Utilizing Advanced Tools to Analyze Breast Cancer Mitochondrial DNA Mutations

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Introduction:

Breast cancer is the most common cancer in women in the United States, accounting for about 30% (1 in 3) of all new female cancers each year (1). In 2022, there were 2,296,840 new cases of breast cancer reported among women. Many breast cancers are carcinomas originating from the epithelial cells lining the breast ducts, which are the channels that transport milk to the nipple (2). Breast cancer that starts in the ducts is called invasive ductal carcinoma. Breast cancer can also start in cells in the milk glands. These glands, called lobules, are designed to make breast milk. Cancer that occurs in the lobules is called invasive lobular carcinoma (3).

Studying how human cells replicate mitochondrial DNA (mtDNA) and its impact on our health is crucial. Many diseases are linked to problems with mtDNA replication. Exploring the different mechanism which involves mtDNA replication can provide valuable findings for diagnosing and treating diseases. *M.P. King, G. Attardi et al, 1996*, created special human cell lines (rho zero or rho 0 cells) which lack mtDNA by using the DNA intercalating dye, ethidium bromide (EtBr) (4). These cell lines help researchers investigate the effects of mtDNA defects. *Picard et al, 2014,* transferred wild type and mutant mtDNA (3243A>G) into a rho 0 cell line to create stable cybrids harboring different levels of mtDNA mutations (5). Our long-term goals are to determine breast cancer (BC)-specific mitochondrial DNA (mtDNA) mutations in a dataset of 32 patients and develop cell line models of cancer by separately transferring BC mtDNA mutations into rho zero cells depleted of mtDNA and determine their physiological consequences.

I am currently studying the research paper by Michael P. King and Giuseppe Attardi, where they utilized the 143B.TK cell line and, exposed it to EtBr, yielding a rho zero cell line. This study and the 143B.TK cell line are significant as others have failed to generate rho 0 cells using common human cell line like HeLa and Hep3B (4). Here we investigated BC mtDNA *ND3* gene mutations from 13 of 32 patients and treated the C2C12 cell line with EtBr to determine if mtDNA could be depleted.

Research Questions and Hypothesis:

➢ **Research Questions:**

1. Are there *ND3* tumor-specific mtDNA mutations in BC?
2. Can C2C12 cells be depleted of mtDNA?

➢ **Hypothesis:**

H_1: BC-specific mtDNA genome mutations alter cancer cell metabolism.

H_2: C2C12 cells can be successfully depleted of mtDNA through the use of specific chemical or genetic interventions.

The human mtDNA genome

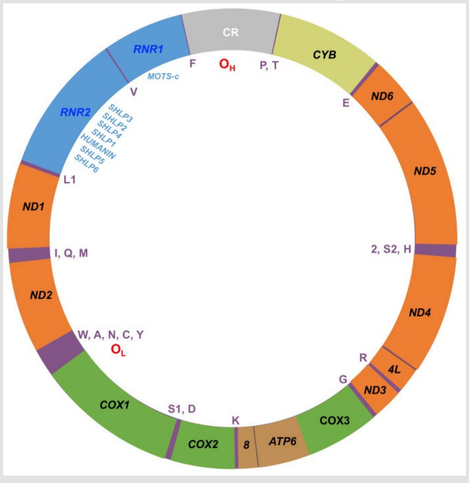


Figure 1. Map of human mtDNA.
•Orange: Complex I genes (*ND1*, *ND2*, *ND3*, *ND4L*, *ND4*, *ND5*, *ND6*)
•Yellow: Complex III genes (*CYB*)
•Green: Complex IV genes (*COX1*, *COX2*, *COX3*)
•Brown: Complex V genes (*ATP6*, *ATP8*)
•Blue: Small (12S) and large (16S) ribosomal RNA genes
•Gray: The non-coding control region (CR)
•OH (Origin of Heavy strand replication): This is the starting point for the replication of the heavy (H) strand of mtDNA.
•OL (Origin of Light strand replication): This is the starting point for the replication of the light (L) strand of mtDNA.
•SHLP1-6 (Small Humanin-like Peptides 1-6): These are a group of peptides encoded in 16S rRNA region of mitochondria.
•tRNA genes (F, P, T, E, L2, S2, & H): These genes encode transfer RNAs (tRNAs), which are essential for the translation of proteins within the mitochondrion. Each letter represents a different amino acid for which the tRNA is specific:

➢F: Phenylalanine
➢P: Proline
➢T: Threonine
➢E: Glutamic acid
➢L2: Leucine (second type)
➢S2: Serine (second type)
➢H: Histidine (6)

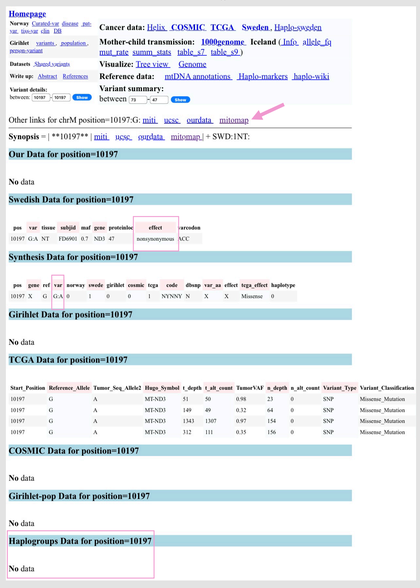
Materials and Methods

Part1, Rho 0 cell line

- Proliferating C2C12 cells were seeded at 2.53E3 cells/cm^2 in a tissue culture dish
- After 2 days the cells were treated with 0.1 µg/ml Ethidium Bromide (EtBr)
- The cells were grown for 6 weeks by changing media every 3 days and splitting them when they are 70-80% confluent
- Later they were trypsinized and seeded at 0.5 cells per well in a 96 well tissue culture plate with regular growth medium (DMEM)
- After 2 weeks two of the wells among 96 wells had cells in them, they were trypsinized and seeded in a separate 100mm dish with regular growth medium
- The cells in the plates were harvested after 80% confluency to obtain cell pellets of 5E6 cells/Eppendorf tube
- Whole-cell genomic DNA was extracted from the cell pellets using our in-house DNA extraction method and ethanol precipitation then quantified using a Qubit Fluorometer
- PCR was done using mouse mtDNA specific primers (MmDloop Forward and MmDloop Reverse) to confirm the presence or absence of mtDNA
- PCR products were subjected to electrophoresis in a 1% agarose gel in 1x TAE buffer at 100V for 1hr 30 minutes, stained with EtBr solution and a picture was taken using a G-box.

Part2, Using the BC Mseek database to investigate *ND3* mutations

Search for mtDNA mutation position of interest



mtDNA mutation information from MITOMAP

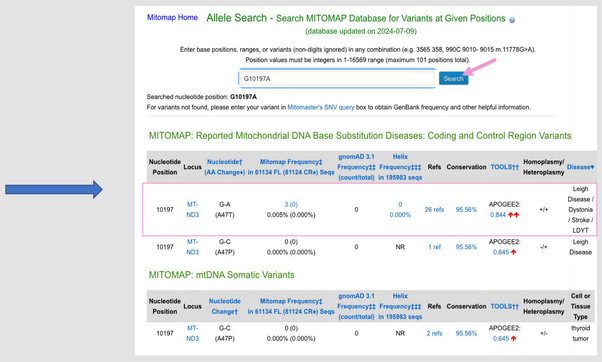


Figure 2. All the required links and information for a particular position in the database
† HGVS 3' Rule: we describe changes in genetic sequences (like deletions, duplications, or insertions) by following this rule
‡ Mitomap Frequency: Mitomap analyzed 61,134 FL sequences and found that 68% belong to haplogroup N (East Asian), 10% to haplogroup L (African), and 20% to haplogroup M (Asian).
‡‡ gnomAD v3.1 Frequency: This data comes from the gnomAD database. In the 56,434 sequences 70% belong to haplogroup N, 25% to haplogroup L, and 5% to haplogroup M.
‡‡‡ Helix Frequency: Distribution in Helix as N of 91% and 9% to haplogroups L and M combined. The rank NR means that there's no record in the database.
APOGEE2:APOGEE2 is a tool in MitImpact that combines data from 13 different predictors and 6 meta-predictors to assess the pathogenicity of genetic variants.
>0.75 likely pathogenic
>0.50-0.75 possibly pathogenic
>0.25-0.50 neutral/possibly benign
0-0.25 neutral/likely benign

Results:

Table 1: *ND3* Mutations identified in thirteen breast cancer patients.

