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Mitochondrial DNA Alterations in Colorectal Cancer Cell Lines

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Abstract

Somatic mutations of mitochondrial DNA (mtDNA) have been reported in different types of cancers and are suggested to play roles in metastasis, cancer development and response to anticancer agents. To predict potential roles of mtDNA alterations in colorectal cancer, we determined the entire mtDNA sequence of eleven human-derived colorectal cancer cell lines and compared with the revised Cambridge Reference Sequence to identify nucleotide alterations. Four homoplasmic and six heteroplasmic alterations were found to be novel. Among them, homoplasmic G6709A (*MT-CO1*) and G14804A (*MT-CYB*) alterations cause amino acid changes in the highly conserved residues. Heteroplasmic G1576A (*MT-RNR1*) and G2975A (*MT-RNR2*) alterations are expected to make the stem structure of mitochondrial ribosomal RNAs unstable. These nucleotide alterations are candidates that could play important roles in cancer.
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Key words: mtDNA, colorectal cancer, mitochondria, nucleotide sequence

Introduction

Mammalian cells have two genomic systems; the nuclear and mitochondrial genomic systems. The mtDNA is a 16,568 base-pair (bp), double-stranded, circular DNA molecule that contains genes coding for 13 polypeptides involved in respiration and oxidative phosphorylation, and 2 rRNAs and a set of 22 tRNAs essential for protein synthesis in mitochondria[1]. The mtDNA has also non-coding region, which is called the control region, containing the replication origin and the promoters for transcription[1].

Colorectal cancer is one of the most common causes of death of human malignancies throughout the world (Centers for Disease Control and Prevention, http://www.cdc.gov/cancer/colorectal/). Recently, mutations of mitochondrial DNA (mtDNA) have been reported to be frequently observed in different types of cancers[2], including colorectal carcinomas[3,4]. Mitochondria are important cellular

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organelle responsible for energy production and also play key roles in generation of reactive oxygen species (ROS) and regulation of apoptosis. These factors have been implicated in the development of cancers [5]. Mitochondrial DNA encodes key components of mitochondrial functions, and those mutations modulate mitochondrial functions. Although it is unclear whether mtDNA mutations found in tumor tissue are causes or consequences of cancer developments, it is plausible that mtDNA mutations may contribute cancer developments. Indeed, close attention is currently being paid to the contribution of somatic mtDNA mutations in the development of cancer and tolerance to anticancer drugs[6]. In addition, Some mtDNA mutations, which affect mitochondrial ROS generation, determine the metastasis potential[2,7].

To find potential roles of mtDNA alterations in colorectal cancer, we determined the entire mtDNA sequence of eleven human-derived colorectal cancer cell lines.

Materials and Methods

Cells and Cell Cultures

Ten colorectal cancer cell lines of human origin CCK-81, CoCM-1, COLO201, DLD-1, HCC-56, LoVo, OUMS-23, SW837, RCM-1 and WiDr were obtained from the Health Science Research Resources Bank (Osaka, Japan). DLD-1/5-FU, kindly obtained from Dr. Togo (Yokohama City University Graduate School of Medicine), is resistant to 5-FU and was established by repeated exposure of DLD-1 cells to escalating concentrations of 5-FU[8]. Cells were cultivated in DMEM/F-12 (Invitrogen, Carlsbad, CA) medium supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37℃ under an atmosphere of 5% CO_2/95% air.

