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Mitochondrial ND3 Gene Mutations in Breast Cancer Patients: Experimental and Computational Analysis of Mt: A10398G

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ABSTRACT

Breast cancer is the most common malignancy in women and a leading cause of mortality, despite advancements in screening and treatment. The mitochondrial genome shows high variability in breast cancer patients, with mutations linked to altered energy production in tumor cells. This study investigates molecular alterations in the MT-ND3 gene, part of NADH dehydrogenase (Complex I of the respiratory chain), in women with breast cancer. Blood and tissue samples were collected from breast cancer patients, with blood samples from selected healthy individuals. The MT-ND3 gene was amplified using PCR and analyzed via Sanger sequencing. Bioinformatics tools, including PolyPhen-2, PhD-SNP, PANTHER, Align-GVGD, and SNPs and GO, evaluated the pathogenicity of the mutations. Sequencing revealed a mutation at position 10398 (A>G), resulting in a threonine to alanine substitution, along with two synonymous mutations (MT: 10400 and MT: 10253). This mutation was found in 75% of breast cancer patients, consistent with studies from Sri Lanka and Bangladesh, but absent in Polish women. *In silico* analysis indicated this mutation is likely benign but decreases protein stability (free energy change of -0.5 kcal/mol) and increases hydrophobicity. These findings suggest that the MT-ND3 mutation may contribute to breast cancer development and underscore the need for further research to clarify the relationship between MT-ND3 mutations and breast cancer, particularly regarding gene expression and cancer biology implications.

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Authors' Contribution

MM conceived the idea and supervised the study, BR performed experimentation and drafted the manuscript, ZH assisted with the manuscript and evaluated results, HM performed the computational analysis.

Key words

Breast cancer, MT-ND3, A > G mutation, Synonymous mutation

INTRODUCTION

Carcinogenesis is characterized by the uncontrolled proliferation of cells that have the capacity to invade surrounding tissues (Azamjah *et al*., 2019). Among all malignancies, breast cancer is the most lethal cancer affecting women globally (Brown *et al*., 2023). The 2020 GLOBOCAN report recorded 2,261,419 diagnosed cases of breast cancer in women worldwide, resulting in 684,996 fatalities. In Pakistan, breast cancer is often diagnosed approximately a decade earlier than in Western countries (Castañeda *et al*., 2022). The same report indicates that Pakistan had 25,928 new cases of breast cancer and 13,725 deaths among women, representing a higher incidence than all other cancers affecting women in the country. Currently, nearly 79% of breast cancer cases occur in women aged 50 and older, with more than 40% in those over 65. The risk of developing breast cancer increases with age: approximately 1.5% after 40 years, 3% after 50, and over 4% after 70 (Chomyn *et al*., 1986). The incidence peaks around menopause, subsequently either declining or stabilizing (Francis *et al*., 2013).

The mitochondrial genome is particularly susceptible to mutations due to the absence of introns and histone proteins. Single deletions in mitochondrial DNA (mtDNA) occur randomly, typically resulting from errors in replication or repair, while multiple deletions are less common (Grzybowska *et al*., 2014). Mitochondria contain a unique genome known as mitochondrial DNA (mtDNA), which encodes essential components such as adenosine triphosphate (ATP) synthase, transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), and core elements of the respiratory chain across most species. mtDNA is maternally inherited, passed down exclusively through gametogenesis and embryogenesis (Habbane *et al*., 2021). Mitochondria have long been implicated in carcinogenesis, as disrupted energy metabolism is a hallmark of cancer (Jayasekera *et al*., 2023). Beyond energy metabolism, mitochondria are crucial for various processes, including biosynthesis, signaling, cellular differentiation, apoptosis, cell proliferation, and cell cycle regulation, all of which

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are intricately linked to cancer (Jin *et al*., 2018).

An emerging field in cancer research examines mitochondrial gene variations and their links to breast cancer. The MT-ND3 gene, which encodes mitochondrial NADH dehydrogenase 3, is a key component of Complex I in the electron transport chain. Investigations into the ND3 A10389G polymorphism have shown inconsistent results regarding its association with breast cancer risk, although it has been noted to affect mitochondrial pH and intracellular calcium levels (Kim *et al*., 2015).

