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Original Article

CYTB: A HOT SPOT FOR PATHOGENIC MUTATIONS IN MITOCHONDRIAL GENOME OF BREAST CANCER AND OVARIAN CANCER PATIENTS

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ABSTRACT

Objective: Out of various cancer types, Breast and ovarian cancers are the most commonly occurring malignancies in women. As per literature, a large number of mutations are reported in various mitochondrial genome encoded subunits of respiratory chain complexes in breast and ovarian cancer patients. However, a very few of them are functionally validated till now. Our study is an attempt to highlight the pathogenic potential of all these reported mutations in breast and ovarian cancer patients.

Methods: In order to achieve so, total 109mitochondrial gene mutations of breast cancer and 11 mitochondrial gene mutations of ovarian cancer patients were selected from MITOMAP database as well as various literatures. All these mutations were analyzed using various *in silico* tools such as MUSCLE, PolyPhen-2, SIFT, Mut Pred, Mu Pro, PANTHER, GOR4 and MUSCLE.

Results: As a result of our analysis, 28 out 95 mutations in CytB gene are most pathogenic in the case of breast cancer patients. On the other hand 2 out of 3 mutations of the same gene were predicted to be potentially pathogenic in case of ovarian cancer patients. Mutations in other mitochondrial subunit was also predicted pathogenic but with the low score.

Conclusion: Out of different mitochondrial subunits, CytB seems to most important site for mutations in these two groups of patients. Hence, mutations of CytB subunit, which are predicted to be highly pathogenic as per our analysis, should be functionally validated in future.

Keywords: Mitochondrial subunits, Mutations, *In-silico*., OXPHOS, Breast cancer (BC), Ovarian cancer (OC).

INTRODUCTION

Mitochondria are double membranous, cytoplasmic semi-autonomously functioning organelles. It is responsible for generating approximately 90% of cellular ATP through the process of oxidative phosphorylation (OXPHOS) and plays essential roles in cellular energy metabolism, free radical generation, and programmed cell death [1].

Mitochondria contain its own genome which is a circular double stranded DNA of 16.6 kb in size. It encodes for 2 rRNAs, 22 tRNAs and 13 polypeptide [2]**.** All these 13 mitochondrial peptides, along with different nuclear encoded proteins, are integral subunits of different respiratory chain complexes Complex I has total 7 mitochondrial encoded subunits (ND1-ND6 and ND4L), complex III has one (cytochrome b), complex IV has three (COX I-III) and complex V has two mitochondrial encoded subunits (ATPase6 and ATPase8).

As compared to the nuclear DNA, mitochondrial DNA (mtDNA) is very much prone for mutations as it is known to lack histone proteins and DNA repair machinery [3]. Further, being a major site of ROS production, mtDNA is more vulnerable to oxidative damage and nucleotide changes. Thus because of all these factors, the reported mutations in mitochondria are 10-200 times more than nuclear DNA [3]. A large number of mutations in different protein regions of the mitochondrial genome are reported in association with different diseases including cancer too.

In early 19th century, Otto Warburg hypothesized from his research that the malfunction in the mitochondria may cause an injury to the respiratory machinery, resulting in the compensatory ATP production through glycolytic pathway [4]. As the glycolytic pathway is less efficient than OXPHOS, thus cancerous cells would consume high glucose to fulfill their energy demand as compared to the healthy cells. It is considered as the important characteristic of cancerous cells [4-6]. It is proposed that mitochondrial dysfunction has been one of the major causes for aerobic glycolysis in the cancerous cells. Additionally, mutations in mtDNA are also proposed to cause high ROS production [6], which ultimately stimulates the cell proliferation and cancer progression. A large number of studies show the association between mutated mtDNA and risk of different types of cancers [7-9]. According to the WHO (World Health Organization) and WCRF (World Cancer Research Fund International), Breast cancer (BC), Cervix cancer and Ovarian cancer (OC) are commonly occurring cancers in women [10].

Globally, BC comprises 16% of female cancers [11] and OC constitutes 20% of all genital malignancy accounting for more deaths than any other cancer of the female reproductive system [12]. Cervical cancer is the most common genital cancer among females in developing country and accounts for 15% of all cancers in women [13]. Amongst all these three common cancer types in female, a large number of mutations in different regions of the mitochondrial genome are reported in BC and OC patients only. Association of these mtDNA mutations in BC and OC patients are suggested because of various studies involving amplification and sequencing of complete mitochondrial genomes of cancer patients and comparing the results to those obtained from non-cancerous tissues derived from the same patients [14]. As the number of these reported mutations is very high (109 in BC and 11 in OC), so it is not possible to functionally validate all these mutations by transmit chondrial studies. Hence it is imperative to do the initial analysis by *In-silico* methods. As this approach will help us to narrow down to few most potential pathogenic mutations, *in-silico* which will pave a path for future research to functionally validate them by using *In vivo/In vitro* model systems. However, till now there is no comprehensive study reported for highlighting the importance of these mt DNA mutations in cancer patients. Hence, our current study is an attempt to reveal the pathogenic nature of mtDNA mutations reported in protein coding region of BC and OC patients by using various *in silico* tools.

MATERIALS AND METHODS

Data collection

Though a large number of mutations are reported all across the mtDNA in these BC and OC patients but we have done our analysis on protein coding mitochondrial subunit genes only and the

respective data were collected from various literatures and MITOMAP database. A total of 109 and 11 non-synonymous nucleotide changes across different protein coding mitochondrial genes, associated with the BC and OC, were included in the study [table 1].