Amplification of mtDNA and Sequencing

Cells cultured in dishes were washed with phosphate-buffered saline (PBS), and subjected to total DNA preparation using a QIAmp DNA Mini Kit (Qiagen, Hilden, Germany). Total DNAs were subjected to amplification of mtDNA by PCR using 28 pairs of primers designed by Taylor et al.[9], where these primers generate overlapping fragments of between 450 and 750 bp that span the entire sequence of the human mitochondrial genome and all primers are tagged with the forward or reverse M13 sequence for the direct sequencing of PCR-amplified products. When the amplification of some fragments was poor due to mutations in the primer sequences, the primer sequences were again designed according to the sequences of neighboring fragments. The amplified fragments were purified by a QIAquick PCR Purification Kit (Qiagen). DNA sequencing was performed using a BigDye terminator v3.1 Sequencing Standard Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). A whole region of mtDNA of 11 human colorectal cancer cell lines, including DLD-1/5-FU, was sequenced to identify mutations, and compared with the human mitochondrial DNA revised Cambridge reference sequence[1]. Poly-C length heteroplasmy caused by T310C and T16189C was ascertained by sequence electropherograms[10]. Mitochondrial (sub) haplogroups were determined by the web-based programs Mitomaster (http://mammag.web.uci.edu/bin/view/Mitomaster/WebHome)[11] and Haplogroup Finder (http://www.ianlogan.co.uk/haplogroup/finder.htm). Mitomaster also provide information about the protein coding gene variants: the affect on amino acid sequence and the interspecific species conservation index (CI) of the mutated amino acid. Mitochodrial DNA polymorphisms relating (sub) haprogroups were excluded according to the huge Mitomap phylogenetic tree (http://www.mitomap.org/mitomap-phylogeny.pdf) and Ian Logan's haprogroup descriptions (http://www.ianlogan.co.uk/mtdna.htm). A novel mtDNA variant search was performed according to Bandelt et al.[12].

Determination of Heteroplasmic Mutation Load

Heteroplasmic mutations were detected by sequenceing analysis (as above). G1576A and 3060A mutations cause a *Bst*1107I site and a *Bsa*AI site loss, respectively. Digested PCR products were quantified to evaluate their mutation load. For the other heteroplasmic mutations, we designed mismatch PCR primers to lead to restriction enzyme site gain

Table 1 The sequences of PCR primers and restriction enzymes to quantify heteroplasmic mutation load. Mismatched bases are indicated by underline

Heteroplasmic mutation	Forward primer (5'-3')	Reverse primer (5'-3')	Restriction enzyme
G2975A	CCAATAACTTGACCAACGGA	TGTCCTGATCCAACATCGAGGTCGTAAAGC	*Hind*Ⅲ
G8873A	ACTAACCTCCTCGGACTCCT	GCTAGGGCATTTTTAATCTTAGAGCGAAGG	*Stu*I
T9510C	TTCTTCGCAGGATTTTTCGAAGCCTTT	CTTCTAGGGGATTTAGCGGG	*Xmn*I
C10581A	CCAAATGCCCCTCATTTACA	TTATGAGAGTAGCTATAATGAACAGCAATA	*Ssp*I

or loss by the mutations. The sequences of PCR primers and restriction enzymes are shown in **Table 1**.

Results

The entire mtDNAs of ten human colorectal cancer cell lines and a 5-FU-resistant derivative of DLD-1 (DLD-1/5-FU) were sequenced, compared with the revised Cambridge reference sequence (rCRS)[1], and determined their mitochondrial haplogroups. **Table 2** summarizes all nucleotides that are different from rCRS, except for mtDNA polymorphisms defining mitochondrial (sub) haplogroups. It is noted that the mtDNA sequence of DLD-1/5-FU is identical to that of DLD-1, indicating that the 5-FU resistance of DLD-1/5-FU is attributed to a nuclear genome mutation. Among them, 4 homoplasmic and 6 heteroplasmic variants were novel (nucleotides underlined in **Table 2**).

Three novel homoplasmic nucleotide alterations were found in protein coding region, two of which led to amino acid changes G6709A (G269E) in the *MT-CO1* gene in CCK-81 and G14804A (D20N) in the *MT-CYB* gene in LoVo (**Table 2A**). The conservation indexes (CI) of the mutated amino acids were both 1.00, suggesting that these amino acid residues are vital. Three novel heteroplasmic variants are located in protein coding region, G8873A (G116D) in the *MT-ATP6* gene in CoCM-1, T9510C (Y102H) in the *MT-CO3* gene in WiDr and C10581A (L38M) in the *MT-ND4L* gene in SW837, and their mutation percentages are 31%, 13% and 14%, respectively. All of them cause amino acid changes in highly conserved residues and their CI are 0.97, 0.95 and 0.95, respectively.

One novel homoplasmic alteration G1336A (DLD-1) and three novel heteroplasmic alterations G1576A (SW837), G2975A (LoVo) and C3060A (COLO201) are located in the ribosomal RNA genes (**Table 2B**). The heteroplasmic mutation percentages are 81%, 55% and 92%, respectively. G1336A, G1576A and G2975A are found in the stem region.

