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ORIGINAL ARTICLE

Mutations in mitochondrial NADH dehydrogenase subunit 1 (mtND1) gene in colorectal carcinoma

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Abstract

Mitochondrial Subunit ND1 (mtND1) gene is involved in the first step of the electron transport chain of oxidative phosphorylation (OXPHOS). Alteration of the electron transport components by mutations in mtDNA may compromise the normal electron flow. This could lead to an increase of bifurcation and generation of superoxidase radicals and increase oxidative stress in various types of cancer cells. Genomic DNA was extracted from thirty matched primary colorectal tumour tissues and matching non-tumour tissues. Blood samples were obtained from twenty-five normal people. The mtNDI coding region was amplified by step-down PCR. The purified products were then subjected to direct sequencing and subsequently, the DNA sequences obtained were compared with the revised Cambridge Reference Sequence (rCRS) and MITOMAP. From the analysis, the mtND1 gene showed 11 (45.8%) different mutations and also 13 (54.2%) polymorphisms. The heteroplasmic mutation A4123A/G (I273I/V) might have a pathogenic significance as it fulfills various pathogenic criteria. Three mutations, T3394C (Y30H), A3434G (Y43C) and C3497T (A64V) which occur in a highly conserved region were likely to alter the structure and function of the ND1 protein. We suggest that these mutations, and in combination with the polymorphic variance in mtDNA, may cause slight changes that generate subtly higher levels of toxic reactive oxygen species (ROS).

Keywords: Mitochondrial DNA mutations, colorectal carcinoma, mtND1 gene, OXPHOS

INTRODUCTION

Colorectal cancer is the second most common cancer in Malaysia, after lung cancer in men and the third most common cancer in women, after cervical and breast cancer according to the Second Report of the National Cancer Registry.[1] To date, a large number of different mutations of various genes have been reported in colorectal cancer. In human tumours, however, mitochondrial DNA (mtDNA) has not been analyzed in great depth. The mtDNA is susceptible to mutations and the ensuing alterations are associated with various cancer types.

Mitochondrial DNA has the potential to be used as a cancer-specific biomarker for several reasons. The mtDNA genome is well characterized with 37 densely packed genes and a lack of introns. Thus, any mutation occurring in the coding sequence will lead to biological consequences.[2] Secondly, its high copy number makes sequence variance easier to detect and less sample is required for analysis. Due to its small size and closed-circular structure, mtDNA is more resistant to damage (caused by isolation and storage) than nuclear DNA. It also has less efficient DNA repair mechanisms than nuclear genome and for that reason, mutations are more likely to persist.[3] A lack of protective histones and its close proximity to a major source of reactive oxygen species (ROS) generated during oxidative phosphorylation (OXPHOS) make mtDNA far more susceptible to DNA-damaging agents, and hence mutation.[4,5]

Recently, there have been reports of the potential use of the mtDNA mutations as biomarkers of solid tumours in other cancers such as breast cancer,[6] lung cancer,[3] oesophageal carcinoma[7] and pancreatic cancer.[8] A study by Polyak et. al[9] in mtDNA genome revealed 12

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somatic homoplasmic mutations (one which occurred in mtND1) in seven (70%) human colorectal cell lines. They also reported that most of these mutations were transition at purines, consistent with an ROS-related derivation.[9]

The human mitochondrial genome is a double-stranded circular molecule of 16,568 base pairs encoding 13 of the 87 essential proteins required for oxidative phosphorylation. Also encoded are two rRNAs and 22 tRNAs which are required for protein synthesis in the mitochondria.[10] One of those essential proteins is NADH dehydrogenase subunit 1 (mtND1) which encompasses 955 base pairs coding sequences. The mtND1 gene encodes one of the seven subunits of respiratory complex 1 and is involved in the first step of the electron transport chain of the mitochondrial energy-generating pathway, OXPHOS. In this process, the electrons from NADH molecule are transferred to another molecule called ubiquinone. The electrons are then passed from ubiquinone through several other enzyme complexes to provide energy for ATP generation.