***In-silico* analysis**

In order to understand the effect of all these above-mentioned mutations on structure and function of respective mitochondrial protein, different bioinformatics tools have been used in this study.

MUSCLE (M Ultiple sequence comparison by log-expectation)

To analyze the significance of a specific mutation, its conservation across the higher to lower eukaryotes was explored using multiple sequence alignment tool MUSCLE. MUSCLE is claimed to achieve both better average accuracy and better speed than ClustalW2 or T-Coffee. [http://www. ebi. ac. uk/Tools/msa/muscle/]

Poly Phen-2 (Polymorphism Phenotyping v2)

This is an automatic tool which uses the machine-learning approach for the prediction of possible impact of amino acid substitution on the structure and function of human protein. Prediction is based on straightforward rules which include sequence based characterization of substitution sites and structural parameters and contacts. The output of the tool predicts mutation to be either probably damaging i.e. mutation is supposed to affect with high confidence, possibly damaging i.e. mutation is supposed to affect but not with high confidence and benign i.e. mutation is not likely to produce any phenotypic effect. [http://genetics. bwh. harvard.edu/pph2/]

SIFT (Sorting intolerant from tolerant)

SIFT uses the approach of degree of conservation of amino-acids in the protein to predict the pathogenicity of the mutation. The tool assumes that the more conserved the site, more important the site becomes from the evolutionary perspective. It searches for closely related sequences through PSI-BLAST, performs multiple-sequence alignment, scans each position of the alignment and calculates the normalized probabilities for all 20 amino acids at that position. The output of the tool is a score between 0 to 1. Mutation is said to be damaging if the score is less than 0.05 and tolerable if score is greater than 0.05. SIFT also evaluates the confidence level of the prediction according to the diversity of the sequences taken for comparison. [http://sift.jcvi.org/www/SIFT_seq_submit2.html].

MUTPRED (Mut Pred)

This tool predicts if the amino acid substitution in the respective protein is disease associated or neutral in nature. The output of MutPred contains a general score (g), *i.e.,* the probability that the amino acid substitution is deleterious/disease-associated, and top 5 property scores (p), where p is the P-value that certain structural and functional properties are impacted. Certain combinations of high values of general scores and low values of property scores are referred to as hypothesis. Scores with $g>0.5$ and $p<0.05$ are referred to as actionable hypotheses, $g>0.75$ and $p<0.05$ as confident hypotheses and $g>0.75$ and $p<0.01$ as very confident hypotheses. [http://mutpred. mutdb. org/].

MuPro

To predict the possible impact of amino-acid substitution on the stability of the protein, MuPro was used. The tool is based on machine-learning approach including support vector machine (SVM) and artificial neural network (ANN) based on 20 pairs of testing and training datasets. Input can be either the amino-acid sequence or tertiary structure of the protein and the output is score between-1 and 1. Score less than 0 indicates a decrease in stability of the protein and score greater than 0 indicates an increase in stability. Change in protein stability lead to altered contacts with other protein subunits, which is can be pathogenic. [http://mupro.proteomics.ics.uci.edu/].

PANTHER (Protein A nalysis through evolutionary relationships)

It calculates the subPSEC (substitution position-specific evolutionary conservation) score based on an alignment of evolutionarily related proteins. subPSEC scores are continuous values from 0 to-10, where 0 implies a very conservative change which is unlikely to affect protein function, and more negative scores are increasingly damaging. A subPSEC score of less than-3, is taken as the cutoff value, indicating a high probability of substitution having a severe impact on the protein function [http:// www.pantherdb.org/tools/].

GOR4

The Garnier–Osguthorpe–Robson (GOR) method analyzes sequences to predict the secondary structures of protein from amino acid sequence and classify it into alpha helix, beta sheet, turn, or random coil secondary structure at each position based on 17-amino-acid sequence windows. It takes into account the tendency of individual amino acids, along with its conditional probability to form particular secondary structures. [https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html].

RESULTS AND DISCUSSION

Breast cancer

After doing *in silico* analysis of all 109 mutations [table1], Cytochrome B (CytB) subunit appears to be the major site for an accumulation of mutations in these patients as it was found to carry largest number of mutation (95 out of 109). However, a few numbers of mutations are also localized to different mitochondrial proteins which are integral component of different respiratory chain complexes such as complex I, IV and V.

Table 1: List of mitochondrial protein mutations reported in association with breast and ovarian cancer (Data obtained from mito map and literature)

Cancer Type	Mitochondrial subunits (No. of mutations)	Nature of mutations
1. Breast Cancer	ND3 (1)	T114A
	ND4 (1)	V381M,T337A
	ND5 (1)	N446K
	CytB (95)	N263K,T264A,T264N,H267P,I268V,P270T, E271A,E271G,E271D,W272L,Y273N,Y273S, Y273C,F276V,F276L,T279A,L281R,V284I, N286D,L288M,L288V,G290S,L292V,L292R, L295V,L295W,S297F,L299V,L299F,L301M, M303V,M303T,M303I,I304M,P305A,I306V, I306M,H308L,H308E,M309L,M309T,S310P, K311N,M315L,M316T,F317I,F317L,R318G, L320M,S321N,L324I,L324F,Y325H,L327V, L327P,L328R,A329G,A329V,A330T,D331G, L242R,L333F,L333H,I334L,L335Q,T336A, W337R,I338M,Q341K,V343E,Y345H,Y345D, P346S,F347V,F347C,F347L,T348N,T348I,