There are many alterations in the control region of mtDNA (nucleotide position (np.) 16023 − 576) (**Table 2C**). T310C (CCK-81) variant generates homopolymeric cytosine tract between np. 303 and 315, resulting poly-C length heteroplasmy. T16189C also causes poly-C length heteroplasmy in CCK-81 and OUMS23, but not in DLD-1 as C16186T interrupts homopolymeric cytosine stretch[13].

Discussion

In this study, we sequenced the entire mtDNA from 11 colon cancer cell lines that are widely distributed and used for *in vitro* study. The mtDNA sequences were compared with the revised Cambridge Reference Sequence (rCRS) used to evaluate somatic mtDNA mutations because matched non-cancerous cells were not available. The sequence determination concluded that mtDNA is not responsible for the resistance to 5-FU of DLD-1/5-FU, because the mtDNA sequence of DLD-1 and DLD-1/5-FU is completely identical. Polymorphisms relating mitochondrial haplogroups, which may or may not associated with cancer risk[14,15], were excluded in this study because our sample number is too small to evaluate such association.

The control region, especially D310 (np. 303–315), is a frequent hot spot of mutations in cancer, including colorectal cancer[16–22]. The control region is the major non-coding region containing the replication origin of the H-strand and the promoter

Table 2A Nucleotide alternations detected in coding regions of proteins

		cell line		rCRS	CCK-81	CoCM-1	COLO201	DLD-1	HCC-56	LoVo	OUMS23	RCM-1	SW837	WiDr
		haplogroup		H2	F1	D4a	V	T1	M7a	UK2	D5	D4	V	UK1
positon*	gene	amino acid	CI											
3672	*MT-ND1*	syn		A									G	
4732	*MT-ND2*	N88S	0.31	A	G									
5049	*MT-ND2*	syn		C	T									
5147	*MT-ND2*	syn		G	A									
5153	*MT-ND2*	syn		A							G			
5231	*MT-ND2*	syn		G						A				
5973	*MT-CO1*	A24T	0.92	G			A							
6146	*MT-CO1*	syn		A							G			
6709	*MT-CO1*	G269E	1.00	G	A									
7979	*MT-CO2*	D132N	0.31	G				A						
8873	*MT-ATP6*	G116D	0.97	G		A#								
9180	*MT-ATP6*	syn		A							G			
9254	*MT-CO3*	syn		A			G						G	
9299	*MT-CO3*	syn		A					G					
9368	*MT-CO3*	syn		A			G						G	
9377	*MT-CO3*	syn		A							G			
9510	*MT-CO3*	Y102H	0.95	T										C#
9554	*MT-CO3*	syn		G							A			
9899	*MT-CO3*	syn		T				C						
9932	*MT-CO3*	syn		G									A	
10581	*MT-ND4L*	L38M	0.95	C								A#		
10976	*MT-ND4*	syn		C	T									
10978	*MT-ND4*	syn		A										G
11017	*MT-ND4*	syn		T					C					
11084	*MT-ND4*	T109A	0.87	A					G					
11215	*MT-ND4*	syn		C								T		
11266	*MT-ND4*	syn		C				T						
11470	*MT-ND4*	syn		A										G
11722	*MT-ND4*	syn		T					C					
11781	*MT-ND4*	I341T	0.26	T			C							
11869	*MT-ND4*	syn		C						A				
11914	*MT-ND4*	syn		G										A
12358	*MT-ND5*	T8A	0.33	A								G		
12507	*MT-ND5*	syn		A						G				
12633	*MT-ND5*	syn		C	T									
12954	*MT-ND5*	syn		T										C
12975	*MT-ND5*	syn		A		G								
13135	*MT-ND5*	A267T	0.10	G						A				
13614	*MT-ND5*	syn		A							G			
13617	*MT-ND5*	syn		T							C			
13928	*MT-ND5*	S531T	0.10	G	C									
14037	*MT-ND5*	syn		A						G				
14364	*MT-ND6*	syn		G					A					
14476	*MT-ND6*	syn		G	A									
14804	*MT-CYB*	D20N	1.00	G						A				
15724	*MT-CYB*	syn		A							G			
15769	*MT-CYB*	Q341H	0.92	A							C			
15874	*MT-CYB*	syn		A								G		

*The nucleotide numbering system is based on the human mitochondrial revised Cambridge Reference Sequence.

#Heteroplasmic alternations. The mutation percentages of G8873A (CoCM-1) , T9510C (WiDr) and C10581A (RCM-1) are 31%, 13% and 14%, respectively.