Variations in MT-ND3 have been reported across diverse populations, including those in Bangladesh, Sri Lanka, Poland, and South India, suggesting potential connections to breast cancer (Li *et al*., 2019). Additionally, mutations in the MT-ND3 gene have been documented in African, American, and Asian cohorts, with the highest mutation frequencies observed in the *CO1* and *ATP6* genes. However, these studies have not established significant associations between the mutations and specific breast cancer subtypes (May *et al*., 2021). In Bangladesh, the A8812C variation in the *ATPase 6* gene leads to a threonine-to-proline substitution, proposed as a potential biomarker for breast cancer (McGuire *et al*., 2015). Pérez *et al*. (2020) identified 709 variants in breast cancer patients across various positions in the mitochondrial genome, including 685 single nucleotide polymorphisms (SNPs), 12 small deletions, and 12 small insertions. Notably, 438 variants were found in coding regions, with 17 variations located in the MT-ND3 gene. Their meta-analysis found no significant association between the MT-ND3 10398 polymorphism and breast cancer risk (Otaegui *et al*., 2004). In Sri Lanka, however, mutations such as A10398G in MT-ND3 and A8701G and A8860G in MT-ATP6 were present in over 50% of both breast cancer patients and control subjects (Li *et al*., 2019).

In this study, we collected breast tissue and blood samples from breast cancer patients to analyze the mutational landscape of MT-ND3 and further elucidate its relationship with breast cancer.

MATERIALS AND METHODS

Sample collection

Tissue samples were collected immediately after surgery and stored in biopsy jars at -22°C without any chemicals. Blood samples were collected in EDTA tubes and similarly stored at -22°C. Tissue samples were specifically used to identify somatic mutations, while blood samples were analyzed for germline mutations. All patient cases involved ductal carcinoma. The patients were from various regions of Punjab, representing a diverse demographic across different age groups. Each patient was diagnosed with varying grades and symptoms of cancer, but all had undergone modified radical mastectomy (MRM). Control samples were also collected from a random population and the family members of the patients to establish a comparative baseline.

DNA extraction

DNA extraction from tissue samples was performed using the organic method, involving stages of cell lysis, protein precipitation, and alcohol purification. For blood samples, DNA was isolated using a commercial DNA isolation kit following the manufacturer's protocols. The extracted DNA from both control and breast cancer samples was quantified via 2% agarose gel electrophoresis and subsequently visualized using a UV gel documentation system. The reaction mixture for PCR included 2µl of template DNA from breast tissue or blood samples. 1.5 µl of each primer,15µl injection water and 10µl of prepared 2X PCR Master Mix added to reach a final volume of 30µl. A negative control was also used to check the credibility with same concentrations but rather than DNA template water template was added. The thermal cycling program consisted of an initial denaturation at 95^{o} for 5 min, followed by 35 cycles of denaturation at 94^{o} for 45 seconds, annealing at 59^{0} for 45 seconds, and extension at 72^{o} for one min. A final extension step was conducted at 72^{o} for 10 min.

The PCR products were then analyzed using gel electrophoresis to verify amplification success and check fragment size. The amplified products were mixed with loading dye and loaded onto a 2% agarose gel in 1X TAE buffer stained with ethidium bromide for visualization. Electrophoresis was performed at 120 volts for 25-35 min, and the gel was examined under UV light.

The size of the PCR products was observed to be almost 450 base pairs and compared to a DNA ladder to ensure they matched the expected size of the MT-ND3 fragment. Successful PCR products were subsequently purified using a purification kit (Invitrogen by thermo fisher scientific Pure Link PCR Purification Kit).The forward primer was used for the sequencing and commercial unidirectional sanger sequencing was done.

Bioinformatics analysis

The amplification products were subjected to comprehensive bioinformatics analysis to identify potential mutations within the MT-ND3 gene and their implications in breast cancer. The analysis commenced with sequence alignment using the Basic Local Alignment Search Tool (BLAST), which allowed for the comparison of the obtained sequences against reference sequences in the nucleotide database. This step was crucial for

identifying any discrepancies or mutations present in the patient samples.

Following the initial alignment, various specialized bioinformatics tools were employed to further evaluate the identified mutations. Panther was utilized to classify the mutations based on their functional characteristics and to predict their potential impact on protein function. This tool provided insights into the biological pathways affected by the mutations, highlighting their relevance in cancer biology. PhD-SNP and SNAP2 were then used to assess the potential pathogenicity of the mutations. PhD-SNP predicts the effect of amino acid substitutions on protein stability, while SNAP2 evaluates the impact of non-synonymous SNPs on protein function and structure. Both tools contribute valuable information regarding which mutations may influence disease progression or treatment response.

