# **Utilizing Advanced Tools to Analyze Breast Cancer Mitochondrial DNA Mutations**

Ashlishya Ghosh<sup>1,2</sup>, Pabitra Khadka<sup>2</sup>, Ravi Sachidanandam PhD<sup>3</sup>, Matthew J. Young PhD<sup>2</sup> <sup>1</sup>SI Bridges to the Baccalaureate, Southern Illinois University Carbondale <sup>2</sup> Department of Biomedical Sciences, Division of Biochemistry & Molecular Biology, SIU Carbondale School of Medicine <sup>3</sup> New York Medical College

## **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?

## > <u>Hypothesis</u>:

H<sub>1</sub>: BC-specific mtDNA genome mutations alter cancer cell metabolism.

H<sub>2</sub>: C2C12 cells can be successfully depleted of mtDNA through the use of specific chemical or genetic interventions.



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 ≻L2: Leucine (second type) ≻P: Proline

≻T: Threonine ≻E: Glutamic acid ≻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<sup>2</sup> in a tissue culture dish
- $\blacktriangleright$  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.

Homepage Norway <u>Curated-var</u> dis	case pat-	data: Helix C	OSMIC	C TCGA	Sweden	. Haplo-s	weden		Mitomap Home Allele Search - 9
<u>var tiss-var clin DB</u> Girihlet <u>variants</u> , pop	pulation, Mother	-child transmis	sion:	1000geno	<u>me</u> Icela	nd ( <u>Info</u>	<u>allele_fq</u>		
person-variant	mut_rate	e <u>summ_stats</u>	table_s	7 table_s	<u>9</u> )				Enter base positions, ranges, or var
Write up: Abstract Re	terences Referen	ce data: mtD	ONA ani	notations	Haplo-m	arkers ha	plo-wiki		Position val
Variant details:	Variant	summary:			<u></u>		<u></u>		G10197A
between: 10197 - 10197	show between	l 73 - 47 S	how						Searched nucleotide position: G10197A
Other links for chi	M position=1019	7:G: <u>miti</u> ucso	<u>ourd</u>	<u>ata mitc</u>	omap				For variants not found, please enter your varian
Synopsis =   **10	197**   <u>miti</u> u <u>cs</u>	<u>e ourdata m</u>	<u>itomap</u>	+ SWD:	1NT:				MITOMAR: Reported Mitochand
Our Data for po	osition=10197								MITOMAP. Reported Mitochond
No doto									Nucleotide Nucleotide† Mitom
		-							Position (AA change) in 61134 P
pos var tissue sub 10197 G:A NT FD6 Synthesis Data	jid maf gene protein 901 0.7 ND3 47 for position=10	loc effect nonsynonymous 197	varcodon ACC						10197 MT- G-C ND3 (A47P) 0.00
pos gene ref var 1	orway swede girihlet	cosmic tcga code	dbsnp	var_aa effe	ct tcga_effect	haplotype			MITOMAP: mtDNA Somatic Vari
Girihlet Data fo	r position=1019	<b>0</b> 7		л л	wissense	U			Nucleotide Nucleotide Mitom
	- p								Position Change† in 61134 F
No data									10197 MT- G-C ND3 (A47P) 0.00
TCGA Data for	position=10197	1							
									Figure 7 All the required links and inform
	ce_Allele Tumor_Seq_A	Allele2 Hugo_Symbo MT-ND3	51 t_depth	t_alt_count	TumorVAF 0.98	<b>n_depth n_al</b> 23 0	t_count Variant_Typ SNP	Wariant_Classification Missense Mutation	* UCVS 3! Dula: we describe changes in constitution
Start_Position Referen 10197 G	A	MT-ND3	149	49	0.32	64 0	SNP	Missense_Mutation	<sup>†</sup> <b>HGvS 5</b> <sup>+</sup> <b>Kule:</b> we describe changes in genetic seq
Start_Position         Referen           10197         G           10197         G		MT-ND3	1343	1307	0.97	154 0	SNP	Missense_Mutation	following this rule
Start_Position         Referent           10197         G           10197         G           10197         G           10197         G	A	MENDO		111	0.35	156 0	SNP	Missense_Mutation	<b>Thitomap Frequency:</b> Mitomap analyzed 61,134 F
Start_Position         Referent           10197         G           10197         G           10197         G           10197         G           10197         G	A A	MT-ND3	312						
Start_Position         Referent           10197         G           10197         G           10197         G           10197         G           10197         G           10197         D           10197         G	A A for position=10	MT-ND3	312						(East Asian), 10% to haplogroup L (African), and 20%
Start_Position         Referent           10197         G           10197         G           10197         G           10197         G           10197         G           10197         Data	A A for position=10	MT-ND3	312						(East Asian), 10% to haplogroup L (African), and 20% <b>‡</b> gnomAD v3.1 Frequency: This data comes from
Start_Position         Referent           10197         G           10197         G           10197         G           10197         G           10197         G           10197         Data	A A for position=10	MT-ND3	312						(East Asian), 10% to haplogroup L (African), and 20% <b>‡‡ gnomAD v3.1 Frequency:</b> This data comes from to haplogroup N, 25% to haplogroup L, and 5% to ha
Start_PositionReferen10197G10197G10197G10197GCOSMICDataNo dataGirihlet-pop Data	A A for position=10 ta for position=	MT-ND3 197 =10197	312						(East Asian), 10% to haplogroup L (African), and 20% <b>‡‡ gnomAD v3.1 Frequency:</b> This data comes from to haplogroup N, 25% to haplogroup L, and 5% to ha <b>‡‡‡ Helix Frequency:</b> Distribution in Helix as N of 9
Start_Position     Referent       10197     G       10197     G       10197     G       10197     G       10197     G       10197     Data	A A for position=10 ta for position=	MT-ND3 197 =10197	312						(East Asian), 10% to haplogroup L (African), and 209 <b>‡‡ gnomAD v3.1 Frequency:</b> This data comes from to haplogroup N, 25% to haplogroup L, and 5% to ha <b>‡‡‡ Helix Frequency:</b> Distribution in Helix as N of 9 NR means that there's no record in the database.
Start_Position     Referent       10197     G       10197     G       10197     G       10197     G       10197     G       10197     Data <b>COSMIC Data No</b> data <b>No</b> data <b>No</b> data	A A for position=10 ta for position=	MT-ND3 197 =10197	312						<ul> <li>(East Asian), 10% to haplogroup L (African), and 209</li> <li><b>‡‡ gnomAD v3.1 Frequency:</b> This data comes from to haplogroup N, 25% to haplogroup L, and 5% to ha</li> <li><b>‡‡‡ Helix Frequency:</b> Distribution in Helix as N of 9</li> <li>NR means that there's no record in the database.</li> <li><b>APOGEE2</b>:APOGEE2 is a tool in MitImpact that compared to the second se</li></ul>
Start_Position         Referent           10197         G           10197         G           10197         G           10197         G           10197         G           10197         G           10197         Data           COSMIC         Data           No data         Start           No data         Haplogroups Data	A A for position=10 ta for position=	MT-ND3 197 =10197 =10197	312						<ul> <li>(East Asian), 10% to haplogroup L (African), and 20%</li> <li><b>‡</b> gnomAD v3.1 Frequency: This data comes from to haplogroup N, 25% to haplogroup L, and 5% to haplogroup L, and 5% to haplicate the second second</li></ul>