Nucleotides underlined are novel alterations.

Table 2B Nucleotide alternations detected in coding regions of ribosomal and transfer RNAs

	cell line	rCRS	CCK-81	CoCM-1	COLO201	DLD-1	HCC-56	LoVo	OUMS23	RCM-1	SW837	WiDr
	haplogroup	H2	F1	D4a	V	T1	M7a	UK2	D5	D4	V	UK1
positon*	gene											
681	*MT-RNR1*	T							C			
789	*MT-RNR1*	T				C						
983	*MT-RNR1*	C							T			
1048	*MT-RNR1*	C							T			
1187	*MT-RNR1*	T								C		
1336	*MT-RNR1*	G				A						
1413	*MT-RNR1*	T										C
1576	*MT-RNR1*	G									A#	
1676	*MT-RNR2*	A			G							
2217	*MT-RNR2*	C						T				
2222	*MT-RNR2*	T			C							
2975	*MT-RNR2*	G						A#				
3060	*MT-RNR2*	C			A#							
5539	*MT-TW*	A							G			
10410	*MT-TR*	T		C								
15924	*MT-TT*	A										G

*The nucleotide numbering system is based on the human mitochondrial revised Cambridge Reference Sequence.
#Heteroplasmic alternations. The mutation percentages of G1576A (SW837) , G2975A (LoVo) and C3060A (COLO201) are 81%, 55% and 92%, respectively.
Nucleotides underlined are novel alterations.

for transcription of the L-strand and synthesis of the RNA primer for the H-strand; therefore, sequence alterations in the control region may alter the copy number and/or gene expression of mtDNA[23]. Indeed, some studies showed that mutations of the control region in tumors increased or decreased the copy number of mtDNA[24–26]. T310C found in CCK-81 was reported in gallbladder[22], colorectal[27], and esophageal cancer[25]. The D310 sequence of all cells tested, except for CCK-81, did not alter the C-stretch identified by comparison with that of normal tissues, which are also within the normal polymorphic range[18–22].

T16189C generates a polymeric cytosine tract between np. 16184 and 16193, resulting in poly-C length heteroplasmy. An alternative mtDNA replication model proposed that a novel origin of mtDNA replication is located much closed to np. 16189[28] and T16189C may affect copy number of mtDNA. The poly-C length heteroplasmy found in CCK-81 and OUMS23, but not in DLD-1 as C16186T interrupts homopolymeric cytosine stretch [13]. T16189C somatic mutation was reported in prostate[17], endometrial and breast cancers[29]. T16189C polymorphism has been reported to be associated with type 2 diabetes mellitus[30,31] and susceptibility to endometrial and breast cancers[29,32].

A mutation, G14804A, in the *MT-CYB* gene of LoVo causes an amino acid change, D20N, of Cyt *b* protein. The asparagine residue is evolutionally conserved among mammals and yeast (CI = 1.00; **Fig. 1a**), suggesting its importance for Cyt *b* protein activity. Cyt *b* is the central catalytic subunit of ubiquinol: cytochrome c reductase (bc_1 complex, or complex III) which is a component of the respiratory chain[33]. In addition to two heme groups, the bc_1 complex contains two sites, Q_i (or Q_N: proton input) and Q_o (or Q_P: proton output), where ubiquinone interacts with cyt *b* in the complex. Respiration inhibitors, antimycin A and diuron, block oxidation-reduction at the Q_i site[34]. Genetic study of a yeast, *S. cervisiae*, indicated that cyt *b* mutations, I17F, N31K, and G37V, causes antimycin A- or diuron resistance (**Fig. 1a**, marked with asterisks)[35,36], suggesting that the N-terminal region including the amino acid residue Asp20 in human Cyt *b* is involved in Q_i function.