To further predict the functional consequences of the mutations, PolyPhen-2 and SIFT were employed. PolyPhen-2 analyzes the possible impact of an amino acid substitution on the structure and function of a protein, categorizing mutations as benign, possibly damaging, or probably damaging. SIFT, on the other hand, predicts whether an amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids. Together, these analyses help prioritize mutations for further investigation. Finally, Align-GVGD and SNPs and GO were used to assess the potential clinical significance of the mutations. Align-GVGD classifies variants based on their alignments with known disease-associated mutations, while SNPs and GO provides insights into the functional annotations of SNPs and their potential associations with diseases. The combination of these tools resulted in a comprehensive understanding of the mutations identified in the MT-ND3 gene, offering valuable insights into their roles in breast cancer growth, proliferation, development, aggression, and progression.

RESULTS

Sequencing analysis

Sequencing analysis revealed a missense mutation, A to G at MT: A10398G, present in tissue samples from three patients with varying grades of breast cancer. This A>G variation resulted in an amino acid substitution, changing threonine to alanine. Initially presumed to be a somatic mutation, its presence in the blood samples confirmed it as a germline mutation. Notably, the same mutation was detected in a family member (control sample) of one patient, suggesting a hereditary component. Additionally, a single nucleotide polymorphism (SNP), C to T at MT: T10400C, was identified in both tissue and blood samples from these patients; however, this SNP did not lead to any protein change. Another variation was observed in a different patient's tissue sample at MT: 10253, characterized as a synonymous substitution where both TTT and TTC code for phenylalanine. Importantly, no mutations were found in the blood samples of the random control population.

In silico *analysis of mutation MT:10398*

To assess the pathogenicity of the mutation MT: 10398, five bioinformatics tools were employed: PolyPhen-2, PhD-SNP, PANTHER, Align-GVGD, and SNPs and GO (Table I). These tools collectively predicted that the missense variation would have a neutral impact on the protein. Align-GVGD classified the mutation as Class C55, indicating no functional impact. Moreover, both PhD-SNP and SNPs and GO corroborated these findings, predicting the effect of the mutation to be neutral as well.

Table I. Predicted effect of MT: 10398 (MTND3:T114A) variation on protein stability using different *in silico* approaches.

Bioinformatic tools	Predicted impact
PANTHER	Probably benign
PhD-SNP	Neutral
ALIGN GVGD	Probably benign
PolyPhen-2	Benign
SNPS AND GO	Neutral

Assessment of protein stability change

Further analysis using bioinformatics tools like I-Mutant, mCSM, and MUpro suggested that the mutation MT: 10398 decreases the protein's stability (Table II). These tools estimated the free energy change associated with the mutation, indicating a potential destabilizing effect on the protein structure.

Table II. Predicted effect of observed variations on protein stability by in silico approaches.

BI Approaches	Stability	$^{\triangle\triangle}$G value (kcal/mol)
mCSM	Destabilizing	-0.564
MUpro	Decrease	-0.928
I-Mutant	Decrease	0.02

Conservatory role of deleterious variations in the MT-ND3 gene

The conserved regions within the MT-ND3 protein sequence were identified using the ConSurf technique, which assesses evolutionary conservation at each position

in the sequence (Fig. 1). The detected mutation MT: 10398 (MTND3: T114A) was located in a region of average conservation, as indicated by the scale provided. This position, T114A, was represented in white in the conservation map, suggesting that while it is not highly conserved, it may still play a role in protein function.

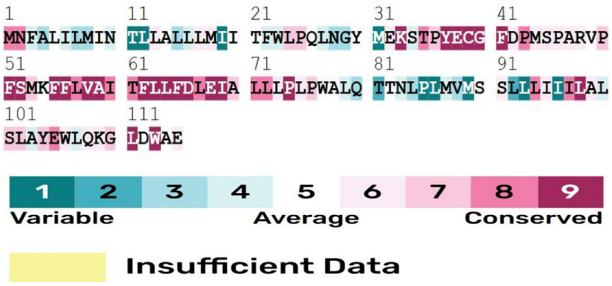

Fig. 1. Consurf mutant residue of MRND3.

Evaluation of protein by 3D structural models

Using Swiss Model, automated 3D models of both the mutant and wild-type MTND3 proteins were generated. The structural comparison revealed slight variations between the two models, each with different confidence levels concerning their reliability. The 3D protein structures for both the wild-type and mutant proteins were visualized using PyMOL. This structural visualization provided insights into the conformational differences between the two variants (Fig. 2).