## **Results:**

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

	Variant	Cohort <sup>1</sup>	NSG <sup>2</sup>	A.A. <sup>3</sup> Change	Notes
	T10075C	SWD:1NT	0Y0	I6T	Nonsynonymous mutation. Likely Benign. No variant four
	T10105C	SWD:1NT	0Y0	L16P	Nonsynonymous mutation. Not in haplogroup. Found in ge
	A10154G	SWD:1NT	0Y0	E32E	Synonymous mutation. Present in haplogroup. Referenced 16404693.
	C10192T	SWD:1NT	0Y0	S45F	Nonsynonymous mutation. Present in haplogroup. Found in
	G10197A	SWD:1NT	0Y0	A47T	Nonsynonymous mutation. Not in haplogroup. Likely path study (PubMed ID 38465286)
	T10265C	SWD:1NT	0Y0	I69I	Synonymous mutation. Present in haplogroup. Western Pyghaplogroup 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 seven LHON mutations and novel missense mutations (PubMed 2)
	T10326C	SWD:1NT	0Y0	S90P	Nonsynonymous mutation. Present in haplogroup. Found in
	G10386T	SWD:1NT	0Y0	G110W	Nonsynonymous mutation. Not present in haplogroup. The tumors of the pituitary and head-and-neck regions (PubMe
	C10400T	SWD:2NT	0Y0	T114T	Synonymous mutation. Present in haplogroup. The studied mitochondrial cytopathies (CPEO, MERRF, MELAS, and
C	WD NT SWI	Detand for the Swa	don data in i	our data sat whoreas th	a N stands for normal and T stands for tumor, and the numbers in front of them star

<sup>2</sup>NGS (0Y0), - Our data set have 3 sets of data Norway (N), Sweden (S) and Girhilet (G) <sup>3</sup>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

>0.50-0.75 possibly pathogenic >0.25-0.50 neutral/possibly benign

0-0.25 neutral/likely benign

affect the protein's structure and function).