Variant	Cohort[1]	NSG[2]	A.A.[3] Change	Notes
T10075C	SWD:1NT	0Y0	I6T	Nonsynonymous mutation. Likely Benign. No variant found in MitoMap
T10105C	SWD:1NT	0Y0	L16P	Nonsynonymous mutation. Not in haplogroup. Found in gene bank.
A10154G	SWD:1NT	0Y0	E32E	Synonymous mutation. Present in haplogroup. Referenced in Eastern ancestry. PubMed ID 16404693.
C10192T	SWD:1NT	0Y0	S45F	Nonsynonymous mutation. Present in haplogroup. Found in gene bank.
G10197A	SWD:1NT	0Y0	A47T	Nonsynonymous mutation. Not in haplogroup. Likely pathogenic. Mentioned in population genetic study (PubMed ID 38465286)
T10265C	SWD:1NT	0Y0	I69I	Synonymous mutation. Present in haplogroup. Western Pygmy populations likely have the L1c haplogroup mutation (PubMed ID 21041797)
T10275C	SWD:1NT	0Y0	L73L	Synonymous mutation. Not present in haplogroup.
A10283G	SWD:2NT	0Y0	L75L	Synonymous mutation. Present in haplogroup. Found several sequence variants, including secondary LHON mutations and novel missense mutations (PubMed ID 11820805).
T10326C	SWD:1NT	0Y0	S90P	Nonsynonymous mutation. Present in haplogroup. Found in gene bank.
G10386T	SWD:1NT	0Y0	G110W	Nonsynonymous mutation. Not present in haplogroup. These mutations are present in oncocytic tumors of the pituitary and head-and-neck regions (PubMed ID 20028790)
C10400T	SWD:2NT	0Y0	T114T	Synonymous mutation. Present in haplogroup. The studied population includes patients with mitochondrial cytopathies (CPEO, MERRF, MELAS, and LHON)- PubMed ID 7874114.

[1]SWD, NT, - SWD stand for the Sweden data in our data set whereas the N stands for normal and T stands for tumor, and the numbers in front of them stands for how many people have the same mutations in normal and tumor.
[2]NGS (0Y0), - Our data set have 3 sets of data Norway (N), Sweden (S) and Girihlet (G).
[3]A.A. – It stands for Amino Acid changes which describes that what type of mutation caused it. It can be synonymous (A synonymous mutation is a change in the DNA sequence that does not alter the amino acid sequence of the protein due to the redundancy in the genetic code) or nonsynonymous (a mutation which changes the DNA sequence in a way that results in a different amino acid being incorporated into the protein, which can affect the protein's structure and function).

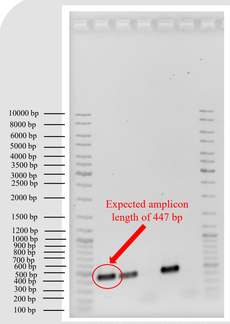


Figure 3. PCR amplification of C2C12 mtDNA
Lane 1- Gene ruler DNA ladder mix
Lane 2- Potential clone G6
Lane 3- Potential clone G7
Lane 4- Negative control reaction
Lane5- Positive control reaction
Lane 6- Negative control reaction
Lane 7- Gene ruler DNA ladder mix

Discussion and Conclusions:

- No tumor-specific mutations were identified in the one gene chosen to study here (*ND3* gene); however, the patients harbor other mtDNA mutations that need to be further investigated
- mtDNA PCR amplification was observed in G6 and G7 clones indicating mtDNA was incompletely depleted
- For future study I could use a different cell line (For example: 143TKB) or try using a different EtBr concentration or incubating the cells in EtBr for a longer time

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