In the *MT-ND4* gene, a C11266T synonymous

Table 2C Nucleotide alternations detected in non-coding regions

cell line	rCRS	CCK-81	CoCM-1	COLO201	DLD-1	HCC-56	LoVo	OUMS23	RCM-1	SW837	WiDr
haplogroup	H2	F1	D4a	V	T1	M7a	UK2	D5	D4	V	UK1
position*											
5899	—					C ins					C ins
15954	A	G									
16129	G	A	A								
16163	A				G						
16176	C							T			
16182	A	C									
16183	A	C						C			
16184–16193	C_5TC_4	C_n#	C_5TC_4	C_5TC_4	C_2TC_7	C_5TC_4	C_5TC_4	C_n#	C_5TC_4	C_5TC_4	C_5TC_4
16222	C						T				
16232	C	A									
16234	C										T
16249	T	C									
16256	C								T	T	
16270	C						T				
16292	C							G			
16298	T			C						C	
16300	A							G			
16311	T	C						C			
16324	T					C					
16343	A								G		
16344	C	T									
16390	G				A						
16519	T	C	C	C	C		C			C	C
16527	C	T									
72	T			C						C	
114	C										T
146	T	C					C				
150	C							T			
152	T	C	C		C						
195	T				C						
249	A	1bp del									
281	A							G			
303–315	C_7TC_5	C_n#	C_7TC_6	C_8TC_6	C_8TC_6	C_9TC_6	C_7TC_6	C_8TC_6	C_7TC_6	C_9TC_6	C_7TC_6
456	C							T			
497	C										T
522–523	CA	2bp del				2bp del					

*The nucleotide numbering system is based on the human mitochondrial revised Cambridge Reference Sequence.
#poly-c length heteroplasmy.

alteration of DLD-1 is novel. A T11781C mutation of COLO201, which was also found in a pancreatic cancer cell line, COLO357[37], leads to an amino acid change (I341T). The amino acid residue at position 341 of ND4 protein is I or T in mammals, but definitively I in primates (mtSNP database).

A novel non-synonymous alteration G6709A (G269E) in *MT-CO1* gene was found in CCK-81. This amino acid residue is completely conserved (CI = 1.00) and this mutation is likely to lead to a conformational change of COI, especially the secondary structure of α-helix VII, which is predicted to start at amino acid residues 271 to 293 (UniProtKB database: Swiss-Prot P00395) (**Fig. 1b**).

One novel homoplasmic alteration G1336A (DLD-1) and three novel heteroplasmic alterations G1576A (SW837), G2975A (LoVo) and C3060A (COLO201) are located in the ribosomal RNA genes (**Table 2B**). The heteroplasmic mutation percentages are 81%, 55% and 92%, respectively. G1336A, G1576A and G2975A