Therefore, mutations in mtDNA might cause an alteration of the electron transport components and compromise the normal electron flow. This can lead to an increase of bifurcation and generation of superoxidase radicals and increase oxidative stress that have been observed in various types of cancer cells in several independent studies.[11]

In this study, we examined mtND1 mutations/polymorphisms in thirty colorectal tumour tissues and the matching non-tumour tissues. We also report point mutations that have been detected in 33% of the colorectal cancer patients analyzed, which may affect the function of the mtND1 electron transport gene.

MATERIALS AND METHODS

Tissue samples and DNA isolation

Thirty primary colorectal tumour tissues and matching normal tissues were obtained following informed consent from patients in the Hospital Universiti Kebangsaan Malaysia (HUKM). They were males and females patients aged between 28 to 94 years. Extraction of DNA from the tissues was performed (QIAGEN Blood Mini DNA Extraction Kit). We also isolated genomic DNA from the blood of twenty-five normal individuals to serve as controls.

PCR amplification

The entire mitochondrial ND1 gene spanning across nucleotide positions 3307-4263 was successfully amplified using two sets of overlapping primers: 5'-CAG AGC CCG GTA ATC GCA TAA- 3' with 5'- GGA GAG GTT AAA GGA GCC ACT-3' and 5'-ATG AAG TCA CCC TAG CCA TCA-3' with 5'-AGG GAT GGG TTC GAT TCT CAT-3'. Briefly, the reaction mixture was subjected to step-down PCR protocol[12] with some modification. The PCR began with pre-denaturation at 95oC for 2 min; 95oc for 30 sec, 64oC for 1 min, 70oC for 2 min, 3 cycles; 95oC for 30 sec, 60oC for 1 min, 70oC for 2 min, 2 cycles; 95oC for 30 sec, 59oC for 1 min, 70oC for 2 min, 30 cycles; and a final extension at 70oC for 2 min. PCR products were subsequently purified using a spin column to remove unincorporated primers and excessive dNTPs (QIAGEN QIAquick Purification Kit).

DNA sequencing

The cycle sequencing involved the amplification of the primary PCR product and the primers, using the standard protocol. Dye-Ex v.2.0 kit (QIAGEN) was used to clean-up the PCR products. Next, the reactions were sequenced bi-directionally using Big Dye Terminator version 3.1 cycle sequencing chemistries (Applied Biosystems) on a 4-channel capillary ABI 3100-Avant Genetic Analyzer.

mtND1 Mutational Analysis

1. Identification of nucleotide changes or variants

The sequences generated were aligned with the published human mitochondrial DNA revised Cambridge Reference Sequence (rCRS)[13] using SeqScape version 2.0 software (Applied Biosystems). The data were then compared to the MITOMAP: a human mitochondrial genome database[13] to identify the mitochondrial genome sequence variants. Sequence variants that were not found in that database were classified as new polymorphism/mutation, whereas others already reported were classified as reported polymorphism/mutation. A sequence variant that differed in tumour mtDNA from its corresponding non-tumour mtDNA was classified as a somatic mutation, whereas a variant found in both tissues was classified as a germ-line origin.

2. *Evaluation of the severity of the amino acid changes associated with disease*

The degree of chemical similarity or differences between alternative residues of amino acids was measured by the commonly used Grantham value for amino acid substitution matrix.[14] The average of the Grantham values of different amino acid replacement is 50. The nucleotide changes with Grantham values larger than 50 are regarded as radical amino acid replacements (i.e. more likely to alter the structure and function of protein); and those with Grantham values of less than 50 are regarded as conservative replacement.