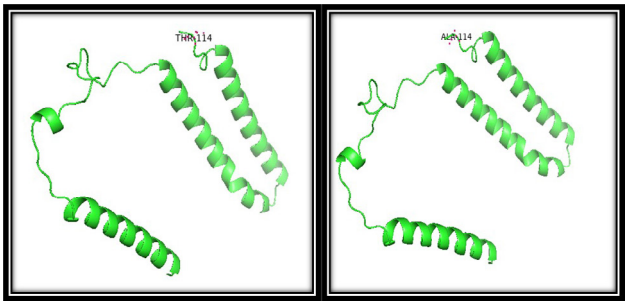

Fig. 2. Structure of wild (A) and mutant (B) MTND3 obtained from pyMOL.

Fig. 3. HOPE structural assessment (protein change from Threonine to Alanine).

HOPE analysis indicated that the mutation creates an empty space within the protein core. The mutant residue is smaller than the wild-type residue, resulting in the loss of hydrogen bonds that are vital for maintaining proper folding. Consequently, the mutant protein exhibited increased hydrophobicity, which could further disrupt its stability and function (Fig. 3).

Analyzing the interaction of ND3 by STRING

STRING database analysis revealed that MTND3 interacts with several proteins, including NDUFB10, NDUFA2, NDUFS4, NDUFS6, NDUFB7, NDUFS3, NDUFA10, NDUFA9, NDUFS7, and NDUFB9 (Fig. 4). Most of these proteins are accessory subunits of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I), which plays a crucial role in catalysis. Any alterations in these interacting proteins may impact the structural integrity and functional efficacy of the MTND3 protein, potentially influencing mitochondrial respiration and energy production.

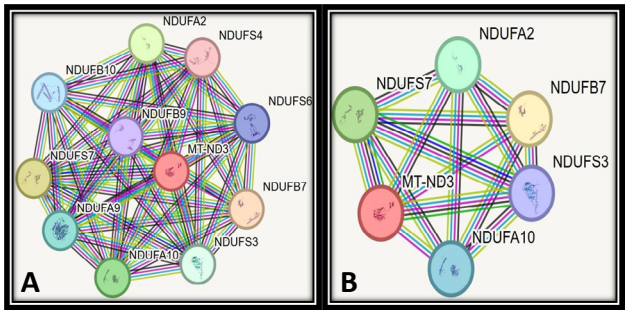

Fig. 4. Protein-Protein Interaction shown by STRING Database (A) interaction of MTND3 with naturally added proteins (B) Protein-protein interaction of MTND3 with different manually added proteins.

DISCUSSION

Mitochondria play a crucial role in energy production through respiration and oxidative phosphorylation, as well as in the production of reactive oxygen species (ROS) and the regulation of apoptosis. Cytotoxic by-products, such as ROS, can damage both cellular DNA and the mitochondrial genome, potentially contributing to tumorigenesis, as well as the growth, invasion, and metastasis of cancer cells (Otaegui *et al.*, 2004). Furthermore, alterations in the oxidative phosphorylation pathway, modifications to mitochondrial proteins, and increased ROS production, along with inherited variations in mitochondrial genes, may significantly influence the development of cancer (Richard *et al.*, 2000).

The circular mitochondrial genome, although small

at 16.6 kilobases, encodes 13 proteins that interact with nuclear-encoded proteins to form respiratory chain complexes. Among these, the mitochondrial NADH dehydrogenase subunit 3 (ND3) is one of seven mitochondrial-encoded proteins (ND1, ND2, ND3, ND4, ND5, ND6, and ND7) involved in the respiratory chain (Smullen *et al*., 2023).

In the present study, we identified a missense mutation at MT: A10398G, which results in a protein change from threonine to alanine, along with two single nucleotide polymorphisms (SNPs) at MT: 10400 and MT: 10253 in breast cancer patients. These findings contrast with those of Grzybowska *et al*. (2014), who reported no correlation between changes in the MT-ND3 gene and breast cancer. Their research, which involved fifty Polish patients, identified 28 changes in the mitochondrial genome but found none in the MTND3 gene (Soomro *et al*., 2018). Smullen *et al*. (2023) noted that the mutation MT: A10398G is significantly linked to heteroplasmy at five different loci. Their study utilized MITOMAP to examine each variant for associated disease phenotypes, revealing that among all reported MT variants, 10398A>G was linked to the greatest number of unique symptoms, including Type 2 diabetes, breast cancer, Parkinson's disease, and Alzheimer's disease. The presence of this same mutation in the breast cancer patients in our study aligns with the findings documented in MITOMAP (Sultana *et al*., 2011).

Interestingly, both breast cancer and Parkinson's disease patients share the mitochondrial 10398 variation. In the context of Parkinson's disease, this polymorphism has been identified as a protective factor (Touhidul Islam *et al*., 2021). A meta-analysis indicated that this variation was present in both diseased patients and control samples across most populations. However, in the Basque population, it was identified as a risk factor, appearing more frequently in diseased individuals than in controls (Yuan *et al*., 2020).