		I349T,I350M,V353E,A354V,L357M,L357V, T360A,T361M,I362V,L363R,I364L,I364F, L365V,M366L,M366I,T368N,T368I
	COX III (1)	F227I
	ATPase6 (8)	F6L, A11T, I14T, I24T, T53I, T59A, A177T, P187S
	ATPase8 (1)	T7A
2. Ovarian cancer	ND1(1)	D248N
	ND4(2)	A131T,N424S
	ND5(2)	S523P,M1T
	CytB (3)	S213P,K217E,A302V
	COX I(1)	M417T
	COX III (1)	A95V
	ATPase8 (1)	P36L

CytB

Out of 95, a total of 28 mutations in CytB subunit are predicted to be pathogenic by the maximum of the tools used [table2]. All these mutations have shown to thermodynamically decrease the structural stability of the protein as predicted by MuPro. 21 out of these pathogenic mutations are found to affect the protein function as predicted by PolyPhen-2, SIFT and PANTHER. Similarly total 22 mutations are predicted to cause a change in the overall percentage content of secondary structure in the protein at the specific position. The common secondary structure change seen was from coiled coil to helix and from helix to coil strand.

Table 2: Reported non-synonymous mutations in protein-coding mitochondrial gene subunits associated with Breast cancer

Mutations	**PolyPhen-2**	**SIFT**	**MuPro**	**MutPred**	**PANTHER**	**GOR4**	**MUSCLE**
	Prediction (score)	**Prediction (score)**	**Prediction (score)**	**Hypothesis**	**Prediction (subPSEC)**		**Prediction**
ND3							
T114A	B (0)	Tol (0.86)	Decrease Stability (-0.92)	No Impact	Not Damaging (-0.65)	e->e	NC
ND4							
V381M	Prb (0.99)	Affect (0)	Decrease Stability (-0.44)	No Impact	Damaging (-4.10)	c->e	PC
T337A	Prb (0.99)	Tol (0.13)	Decrease Stability (-1)	No Impact	Not Damaging (-2.58)	c->h	NC
ND5							
N446K	Prb (0.99)	Affect (0.01)	Decrease Stability (-1)	Confident (gain of methylation, ubiquitination)	Damaging (-3.01)	h,e->c	NC
CytB							
N263K	B (0.02)	Affect (0)	Decrease Stability (-0.70)	No Impact	Not Damaging (-2.02)	c->h	NC
T264A	B (0.20)	Affect (0)	Decrease Stability (-1)	Actionable (loss of glycosylation)	Damaging (-3.62)	c->c	C
T264N	Pos (0.64)	Affect (0)	Decrease Stability (-0.99)	Actionable (loss of glycosylation)	Damaging (-4.88)	c->c	C
H267P	Pos (0.47)	Affect (0)	Increased Stability (0.02)	Actionable (gain of glycosylation)	Not Damaging (-2.90)	c->c	C
I268V	B (0.01)	Affect (0)	Decrease Stability (-0.56)	Actionable (Loss of catalytic residue)	Not Damaging (-2.79)	c->c	C
P270T	Prb(0.96)	Affect (0)	Decrease Stability (-0.92)	No Impact	Damaging (-3.93)	c->h	C
E271A	Pos (0.60)	Affect (0)	Decrease Stability (-0.12)	No Impact	Damaging (-4.32)	c->c	C
E271G	Pos (0.92)	Affect (0)	Decrease Stability (-1)	No Impact	Damaging (-4.56)	h->e	C
E271D	B (0.07)	Affect (0)	Increased Stability (+0.3)	No Impact	Damaging (-3.72)	h->c	C
W272L	Pos (0.94)	Affect (0)	Decrease Stability (-0.40)	No Impact	Damaging (-3.68)	c->h	C
Y273N	Prb (0.99)	Affect (0)	Decrease Stability (-1)	No Impact	Damaging (-4.78)	h->h	C
Y273S	Prb (0.99)	Affect (0)	Decrease Stability (-1)	No Impact	Damaging (-3.98)	h->h	C
Y273C	Prb (0.99)	Affect (0)	Decrease Stability (-0.93)	No Impact	Damaging (-5.08)	h->c	C
F276V	Pos (0.86)	Affect (0)	Increased Stability (0.34)	No Impact	Not Damaging (-2.82)	h->e	NC
F276L	Pos (0.62)	Affect (0)	Increased Stability (0.09)	No Impact	Not Damaging (-2.73)	h->c	NC
T279A	Error	Tol (1)	Increased Stability (1)	No Impact	Not Damaging (-1.97)	c->h	PC
L281R	Prb (0.99)	Affect (0)	Decrease Stability	Very Confident (gain of catalytic residue)	Damaging	h-	C