3. *Prediction of the nucleotide changes on a 2-dimensional structure*

The effects of nucleotide changes on the functioning of the mitochondrial respiratory chain were evaluated using the 2D structures of NADH dehydrogenase subunits (ND1-6, ND4L) based on mtSNP, a database of human mitochondrial genome polymorphisms.[15]

4. *Conservation of nucleotide changes among species*

The conservation of the 11 mutated amino acid residues detected in the patients were examined and compared with other 61 mammalian species. The entire sequences of the mitochondrial genome for these 61 mammalian species are registered in genome database (http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/40674.html).[15]

RESULTS

Table 1 summarises the mtND1 mutations encountered in the colorectal carcinoma patients. All were missense mutations. Fig. 1 illustrates the mutations in the ND1 gene.

A prediction of a 2-D structure of ND1 protein[15] showing 6 mutations residing in transmembrane α-helical segments in a membrane domain is illustrated in Fig. 2. This domain is important for the electron transfer (proton pumping) and ubiquinone (Q) binding. The other 5 mutations are residing in the loop regions. Mutation of T3394C (Y30H), A3434G (Y43C) and C3497T (A64V) cause amino acid changes in the hydrophilic loops between the transmembrane segments I and II that face the mitochondrial matrix. The C3571T (L89F) mutation occurs in the loop between the transmembrane segments II and III, whereas the A3796G (T164A) resides in the loop between segment IV and V.

TABLE 1: Summary of mtND1 mutations in colorectal patients (n=30)

Patient	Position	Mutation	Amino acid change	Grantham value	Comment
1	3497	C → T	A 64 V	64	Reported[a,b]
	3571	C → T	L 89 F	22	Reported[b,c]
2	3796	A → G	T 164 A	58	Reported[a,b]
	4123	A → A/G	1 273 I/V	29	New
6	3337	G → A	V 11 M	21	Reported[b]
	4048	G → A	D 248 N	23	Reported[b]
3	3394	T → C	Y 30 H	83	Reported[a,b,c]
4	4231	A → G	1 309 V	29	Reported[b]
5	3316	G → A	A 4 T	58	Reported[a,b]
7	4048	G → A	D 238 N	23	Reported[b]
8	3316	G → A	A 4 T	58	Reported[a,b]
9	3434	A → G	Y 43 C	194	Reported[b,c]
10	4129	A → G	T 275 A	58	Reported[b]

All of these mutations (except 1 new mutation) have been reported in MITOMAP: a human mitochondrial genome database.[13]

[a] Reported point mutation in mitochondrial disorders.

[b] Reported polymorphism in mitochondrial disorders.

[c] Reported somatic mutation in other cancers.

Abbreviations: A, Alanine; T, Threonine; V, Valine; M, Methionine; Y, Tyrosine; H, Histidine; L, Leucine; F, Phenylalanine; D, Aspartate; N, Asparagine; I, Isoleucine