#### **Figure 3. PCR amplification of C2C12 mtDNA**

SOUTHERN ILLINOIS UNIVERSITY

SI BRIDGES TO

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

# igate ND3 mutations

### **Cormation from MITOMAP**

Search MITOMAP Database for Variants at Given Positions  (database updated on 2024-07-09)											
ants (non-digits ignored) in any combination (e.g. 3565 358, 990C 9010- 9015 m.11778G>A). les must be integers in 1-16569 range (maximum 101 positions total).											
in Mitomaster's SNV query box to obtain GenBank frequency and other helpful information.											
ial DNA Base Substitution Diseases: Coding and Control Region Variants											
ap Frequency‡ <sub>-</sub> (81124 CR <u>+</u> ) Seqs	gnomAD 3.1 Frequency‡‡ (count/total)	Helix Frequency‡‡‡ in 195983 seqs	Refs	Conservation	TOOLS	Homoplasmy/ Heteroplasmy	Disease♥				
3 (0) 5% (0.000%)	0	0 0.000%	26 refs	95.56%	APOGEE2: 0.844 <b>↑↑</b>	+/+	Leigh Disease / Dystonia / Stroke / LDYT				
0 (0) 0% (0.000%)	0	NR	1 ref	95.56%	APOGEE2: 0.645 <b>↑</b>	-/+	Leigh Disease				
ants											
ap Frequency‡ <sub>-</sub> (81124 CR <del>≜</del> ) Seqs	gnomAD 3.1 Frequency‡‡ (count/total)	Helix Frequency‡‡‡ in 195983 seqs	Refs	Conservation	TOOLS	Homoplasmy/ Heteroplasmy	Cell or Tissue Type				
0 (0) 0% (0.000%)	0	NR	2 refs	95.56%	APOGEE2: 0.645 <b>↑</b>	+/-	thyroid tumor				

nation for a particular position in the database uences (like deletions, duplications, or insertions) by

sequences and found that 68% belong to haplogroup N to haplogroup M (Asian). he gnomAD database. In the 56,434 sequences 70% belong 1% and 9% to haplogroups L and M combined. The rank

nbines data from 13 different predictors and 6 meta-

nd in MitoMap ene bank. in Eastern ancestry. PubMed ID

in gene bank. ogenic. Mentioned in population genetic

gmy populations likely have the L1c

ral sequence variants, including secondary ID 11820805).

in gene bank. ese mutations are present in oncocytic ed ID 20028790) l population includes patients with

LHON)- PubMed ID 7874114.

# 1 2 3 4 5 6 7 **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

## **Acknowledgements:**

- > We are grateful for the opportunity provided by SI Bridges (AG), the Simmons Cancer Institute (TSG to MJY), and the NIH (R15 to MJY) for funding the program.
- > I really appreciate my co authors Pabitra Khadka to walk me through the procedures in the lab, Dr. Ravi Sachidanandam for providing the Mseek data and to make this interesting website to work on the sequencing, and Dr. Young for his mentorship and editing the poster.

# **References:**

[1] Key statistics for breast cancer. (2024, January 17). American Cancer Society. Retrieved July 18, 2024 from https://www.cancer.org/cancer/types/breast-cancer/about/how-

common-isbreastcancer.html#:~:text=The%20American%20Cancer%20Societ

y's%20estimates,will%20die%20from%20breast%20cancer. [2] Breast cancer statistics: World cancer research fund *international.*(2024, June 26). WCRF International. Retrieved July 18, 2024 from https://www.wcrf.org/cancer-trends/breast-cancerstatistics/.

[3] *Invasive lobular carcinoma*. (2024, June 26). Mayo Foundation for Medical Education and Research. Mayo Clinic. Retrieved July 20,2024 from https://www.mayoclinic.org/diseasesconditions/invasive-lobular-carcinoma/symptoms-causes/syc-20373973.

[4] King, M. P., and Attardi G. (1996) Isolation of human cell lines lacking mitochondrial DNA. *Methods Enzymol* **264**, 304-313 [5] Picard, M., Zhang, J., Hamcock, S., Derbeneva, O., Golhar, R., Golik, P., O'Heran, S., levy, S., Potluri, P., Lvova, M., Davila, A., Lin, C.S., Perin, J. C., Rappaporat, E.F., Hakonarson, H., Trounce, I. A., Procaccio, V., and Wallace, D.C. (2014) Progressive increase in mtDNA 3243A>G heteroplasmy causes abrupt transcriptional reprogramming. Proc Natl Acad Sci USA 111, E4033-4042. [6]Khadka P, Young CKJ, Sachidanandam R, Brard L and Young MJ (2024) Our current understanding of the biological impact of endometrial cancer mtDNA genome mutations and their potential use as a biomarker. Front. Oncol. 14:1394699. doi: 10.3389/fonc.2024.1394699