			(-1)		(-5.22)	>c	
V284I	Pos (0.51)	Tol (1)	Decrease Stability (-0.38)	No Impact	Not Damaging (-1.90)	c->h	PC
N286D	B (0.30)	Affect (0)	Increased Stability (0.31)	No Impact	Not Damaging (-2.47)	c->c	WC
L288M	B (0.37)	Affect (0)	Decrease Stability (-0.86)	Confident (loss of methylation)	Damaging (-3.41)	c->h	NC
L288V	B (0.03)	Affect (0)	Decrease Stability (-1)	No Impact	Not Damaging (-2.28)	c->c	NC
G290S	Prb (0.99)	Affect (0)	Decrease Stability (-1)	No Impact	Damaging (-3.80)	c->h	C
L292V	B (0.00)	Affect (0.03)	Decrease Stability (-0.15)	No Impact	Not Damaging (-1.88)	h->h	NC
L292R	B (0.23)	Affect (0)	Decrease Stability (-0.65)	Confident (gain of methylation)	Damaging (-4.70)	h->h	NC
L295V	B (0.04)	Tol (1)	Decrease Stability (-0.37)	No Impact	Not Damaging (-1.56)	h->h	NC
L295W	Pos (0.94)	Affect (0)	Decrease Stability (-0.67)	No Impact	Damaging (-4.40)	h->h	NC
S297F	Prb (0.99)	Affect (0)	Increased Stability (+0.73)	No Impact	Damaging (-3.43)	h->h	NC
L299V	B (0.13)	Affect (0.02)	Increased Stability (+0.29)	No Impact	Not Damaging (-2.20)	h->h	NC
L299F	Pos (0.89)	Affect (0)	Decrease Stability (-0.54)	No Impact	Damaging (-3.27)	h->h	NC
L301M	Pos (0.93)	Affect (0)	Decrease Stability (-0.19)	No Impact	Not Damaging (-2.95)	h->e	C
M303V	B (0.00)	Tol (0.34)	Decrease Stability (-0.64)	Actionable (gain of catalytic residue)	Not Damaging (-1.34)	c->h	NC
M303T	B (0.00)	Affect (0.02)	Decrease Stability (-1)	Actionable (loss of stability)	Not Damaging (-1.51)	c->h	NC
M303I	B (0.00)	Tol (1)	Decrease Stability (-0.67)	Actionable (gain of catalytic residue)	Not Damaging (-1.54)	c->h	NC
I304M	Pos (0.77)	Tol (0.66)	Decrease Stability (-0.68)	No Impact	Not Damaging (-1.98)	h->h	WC
P305A	Pos (0.87)	Affect (0)	Decrease Stability (-0.27)	No Impact	Not Damaging (-2.98)	c->h	C
I306V	B (0.00)	Affect (0.04)	Decrease Stability (-0.21)	No Impact	Not Damaging (-1.72)	h->h	PC
I306M	B (0.21)	Tol (0.2)	Decrease Stability (-0.62)	Actionable (gain of disorder)	Not Damaging (-2.04)	h->h	PC
H308L	Pos (0.73)	Affect (0)	Decrease Stability (-0.29)	Actionable (loss of disorder)	Not Damaging (-2.88)	c->h	PC
H308E	Prb (0.55)	Affect (0)	Decrease Stability (-0.67)	Actionable (gain of ubiquitination)	Not Damaging (-1.69)	c->h	PC
M309L	B (0.00)	Tol (0.08)	Increase Stability (0.10)	No Impact	Not Damaging (-1.52)	h->h	NC
M309T	B (0.00)	Tol (1)	Decrease Stability (-1)	No Impact	Not Damaging (-1.56)	h->c	NC
S310P	Prb (0.97)	Affect (0)	Decrease Stability (-0.25)	Actionable (gain of glycosylation)	Damaging (-3.86)	h->c	NC
K311N	Pos (0.89)	Affect (0.04)	Decrease Stability (-0.45)	Actionable (loss of methylation, ubiquitination)	Not Damaging (-2.63)	h->e	NC
M315L	B	Tol (0.08)	Decrease Stability (-0.17)	Actionable (loss of catalytic residue)	Not Damaging (-1.82)	e->h	NC
M316T	B (0.00)	Tol (0.1)	Decrease Stability (-1)	No Impact	Not Damaging (-1.76)	h->c	NC
F317I	Pos (0.89)	Affect (0)	Decrease Stability (-0.84)	Actionable (gain of catalytic residue)	Damaging (-3.55)	h,c->e	C
F317L	Pos (0,62)	Affect (0)	Increase Stability (0.08)	No Impact	Not Damaging (-2.81)	c->h	C
R318G	Prb (0.99)	Affect (0)	Decrease Stability (-1)	No Impact	Damaging (-4.14)	h->e	NC
L320M	Pos (0.70)	Tol (0.09)	Decrease Stability (-0.24)	No Impact	Damaging (-3.30)	h->e,c	WC
S321N	B (0.3)	Affect (0)	Decrease Stability (-0.46)	No Impact	Not Damaging (-2.42)	h,e->c	NC
L324I	B (0.26)	Affect (0.01)	Decrease Stability (-0.76)	No Impact	Not Damaging (-2.54)	h,e->c	PC
L324F	Prb (0,.96)	Affect (0.01)	Decrease Stability (-1)	Actionable (loss of catalytic residue)	Not Damaging (-2.87)	h->c	PC
Y325H	Prb (0.93)	Affect (0)	Decrease Stability (-0.90)	No Impact	Damaging (-3.43)	h->e,c	NC
L327V	B (0.00)	Tol (0.17)	Increase Stability	No Impact	Not Damaging	h-	NC