Additionally, our study found that mitochondrial variants 10398 and SNP 10400 are common among gastric cancer patients within the Korean population, suggesting an increased susceptibility to gastric cancer associated with these variants (Zong *et al*., 2016). According to NCBI, numerous variations have been reported in the MT-ND3 gene among patients with Leigh's syndrome, a neurological disorder. While variations in this gene have also been observed in breast cancer patients, the correlation between these mutations and breast cancer remains unclear due to a lack of expression studies. A related study that analyzed both pre- and postmenopausal breast cancer patients and control samples from women in South Asia found no correlation between breast cancer and the MT-ND3 10398 mutation, echoing our findings. Their meta-analysis similarly concluded that there was no association between this polymorphism and an increased risk of breast cancer (Pérez *et al*., 2020).

The results of Sultana *et al*. (2011) who conducted a meta-analysis investigating differences in the mitochondrial ND3 gene in breast cancer patients are consistent with our study. They sequenced 24 blood samples from individuals with breast cancer using MTND3 gene primers and identified two mutations, MT: A10398G and MT: 10400 (C>T), in 18 of the samples. Notably, three affected patients in our current investigation also exhibited the same mutations, 10398 and 10400. The presence of these mutations in both tissue and blood samples suggests that these variants represent germline mutations. Sultana *et al*. also found that this variation is common in Asian and African populations (Li *et al*., 2019).

Our findings align with those of Jayasekera *et al*. (2023), who studied 60 patients with sporadic breast cancer alongside control groups in Sri Lanka. In their study, 75% of the cases were invasive ductal carcinoma, consistent with our findings where all patients had invasive ductal carcinoma (Mao *et al*., 2013). Over half of the patients and controls in their study carried the MTND3 variants 10398 and 10400, which were also present in our study. Additionally, a blood sample from a patient's sister, who was older and healthy, revealed that she also carried the MT: 10398 mutation, although their study did not sequence family members' samples as a control group.

To date, no computational analysis of the MT: 10398 variation has been reported. In this study, we employed several in-silico tools to analyze the variation's potential impact. The deleterious effects of the protein variation were assessed using five different tools: SNPs and GO, PolyPhen-2, PhD-SNP, PANTHER, and Align GVGD. All tools predicted that the missense variation MT: 10398 has a neutral impact on the protein, with PANTHER, PolyPhen-2, and Align GVGD classifying the mutation as probably benign.

To evaluate the impact of the identified variation on protein stability, we utilized three web servers: I-mutant2.0, mCSM, and MUpro. These servers predicted that the mutation would decrease the protein's stability by estimating changes in free energy. HOPE analysis further revealed that this mutation reduced the protein's size, created empty spaces within the protein core, and increased its hydrophobicity. Finally, we modeled the 3D structures of both the wild-type and mutant proteins using Swiss Model and PyMOL, while the STRING database provided insights into the proteins interacting with MT-ND3.

CONCLUSION

This study concludes that mutations in the MTND3 gene are common among patients with invasive ductal carcinoma. Sequencing results revealed the MT: A10398G variation in both tissue and blood samples, alongside two single nucleotide polymorphisms (SNPs), MT: 10400 and MT: 10253. These variants appear to be more prevalent in Asian and African populations, as evidenced by their discovery in studies conducted in Bangladesh and Sri Lanka, while being absent in Polish women with breast cancer. Additionally, these mutations have been noted in patients with Parkinson's disease and may also increase susceptibility to gastric cancer. The presence of the detected variants in both blood and tissue samples suggests that they are germline mutations rather than localized somatic mutations. Although results from various in-silico tools indicate that the MT: A10398G mutation is likely benign, it also suggests a decrease in protein stability and an increase in hydrophobicity. The relationship between MTND3 mutations and breast cancer remains unclear, highlighting the need for further studies focused on the gene's expression and its potential role in cancer development.

DECLARATIONS

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IRB approval

The Advance Study and Research Board at University of Education, Lahore approved the protocol of the present study.

Ethical statement

To identify mutations, blood and tissue samples were collected from breast cancer patients after obtaining informed consent. The study was approved by the Ethical Review Board of the University of Education, Lahore. The informed consent process included detailed explanations of the study's objectives, procedures, and potential risks to ensure that participants were fully aware of their involvement.

Permission from Ethical Committee, University of Education, Lahore, was taken for the research work.

Generative AI or AI-assisted technology statement

No genarative AI or AI-assisted technology was used for this study.

Statement of conflict of interest

The authors have declared no conflict of interest.

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