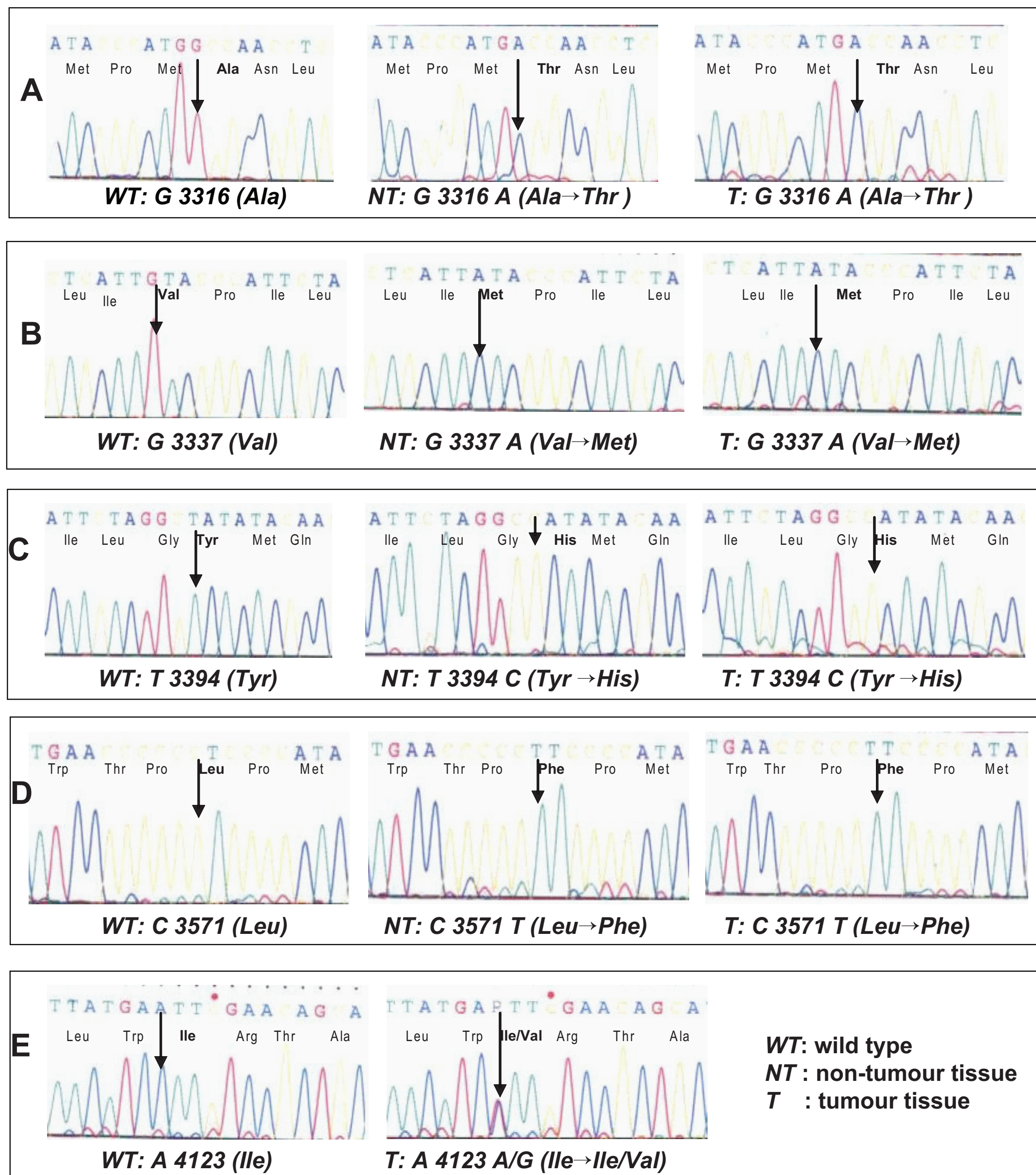

FIG. 1: Mutations in ND1 gene found in colorectal cancer. Fig 1A-D shows the sequence data found in wild-type, tumour and non-tumour tissue which harbors homoplasmic single base substitution respectively. In Fig 1E, the sequence data in the tumour was observed as heteroplasmic with 50% mutant load at the nucleotide position 4123.

A phylogenetic conservation of amino acid residue 4, 11, 30 and 43 within the ND1 protein (shown in 44 of 61 mammalian species) is illustrated in Fig.3 The mutated residues in this study are highlighted. Mutation of Y30H and Y43C are in a highly conserved region. The alignment was carried out using mtSNP database mtSAP.[15]

DISCUSSION

The sequencing analysis of the mtND1 gene in 30 matched samples from colorectal cancer patients identified a total of twenty-four sequence variants. Eleven (45.8%) variants detected in 10 patients were missense mutations and would alter the amino acid sequence of the encoded mtND1 protein. Of these, 2 variants were identified