			(0.17)		(-2.30)	>e,c	
L327P	Pos (0.83)	Affect (0)	Decrease Stability (-1)	No Impact	Damaging (-3.96)	h->c	NC
L328R	Pos (0.99)	Affect (0)	Decrease Stability (-0.26)	No Impact	Damaging (-3.71)	h->c	PC
A329G	B (0.00)	Affect (0)	Increase Stability (0.1)	No Impact	Damaging (-3.11)	h->c,e	NC
A329V	B (0.00)	Tol (1)	Increase Stability (1)	No Impact	Not Damaging (-1.73)	h->c,e	NC
A330T	B (0.00)	Affect (0)	Decrease Stability (-1)	Actionable (loss of stability)	Not Damaging (-2.18)	h->e	NC
D331G	Prb (0.98)	Affect (0)	Decrease Stability (-1)	Actionable (loss of stability)	Damaging (-3.08)	h->e	NC
L242R	Prb (0.96)	Affect (0)	Decrease Stability (-1)	Actionable (loss of stability, gain of methylation, gain of sheet)	Damaging (-3.77)	h->h	NC
L333F	B (0)	Tol(0.33)	Decrease Stability (-0.75)	No Impact	Not Damaging (-2.47)	h->e	PC
L333H	Pos (0.67)	Affect (0)	Decrease Stability (-0.92)	Actionable (loss of stability)	Damaging (-3.98)	c->h	PC
I334L	B (0.00)	Affect (0)	Decrease Stability (-0.72)	No Impact	Not Damaging (-1.41)	h->h	PC
L335Q	Prb (0.99)	Affect (0)	Decrease Stability (-1)	Actionable (loss of stability)	Damaging (-4.51)	h->c	C
T336A	B (0.29)	Affect (0)	Decrease Stability (-0.31)	No Impact	Not Damaging (-2.67)	c->h	NC
W337R	Prb (0.99)	Affect (0)	Decrease Stability (-0.12)	Confident (loss of catalytic residue)	Damaging (-3.57)	h->c	C
I338M	Pos (0.49)	Affect (0)	Decrease Stability (-0.62)	No Impact	Damaging (-3.20)	c->c	PC
Q341K	B (0.34)	Affect (0)	Increase Stability (0.06)	Actionable (gain of methylation, gain of ubiquitination)	Not Damaging (-2.62)	c->c	NC
V343E	Pos (0.82)	Affect (0)	Increase Stability (0.74)	No Impact	Not Damaging (-2.10)	c->c	NC
Y345H	Pos (0.93)	Affect (0)	Decrease Stability (-0.48)	No Impact	Not Damaging (-1.31)	c->c	NC
Y345D	Pos (0.93)	Affect (0)	Decrease Stability (-0.19)	No Impact	Not Damaging (-1.32)	e->c	NC
P346S	Prb (0.99)	Affect (0)	Decrease Stability (-0.86)	No Impact	Not Damaging (-2.41)	c->e	C
F347V	Pos (0.91)	Affect (0)	Increase Stability (0.05)	No Impact	Damaging (-3.37)	e->c	NC
F347C	Prb (0.99)	Affect (0)	Decrease Stability (-0.19)	Actionable (gain of catalytic residue)	Damaging (-4.88)	e->c	NC
F347L	Pos (0.73)	Affect (1)	Increase Stability (0.04)	No Impact	Not Damaging (-2.81)	e->c	NC
T348N	B (0.00)	Affect (0)	Increase Stability (0.07)	No Impact	Not Damaging (-2.22)	e->h	NC
T348I	B (0.00)	Tol (1)	Increase Stability (0.63)	No Impact	Not Damaging (-1.34)	e,c->h	NC
I349T	B (0.00)	Tol (0.32)	Decrease Stability (-1)	No Impact	Not Damaging (-2.07)	c->e	NC
I350M	Pos (0.86)	Affect (0)	Decrease Stability (-0.61)	No Impact	Not Damaging (-2.82)	c->e	C
V353E	Pos (0.93)	Affect (0)	Increase Stability (0.19)	No Impact	position not align	e->h	PC
A354V	Prb (0.99)	Affect (0)	Increase Stability (0.60)	No Impact	position not align	c->e	NC
L357M	Pos (0.81)	Tol (0.08)	Decrease Stability (-1)	No Impact	position not align	c->e	NC
L357V	B (0.20)	Affect (0)	Decrease Stability (-1)	Actionable (gain of catalytic residue)	position not align	c->e	NC
T360A	B (0.10)	Tol (0.25)	Decrease Stability (-0.10)	No Impact	position not align	e->h	NC
T361M	Pos (0.96)	Affect (0.01)	Increase Stability (1)	No Impact	position not align	e->h	NC
I362V	B (0.01)	Affect (0.01)	Decrease Stability (-0.48)	No Impact	position not align	c->e	NC
L363R	Prb (0.96)	Affect (0)	Decrease Stability (-0.89)	No Impact	position not align	c->e	NC
I364L	B (0.00)	Tol (0.22)	Decrease Stability (-0.92)	No Impact	position not align	c->e	NC
I364F	B (0.00)	Tol (0.09)	Decrease Stability (-1)	No Impact	position not align	c->e	NC
L365V	B (0.10)	Affect (0.01)	Decrease Stability	No Impact	position not	e->c	PC