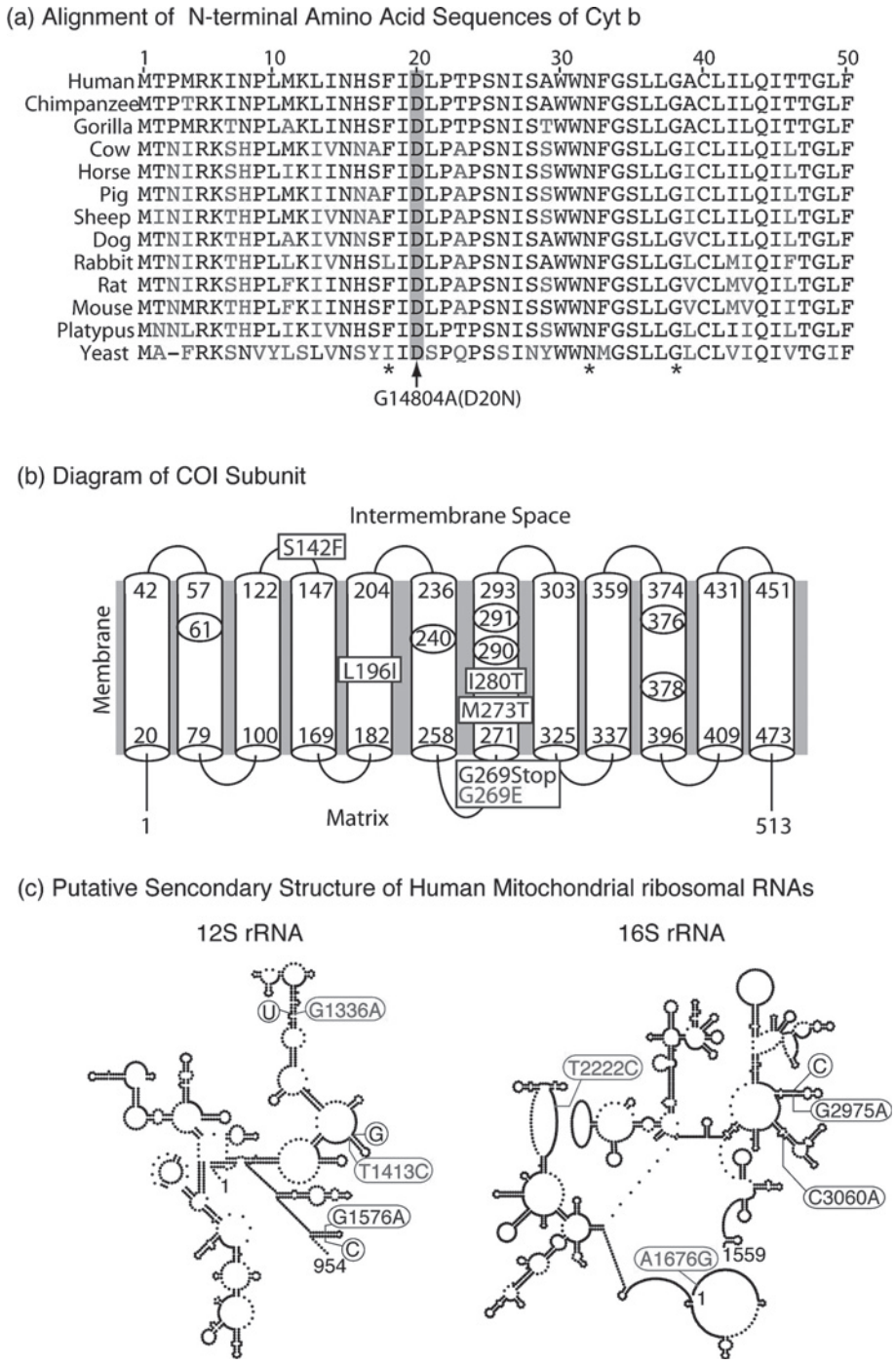

Fig. 1 Novel mutation sites found in this study. **a**: Alignment of the N-terminal amino acid sequences of Cyt *b* was obtained from GiiB-JST Mitochondrial mtSNP database and Ruppert et al.[44] Asterisks indicate the amino acid residues whose mutations were reported to confer resistance to Qi inhibitors in a yeast genetic study. **b**: Diagram of COI, showing mutations (rectangles) identified as a pathogenic mutation[37]. COI has 12 transmembrane regions (cylinders) and the amino acid residues responsible for metal binding are also presented in ellipses, based on UniProtKB database (Swiss-Prot P00395). **c**: The predicted secondary structures of 12S and 16S rRNAs were obtained from GiiB-JST Mitochondrial mtSNP database. Homoplasmic and heterplasmic alterations are presented in red and blue, respectively. Three novel alterations, G1336A, G1576A, G2975A located in the predicted stem regions, where the complementary nucleotides are presented in black circles. An alteration C3060A is also novel.

are found in the stem region (**Fig. 1c**). G1576A and G2975A are clearly expected to make the stem structure unstable, whereas T1413C makes the stem structure more stable. G1336A seems to unaffect the stem structure as judged by terms of free energy, since the complementary nucleotide is U. A1676 G and T2222C alterations of COLO201 are also found in pancreatic cancer cell lines[37].

Many mutational and comparative studies of ribosomal RNAs have been reported and reviewed[38–40]. In the current secondary model[38,39], the stem where mitochondrial mutation G1336A is found in this study corresponds to the stem where the C1054A mutation is found in the 16S rRNA gene of *Escherichia coli*; the two mutations are very close to each other in the models, and the C1054A mutation of *E. coli* causes defects in translation termination[41]. Similarly, nucleotides T1413 and G1576, found to be altered in this study, are close, within 10 nucleotides, to nucleotides G1338/A1339 and A1518/A1519 of the 16S rRNA of *E. coli*, respectively, in the models; these nucleotides of *E. coli* are known to be involved in translation activity[42,43]. Nucleotide 2975 of the mitochondrial 16S rRNA gene seems to be located in the region equivalent to the peptidyltransferase region of domain V of 23S rRNA of *E. coli*: the equivalent *E. coli* nucleotide is surrounded by nucleotides responsible for sensitivity to antibiotics, including chloramphenicol and evernimicin, which inhibit bacterial protein syntzhesis[40]. Taking these considerations into account, it is possible that those mutations cause low mitochondrial translation activity and be associated with cancer development.

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