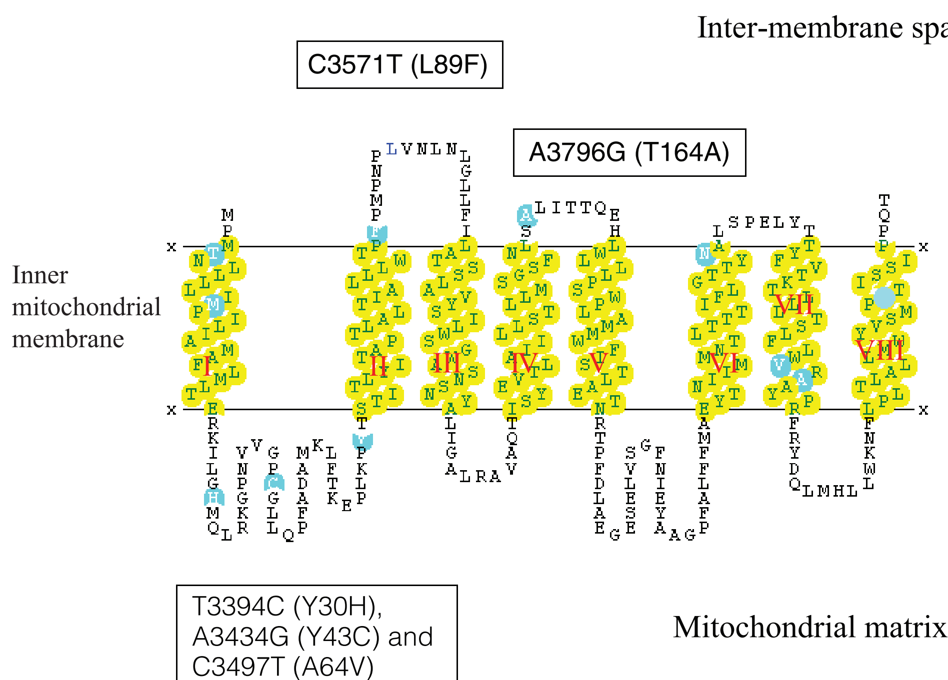

FIG. 2: A prediction of a 2-D structure of ND1 protein

as new mutations; 8 variants were previously reported as either mutations or polymorphisms and one was previously reported as somatic mutation (Table 1). In the present case, the mutation was not somatic since the variant was found in both tumour and non-tumour tissue. Therefore, it may be considered as being of germ-line origin.

All mutations and polymorphisms observed were single base substitution and were homoplasmic except for one heteroplasmic (co-existence of wild-type and mutant mtDNA) in patient 2 showing a 50% mutation load. This mutation converts an A to A/G at nucleotide position 4123, resulting in the substitution of amino acid Ile to lle/Val. Sequencing details of the heteroplasmic mutation is illustrated in Fig. 1. We believe that the presence of A 4123 A/G in this patient is a pathogenic mutation. This particular mutation has fulfilled three out of four criteria for the mtDNA mutation to be considered pathogenic.[16] Firstly it is not detected in all of our normal controls. Secondly, the nature and location of the mutation suggests a logical mechanism of disease and finally, it is detected as heteroplasmic.

We also assessed the evolutionary conservation of the amino acid residues affected by the mutations using a human mitochondrial genome polymorphism database; mtSNP and mtSAP Database.[15] Multiple sequence alignment comprising a panel of 61 mammalians mitochondrial ND1 protein was performed. Four of the eleven mutated residues were conserved in more than 80% of mammalians mtND1. For example in patient 3, the tyrosine residue mutated by the transition at position 3394 was conserved in 97% of mammalians mtND1. The substitution of the hydrophobic tyrosine to the hydrophilic histidine (Y30H) may thus significantly affect the activity of the protein. This mutation may change the secondary structure of the ND1 protein and subsequently decreased its enzyme activity. This may have a parallel effect on the cellular metabolism via increased radical oxygen species (ROS) generation or the promotion of apoptosis.[17] Other mutations that reside in the conserved residues are L 89 F (93%), heteroplasmic I 273 I/V (92%) and Y 43 C (85%). All except the heteroplasmic mutation were previously reported as mutations in other diseases. This heteroplasmic mtDNA mutation may create a subtle functional defect in oxidative phosphorylation resulting in slightly increased ROS levels. It is known that low levels of ROS are mitogenic to the cell and possibly mutagenic to the nuclear DNA.[11]