			(-1)		align		
M366L	B (0.00)	Tol (0.11)	Decrease Stability (-1)	No Impact	position not align	e->c	NC
M366I	B (0.00)	Affect (0)	Decrease Stability (-1)	No Impact	position not align	e->c	NC
T368N	B (0.01)	Affect (0)	Decrease Stability (-0.28)	No Impact	position not align	h->e	NC
T368I	B (0.00)	Tol (1)	Increase Stability (0.74)	No Impact	position not align	e->c	NC
COX III							
F227I	Prb (0.99)	Affect (0.05)	Increase Stability (0.48)	No Impact	Not Damaging (-1.53)	c->e	PC
ATPase 6							
F6L	Prb (0.97)	Affect (0.03)	Decrease Stability (-0.40)	No Impact	Damaging (-3.45)	c->c	PC
A11T	B (0.00)	Tol (0.77)	Decrease Stability (-1)	No Impact	Not Damaging (-2.25)	c->c	NC
I14T	Pos (0.74)	Affect (0.01)	Decrease Stability (-1)	Actionable (loss of stability)	Not Damaging (-2.23)	c->e	PC
T53I	B (0.01)	Tol (1)	Neutral (0)	No Impact	Not Damaging (-2.09)	e->h	NC
I24T	Prb (0.99)	Affect (0.01)	Decrease Stability (-1)	No Impact	Not Damaging (-2.79)	h->h	NC
T59A	B (0.00)	Tol (0.64)	Decrease Stability (-1)	No Impact	does not align	e->h	NC
A177T	Pos (0.91)	Tol (0.16)	Increase Stability (0.05)	No Impact	Damaging (-3.00)	h->e	NC
P187S	B (0.04)	Tol (0.25)	Decrease Stability (-1)	No Impact	Not Damaging (-2.79)	h,c->e	NC
ATPase 8							
T7A	B (0.00)	Affect (0.01)	Decrease Stability (-1)	No Impact	Not Damaging (-1.75)	e->c	NC

B: Benign, Prb: Probably damaging, Pos: Possibly damaging, Tol: Tolerable, c-random coil, e-extended sheets, h-helix, NC: Non-Conserved, PC: Partially Conserved, WC: Weakly Conserved, C: Conserved

Out of important mutations, change at 264 position (T264A, T264N), cause the substitution from Threonine to Alanine and Aspargine, which is strongly conserved in position. As this change is from polar, hydrophilic to hydrophobic amino acid, may lead to the improper protein folding. Loss of glycosylation which is predicted by MutPred and MuPro, may further affect the protein-protein interaction and decrease the stability of protein. However, there is no change in the secondary structure.

Amino-acid position 273 is conserved across species and there are 3 mutations (Y273N, Y273S, Y273C)which cause change from Tyrosine (aromatic) to Asparagine, Serine or Cysteine in different patients of BC. Although GOR4 shows no secondary structure change except in Y273C, but all 3 are predicted to have a functional impact as predicted by PolyPhen-2, SIFT and PANTHER as well as a major decrease in stability, predicted by MuPro.

L281R mutation causes a change from a neutral, non polar amino acid Leucine to a polar, strongly basic amino acid Arginine which may affect the charge distribution on the surface of the CytB protein at the specific position. The result of MuPro also supported the same by predicting this change to decrease the stability of the protein. Moreover, according to MutPred, the mutation caused a gain of catalytic residue which may possibly alter the rate of the catalytic reaction. This mutation seems to be critical as on one hand it is suggested to be damaging by *in-silico* tools in the study and on the other hand it affects the secondary structure from helix to coil. The importance of the position was also shown by its evolutionary conservation.

On the other hand, though L242R, L288M, L292R, L328R mutations are not present at conserved positions but predicted to be damaging with a decrease in stability. Leucine is a hydrophobic amino acid and Arginine is polar, positively charged which might change the ionic environment around the protein at the specific position. Methionine, otherwise being fairly non-reactive but has a propensity to be found in the helical structures, and further hence L288M, L328R, L242R predicted to cause change the secondary structure from coil to helix. For all the mutations, MutPred predicted very opposite effects *i. e* loss of methylation in L288M and gain of methylation in case of L292R whereas PolyPhen-2 predicted it as Benign. PANTHER showed a damaging effect of mutations for L242R and L328R.

The three mutations P270T, E271G, S310P have almost similar damaging effect on Cyt B protein since in both the cases there is a change between polar and non-polar nature of amino acids. The substantial reduction in stability in case of P270T might be due to the branched side chain of Threonine causing steric collisions and destabilizing the protein structure, whereas in case of E271G, charge difference between acidic Glutamic acid and neutral small Glycine may be the causing factor. Also, since E271G causes a secondary structure changes from helix to extended sheet and coil, it might cause altered rigidity/flexibility in the protein structure at specific site.

Both the changes W272L and F317I are predicted to be damaging and unstable which is supported by the fact that Phenyl alanine, Tryptophan being aromatic occupies a larger space than Leucine, and hence the extent of interaction with its neighboring residues might get affected. Moreover, helical confirmation is changed to coils and sheets as predicted by GOR4.

The importance of G290S mutation lies in the fact that Glycine is unique in having much more conformational flexibility than any other amino acid, due to which it can reside in parts of protein structures forbidden to all other amino acids. This means that substitution of a conserved Glycine residue with Serine change could have a drastic impact on function. The tools strongly supported this with a high score value.

