLE* proofreading domain mutations indicate MMR deficiency.

We further sequenced the *APC*, *KRAS*, *TP53* genes, the alterations of which are known to play crucial roles in colorectal tumourigenesis,[51–53] in the IC678 tumour. However, we found no detectable

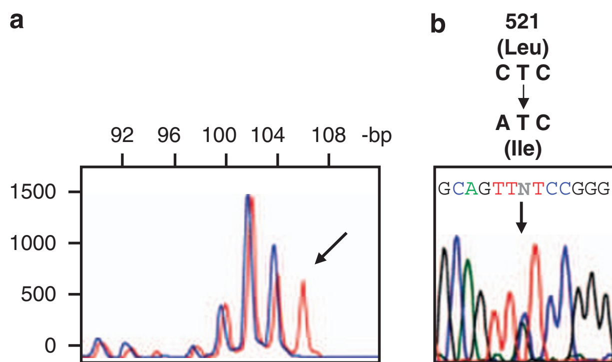

Figure 4 The proofreading-mutant human colorectal tumour, IC678, exhibits a microsatellite-unstable phenotype and harbours a mutation in a DNA *MMR* gene. (**a**) In the genomic DNA samples prepared from the tumour and the corresponding normal tissues, the human microsatellite sequences were amplified by PCR using differentially fluorescence-labelled primers and electrophoresed simultaneously in an automated sequencer. Results obtained in D13S175 are shown: red lines, cancer; and blue lines, normal mucosa. Fragment length alteration indicating microsatellite instability is evident (pointed by arrow). (**b**) Sequencing analyses revealed a base substitution in *MLH1*.

sequence alterations in these genes (Table 2), whereas several mutations have been reported in DLD-1 and LoVo cells and, in particular, DLD-1 cells harbour mutations in all these three genes (Table 2).

DISCUSSION

The genomic sequence of *POLD1* has previously been examined in six human colorectal cancer cell lines and seven clinically obtained colorectal carcinoma tissues, and several sequence alterations, including possible polymorphisms, were reported.[32] However, apart from the previously reported R506H mutation in DLD-1 cells, no sequence alterations resulting in amino-acid substitutions were found in the genomic regions encoding the proofreading domain. In this present study, using a relatively large panel of colorectal carcinomas, we first elucidated that *POLD1* proofreading domain mutations were indeed infrequent in colorectal cancer patients. Nevertheless, in addition to the previously reported R506H mutation in DLD-1 cells, we found a novel *POLD1* mutation in LoVo cells. LoVo was not included in the panel of human colorectal cancer cell lines in the above mentioned report.[32] On the other hand, mutations in the *POLE* gene have never been reported in human individuals, although Matakidou *et al* pointed out the correlation between a *POLE* single-nucleotide polymorphism and poor patient outcomes in lung cancer.[54] In this context, our present study is the first to report *POLE* mutation in human cells, and, therefore, F367S is the first identified *POLE* mutation in human diseases.

One important finding of this study is that all the three proofreading-mutant tumours are MMR deficient. DLD-1 and LoVo cells have been known as MMR-defective cell lines.[36,37,55] In the genomic DNA extracted from the IC678 tumour, an unequivocal MSI phenomenon was observed and a deleterious *MLH1* mutation was indeed found. As discussed above, the abrogation of either polymerase proofreading or MMR inevitably leads to an immense increase of mutations derived from the replication errors left on the genome. In this sense, concurrent defects of both systems are of particular interest, and one important problem is the causal relationship between the two molecular abnormalities. In general, the mutation spectra observed in cells defective in an anti-mutagenic system are known to be distinct depending on the system abrogated. It is now widely accepted that transitions, in particular G:C to A:T, are predominant in cells defective in MMR.[16,56,57] On the other hand, A:T to C:G transversions predominate in proofreading-mutant mouse cells.[31] In *E. coli dnaQ* mutators, all types of base substitutions, in addition to frameshifts, are known to increase dramatically. Among these base substitutions, A:T to G:C transitions are relatively frequent.[58] The two *POLD1* mutations in DLD-1 and LoVo cells were G:C to A:T transitions, and the *POLE* mutation found in the IC678 tumour was also transition (Table 2). On the other hand, *MSH6* mutations in DLD-1 cells are a 1-bp deletion, that is, frameshift mutation, and a large sequence deletion/insertion. Although the former is indeed frequent in MMR-defective cells, the latter is exceptional. The large deletion involving from exons 3–8 of *MSH2* in LoVo cells is also not explicable from MMR deficiency. Considering these facts, it may be more likely that the *POLD1* and *POLE* mutations are the results of MMR deficiency than that defective polymerase proofreading has caused the mutations in the MMR genes. Furthermore, in this present study, we demonstrated that proofreading domain mutations were relatively infrequent in human colorectal cancer, whereas defective MMR is known to be frequently observed.[59] This may also support the above hypothesis.

This problem can also be discussed from another point of view. The mutator phenotype derived from defects in the anti-mutagenic systems is believed to underlie tumourigenesis, causing the accumulation of mutations in various genes including oncogenes and tumour suppressor genes.[60] *APC*, *KRAS* and *TP53* are the representative genes, the alterations of which are regarded as playing crucial roles in the step-by-step tumourigenesis in the human colorectal epithelia.[51–53] Intriguingly, DLD-1 cells, which are defective in both polymerase proofreading and MMR, harbour mutations in all these genes, and the mutations are all G:C to A:T transitions (Table 2). Also in LoVo cells with the *POLD1* mutation, *APC* and *KRAS* are mutated, and the sequence alterations are G:C to A:T transitions (Table 2). Taking all these into account, it may be suggested that MMR deficiency contributes more than defective polymerase proofreading does to the mutator phenotype observed in human tumours.

The reason why proofreading domain mutations were infrequent in colorectal tumours is unknown. Needless to say, the possibility that mutations in small cell populations were not detected is not excluded. However, considering the facts that the mutations rates in the *dnaQ* mutators are two orders higher than those observed in MMR-defective *E. coli* mutators,[4,5] such an extreme increase in the mutation rate may be toxic or, at least, disadvantageous for cells to survive. Indeed, the mutation rates are not widely different between DLD-1 cells, defective in both MMR and polymerase proofreading, and the other MMR-deficient human cell lines.[14,16,17] In addition, the mutation rates observed in proofreading-defective mouse cells are at the same level as those in MMR gene-knockout mouse cells.[31] Mutations occurring in the genomic sequences encoding the proofreading domains may be in fact selected according to their survival disadvantage, particularly in naturally occurring tumours. Indeed, the cell population harbouring the *POLE* F367S mutation is not predominant in the IC678 tumour (Figure 2c). Nevertheless, defective polymerase proofreading may contribute to the mutator phenotype as a 'booster' of MMR deficiency. In this context, the phenotypes of animals (or, possibly, cells) defective in both polymerase proofreading and MMR are of particular interest.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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