The Grantham Difference is one popular method for measuring biochemical distances between pairs of amino acids, by taking

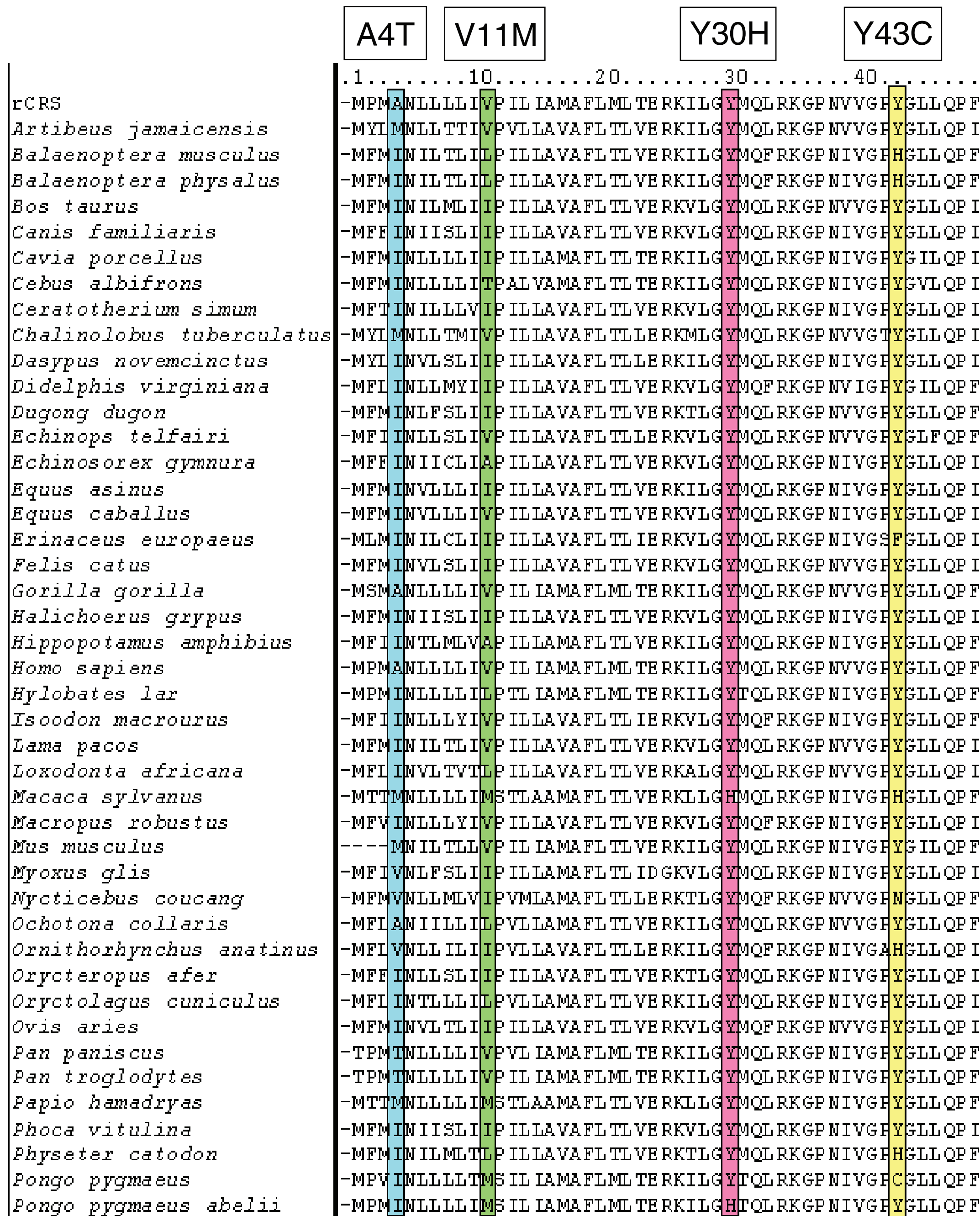

FIG. 3: A phylogenetic conservation of amino acid residue 4, 11, 30 and 43 in an ND1 protein

consideration of the composition, polarity and volume of mutant and wild type amino acids. The amino acid changes with Grantham value larger than 50 are considered as radical amino acid replacements (missense mutation), whereas values of less than 50, as conservative replacements (silent mutation).[14] Interestingly, we found 3 mutations; Y30H, Y43C and A64V that reside in hydrophilic loops between the transmembrane segments I and II facing the mitochondrial matrix with Grantham values of 83, 194 and 64, respectively. These replacements are assumed to be more likely to alter the structure and function of the ND1 protein than conservative replacements.

Although the heteroplasmic I 273 I/V mutation

has the Grantham value of 29 and is therefore considered to be silent mutation, it is possible that the mutation is a potential pathogenic variant. This is because the mutation is heteroplasmic and resides in a highly conserved region of the ND1 subunit.

Analysis of mtND1 gene in twenty-five normal individuals showed six different polymorphisms (data not shown). None of these was detected in the colorectal cancer patients. Most (95%) of the 13 polymorphisms detected (in patients and normal individuals) were either C→T or A→G transitions. We noted no correlation between the sequence variants with either the patient's age or gender. Likewise, no correlation was found between the sequence variants and tumour classification (Table 2).

Conclusion