In case of mutations H308E, K311N and R318G, both K311N and R318G were found to drastically affect the stability of protein except

H308E. However, for H308E and K311N mutations, MutPred predicted to cause ubiquitination of protein. Ubiquitin moieties are responsible for directing the protein to degradation pathway. Additionally, Histidines are the most common amino acids present in active/binding site of proteins so its loss at the specific position in protein might affect the catalysis process of substrate. On the similar lines, all these three substitutions were indicated damaging by PolyPhen-2 and SIFT too. As a result of L324F mutation, non-polar aliphatic amino acid Leucine is being changed to aromatic Phenylalanine. Leucine is known to be the strong helix-favoring residues because of long side chains, whereas in Phenylalanine side chains are branched due to which it prefers to be in β sheets [15]. This is correctly supported by the tool GOR4 which predicted the change from helix to extended sheets. Also, this might cause steric hindrance to interactions with other protein subunits. Moreover, it is also proposed to decrease the stability of protein and loss of catalytic residue by the tools of our study

Y325H mutation is not present at conserved position, but still proposed to be deleterious by *in-silico* tools. Tyrosine residues, being involved in phosphorylation events by various kinases and play an important role in protein function. Therefore, its substitution by Histidine may account for its damaging effects. Also, there is a substantial decrease in stability which may be due to change in aromatic to aliphatic amino acid. As per GOR 4, the helical structure at this position is predicted to be converted in both coil and extended sheet with equal probability. In case of mutations L327P, I350M both amino acids belong to the same neutral, non-polar category. Mur Pred did not predict this change to be deleterious, but PolyPhen-2 and SIFT suggested it to be pathogenic, L327P was further predicted damaging by PANTHER too.

D331G is a change from polar, acidic Aspartic acid to non-polar, neutral Glycine. Tools used in the study have suggested it to be pathogenic. The wide difference between the hydrophobicity and size of Aspartic acid and Glycine may account for the pathogenic nature of this mutation. The negative charge of Aspartic acid means that it can interact with metallic ions carrying opposite charge, while change to Glycine may affect the molecular interactions. This may be the possible explanation towards the decreased stability of the protein which is also supported by the MutPred.

By looking at the scores of PolyPhen-2 and SIFT, L333H and L335Q, have similar damaging effects on the protein structure and function. Also, both mutations are causing decrease in stability with comparable scores which is supported by Mut Pred with an actionable hypothesis of loss of stability. This can be explained, on the basis of the fact that Leucine (small, non-polar molecule) is substituted with Histidine or Glutamine, which are polar in nature and hence will have altered tendency to form bonds with neighboring amino acids. Due to change in nature of amino acids, secondary structure is also changed with respect to these mutations.

The W337R mutation replaces Tryptophan by Arginine. As, Tryptophan has very hydrophobic aromatic side chains, so its replacement with polar strongly basic Arginine might be disastrous at that particular conserved position. This is completely supported by the tools which predicted it to be damaging. Because of this mutation, a regular helical conformation is converted to random coil and the reason might be loss of stacking interactions by the aromatic amino acids. Based on the confident hypothesis of MutPred, this mutation is also found to result in loss of catalytic residue.

F317I and F347C both resulted in a change from aromatic to aliphatic amino acid which might account for its damaging effect. Isoleucine is more restricted in conformation of it's main chain and hence prefers to lie within β sheets. Favoring it, GOR4 predicts change in secondary structure of protein from helix and coil to extended sheets due to F317I mutation. While the mutation F347C is not present at conserved position and almost neutral in its influence on protein's stability, F317I is predicted to result in substantial decrease in stability and also found at conserved position. Both the mutations are hypothesized to cause gain of catalytic residue, which may further alter enzyme activity.

Table 3: Reported non-synonymous mutations in protein-coding mitochondrial gene subunits associated with ovarian cancer (OC)

Mutations	PolyPhen-2	SIFT	MuPro	MutPred	PANTHER	GOR4	MUSCLE
	Prediction (score)	Prediction (score)	Prediction (score)	Hypothesis	Prediction (subPSEC)		Prediction
ND1							
D248N	Error	Tol (0.79)	Decreased Stability (-0.27)	No Impact	Not Damaging (-1.11)	c->c	NC
ND4							
A131T	B (0.03)	Tol (0.05)	Decreased Stability (-0.55)	No Impact	Position not align	e->e	NC
N424S	B (0.00)	Tol (0.42)	Decreased Stability (-0.16)	No Impact	Not Damaging (-2.48)	c->c	NC
ND5							
S523P	Error	Tol (0.08)	Increased Stability (0.37)	No Impact	Damaging (-4.16)	e->c	NC
M1T	Score not available	Affect (0.00)	Decreased Stability (-0.45)	confident (gain C. R, loss stability), very conf (gain glycosylation)	Position not align	e->c	NC
CytB							
S213P	Pos (0.95)	Affect (0.00)	Decreased Stability (-0.11)	No Impact	Damaging (-3.24)	e->c	WC
K217E	Pos (0.60)	Affect (0.00)	Decreased Stability (-0.11)	No Impact	Damaging (-3.79)	e->c	C
A302V	B (0.03)	Tol (0.12)	Increased Stability (0.22)	No Impact	Not Damaging (-2.08)	h->h	NC
COX I							
M417T	Error	Tol (0.64)	Decreased Stability (-1)	No Impact	Not Damaging (-1.88)	e->c	NC
COX III							
A95V	B (0.00)	Tol (1)	Increased Stability (1)	No Impact	Not Damaging (-1.26)	c-e	NC
ATPase 8							
P36L	B (0.00)	Tol (0.23)	Decreased Stability (-0.42)	No Impact	Not Damaging (-2.12)	h,c-e	NC

B: Benign, Prb: Probably damaging, Pos: Possibly damaging, Tol: Tolerable, c-random coil, e-extended sheets, h-helix, NC: Non-Conserved, PC: Partially Conserved, WC: Weakly Conserved, C: Conserved

ATpase6 and ATpase8

Apart from a large number of mutations in CytB subunit, a total of 8 and 1 nucleotide changes are also reported in ATPase6 and ATPase8 subunits of BC patients, respectively (Supplementary table1). F6L and I14T of ATPase6 were suggested pathogenic by most of the *in-silico* tools used in our study. Though Phenylalanine and Leucine both are hydrophobic and hence buried in protein core but

substitution of an aromatic compound by aliphatic amino acid might account for the damaging effect of F6L mutation. Isoleucine is also non-polar whereas Tyrosine is polar-aromatic compound containing a reactive hydroxyl group, thus making it much more likely to be involved in interactions with non-carbon atoms [16]. Although PANTHER score was less for this mutation, it is hypothesized as actionable due to loss of its stability. Furthermore, it changed the secondary structure from coil to extended sheets which may provide rigidity to the protein. In I24T mutation of ATPase6, both amino acids are hydrophobic in nature and found to be buried in protein core. The mutation is not present at conserved position and is not found to have any molecular impact or change in secondary structure. But since Isoleucine is aliphatic and Tyrosine is aromatic, this may have resulted in decrease in the protein stability as predicted by MuPro.

ND3, ND4 and ND5

Mitochondrial subunits such as ND3, ND4 and ND5 which are components of respiratory chain complex I also have limited number of mutations in BC patients and most of them are not predicted to affect the respective proteins. For example, mutations such T114A (ND3) and T337A (ND4) are not found to affect the proteins, but V381M in ND4 is predicted to affect the stability of the protein as per MuPro. The mutation is also suggested to affect the protein by PolyPhen-2, SIFT and PANTHER as well as causes change in the secondary structure from coil to extended sheet. Similarly N446K (ND5) though not present at conserved position, is predicted to affect the protein by all the rest of the tools. Moreover, the prediction by MutPred is confident in hypothesizing the mutation to cause gain in methyl and ubiquitin residue

CO3

It is one of the mitochondrial subunit of respiratory chain complex IV. F227I was the only mutation reported in CO3 subunit of BC patients. It was partially conserved and found to increase the stability of the protein. Although PolyPhen-2 and SIFT suggested it to affect the protein it is not predicted pathogenic by PANTHER and MutPred both.

Ovarian cancer

In contrast to BC, a small number of mutations in different mitochondrial subunits such as ND1, ND4, ND5, CytB, CO1, CO3, and ATPase8 are reported in OC patients [table 3]. As per our analysis, most of these mutations are not predicted to be of any significance except S213P and K217E mutations reported in CytB. Both these mutations were found to affect the protein function as per PolyPhen-2, SIFT and PANTHER. PolyPhen-2 score is observed highest in S213P. The possible reason for this high score might be the presence of hydroxyl group in serine side chain which is fairly reactive and able to form hydrogen bonds with a variety of polar substrates. But because of its substitution with Proline, this position of protein may become less reactive. Study of their effect on secondary structure of protein by GOR4 revealed that due to both the mutations, there is change of secondary structure from extended strand to random coil. However, these two mutations appeared to be somewhat neutral in their influence on protein stability which is also supported by absence of any structural change as predicted by Mut Pred.

CONCLUSION

Mutations and altered expression of the mtDNA encoded proteins have been observed in various tumours and appears to be a general feature of the cancerous cells. Though various studies have focused on identification and characterization of these alterations but have limited insights to its functional and clinical consequences especially when there is large number of mutations reported. Our study highlights the pathogenic importance of different mitochondrial protein mutations in case of BC and OC. As per our analysis, out of all the protein coding mitochondrial genes, CytB gene seems to carry largest number of pathogenic mutation (28) in BC. All these mutations of CytB are predicted to affect the protein in different manner which may ultimately alter the activity of complex III enzyme. On the basis of our vigorous analysis and predictions made by various *in-silico* tools, some most important mutations, which are predicted to significantly pathogenic are T264A, T254N, P270T, E271G, W272L, Y273N, Y273S, Y273C, L281R, L288M, G290S, L292R, H308E, S310P, K311N, F317I, R318G, L324F, Y325H, L327P, L328R, D331G, L242R, L333H, L335Q, W337R, F347C, I350M in BC All these selected mutations may be further analyzed for its functional relevance in *in-vitro* system. Apart from CytB, mutation in other mitochondrial subunits did not show such a high level of significance. The total number of mitochondrial gene mutations is very limited in case of OC patients, however the maximum number of mutations which are predicted to be significantly pathogenic are again localized to CytB only. S213P and K217E are two mutations in CytB which are predicted to be highly pathogenic in OC. It is conceivable that mtDNA variants (especially CytB) might play very important role in influencing the risk of BC and OC risk. New prognostic markers of BC metastasis are urgently needed to avoid overtreatment or under treatment of newly diagnosed patients. This knowledge will allow the development of new diagnostic methods, which might lead to more effective intervention strategies for BC and OC.

CONFLICT OF INTERESTS

Declared None

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