We observed eleven homoplasmic mtND1 mutations, with the exception of one heteroplasmic. The heteroplasmic A4123A/G (I273I/V) might have a pathogenic significance as it fulfilled 3 out of 4 pathogenic criteria. Three mutations; T3394C (Y30H), A3434G (Y43C) and C3497T (A64V) which occur in a highly conserved region are assumed to be more likely to alter the structure and function of an ND1 protein. All mutations detected may be considered as of germ line origin since the variants were found in both matched tumour and non-tumour tissues. Although the functional significance of these mutations remains to be determined, all mutations occurred in mtND1 gene could likely be leading to a defect in mitochondrial protein synthesis and hence oxidative phosphorylation, i.e. the electron transport chain of mitochondrial energy-generating pathway. We believe that these mutations, and in combination with the polymorphic variance in mtDNA, may cause slight changes that generate subtly higher levels of toxic reactive oxygen species (ROS).[9] Nevertheless, investigation of biochemical and functional analysis of the mutated mtND1 need to be carried out to assess the significance of these mutations and also their association in carcinogenesis.

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TABLE 2: Details of subjects harboring mtNDI mutation and polymorphism

Patient	Age	Gender	Ethnic group	Clinical diagnosis	Mutation
1	46	F	Malay	Duke's C	3497 C → T 3571 C → T
2	57	M	Malay	Duke's C	3796 A → G 4123 A → A/G
3	63	M	Malay	NA	3394 C → T
4	66	M	Malay	Duke's B	4231 A → G
5	78	F	Malay	Duke's C	3316 G → A
6	71	M	Chinese	Duke's B	4048 G → A 3337 G → A
7	94	M	Chinese	Duke's B	4048 G → A
8	56	F	Indian	Duke's B	3316 G → A
9	NA	F	Chinese	Duke's B	3434 A → G
10	NA	F	Chinese	Duke's B	4129 A → G

F: Female, M: Male, NA: not available.

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