Differential modulation of cancer-related genes by mitochondrial DNA haplogroups and the STING DNA sensing system

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Abstract
Activation of the Simulator of Interferon Genes (STING) system by mitochondrial (mt) DNA can upregulate type 1 interferon genes and enhance immune responses to combat bacterial and viral infections. In cancers, the tumor-derived DNA activates STING leading to upregulation of IFN-beta and induction of antitumor T cells. The entire mtDNA from the cell lines was sequenced using next-generation sequencing (NGS) technology with independent sequencing of both strands in both directions, allowing identification of low-frequency heteroplasmy SNPs. There were 15 heteroplasmy SNPs showing a range from 3.4% to 40.5% occurrence in the K cybrid cell lines. Three H haplogroup cybrids possessed SNP heteroplasmy that ranged from 4.39% to 30.7%. The present study used qRT-PCR to determine if cybrids of H and K haplogroups differentially regulate expression levels of five cancer genes (BRAC1, ALK, PD1, EGFR, and HER2) and seven STING subunits genes (CGAS, TBK1, IRF3, IkBa, NFkB, TRAF2, and TNFRSF19). Some cybrids underwent siRNA knockdown of STING followed by qRT-PCR in order to determine the impact of STING on gene expression. Rho0 (lacking mtDNA) ARPE-19 cells were used to determine if mtDNA is required for the expression of the cancer genes studied. Our results showed that (a) K cybrids have lower expression levels for BRAC1, ALK, PD1, EGFR, and HER2 compared to H cybrids; (b) STING KD decreases expression of EGFR in both H and K cybrids, and (c) PD1 expression is negligible in Rho0 cells. Our findings suggest that the STING DNA sensing pathway may be a previously unrecognized pathway to target modulation of cancer-related genes and the PD1 expression requires the presence of mtDNA.

Abbreviations: BSA, Bovine Serum Albumin; DC, Dendritic Cell; mtDNA, Mitochondrial Deoxyribonucleic Acid; NK, Natural Killer; OXPHOS, Oxidative Phosphorylation; PCR, Polymerase Chain Reaction; PVDF, Polyvinylidene Difluoride; qRT-PCR, Quantitative Real-time PCR; RIPA Buffer, Radioimmunoprecipitation assay buffer; RPE, Retinal Pigment Epithelium; siRNA, Small Interfering Ribonucleic Acid; STING, Simulator of Interferon Genes; TBST, Tris-Buffered Saline with Tween 20.

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1 | INTRODUCTION

The regulation of immune responses plays a significant role in tumor development and progression. A major inducer of the immune response is the Simulator of Interferon Genes (STING) system, making it a potential therapeutic target for novel drugs to treat various diseases. Most of what is known about the STING system is related to its DNA sensing capacity that activates interferon gamma to combat viral and bacterial infections. In cancers, tumor-derived DNA activates STING leading to up-regulation of IFN-beta and induction of antitumor T cells. Modulation of STING can even affect levels of cancer cell radio-resistance by suppressing inflammation via myeloid cell recruitment. When STING is activated in conjunction with Cetuximab treatment, the natural killer (NK) cell activation and dendritic cell (DC) maturation levels are increased. The cGAS-STING complex can be activated by cytosolic DNA that has been released from damaged nuclei or mitochondria. Permeabilization of the mitochondrial inner membrane enables the release of mtDNA into the cytosol where the cGAS-STING signaling pathway is activated. In a mouse model, the absence of TFAM, a pro-tumor suppressor, results in increased cell death. The cGAS-STING complex can be activated by cytosolic DNA that has been released from damaged nuclei or mitochondria. Permeabilization of the mitochondrial inner membrane enables the release of mtDNA into the cytosol where the cGAS-STING signaling pathway is activated. Further studies have shown that cytosolic mtDNA is associated with either increased risk or protection for human diseases, including cancers, Alzheimer’s disease, age-related macular degeneration (AMD), and diabetes. In humans, there is considerable variability of mtDNA single nucleotide polymorphism (SNPs) patterns, referred to as haplogroups, that define different racial/ethnic populations. Often these haplogroups are associated with either increased risk or protection for human diseases, including cancers, Alzheimer’s disease, age-related macular degeneration (AMD), and diabetes. To date, it is not known if different mtDNA haplogroups might (a) modulate differentially the expression levels of STING complex genes/proteins or known cancer-related genes; or (b) if the mtDNA/STING interaction is required for the expression of specific cancer genes.

Mitochondria (mt) possess unique, maternally inherited, circular DNA. The mtDNA encodes for 37 genes, including 13 protein subunits essential for oxidative phosphorylation (OXPHOS), 2 ribosomal RNAs, and 22 transfer RNAs. The non-coding region of 1121 nucleotides, known as the MT-Dloop, is critical for mtDNA replication and transcription. Small biologically active peptides called Humanin and MOTsSc are encoded from the 16s and 12s rRNA regions of the mtDNA, respectively, and are involved in various pathological processes. The maternal origins of different human populations are classified by their mtDNA SNP profiles into haplogroups and there is growing evidence that the mtDNA haplogroups play a role in disease progression and responses to medications.

The H haplogroups are the most common European mtDNA haplogroup (www.MitoMap.com). The A12308G SNP defines the UK cluster that contains both the U and K haplogroups. The K haplogroups (also known as Uk) are further defined by the G9055A SNP, have a 1%–6% worldwide distribution, and represent approximately 10% of ancestral Europeans. Approximately 32% of the Ashkenazi Jewish population is highly associated with the K haplogroup and can be classified into the K1a1b1a, K2a2a, and K1a9 subsets. The genetic profile of the Ashkenazi Jewish population has become homogeneous because of limited numbers of founders, intermarriage within the group, and population bottlenecks involving decreases in population sizes due to environmental and/or sociological events. As a result, with respect to genetic profiles, the Ashkenazi Jewish population is an excellent well-defined group for studies correlating genetic associations with specific diseases, including BRCA1/BRCA2 genes associated with breast and ovarian cancers, hypercholesterolemia, hyperlipidemia, cardiovascular disease, Gaucher disease type I, Usher Type 3A, and Tay-Sachs disease.

Transmitochondrial cybrids (cytoplasmic hybrids) are used to identify the effects of an individual’s mtDNA upon cellular homeostasis. Previously, using the human retinal pigment epithelial (RPE) cybrid model (cell lines with identical nuclei but mtDNA from either H or K haplogroup subjects) we showed that cybrids with K haplogroup mtDNA have (a) significantly increased expression of APOE, a critical lipid transporter molecule associated with human diseases; (b) more protection from cytotoxic effects of amyloid-β1-42 (active form); (c) increased expression of inhibitors of the alternative complement pathways and important inflammation-related genes; and (d) elevated bioenergetic respiratory profiles compared to the H cybrids. These findings suggest that an individual’s K haplogroup mtDNA contributes to lipid transport, cholesterol metabolism, complement activation, and inflammation, factors critical for cancers and age-related diseases.

Previous studies have shown that cytosolic mtDNA is capable of STING activation, which in turn leads to up-regulated type 1 interferon genes and viral resistance. However, it is unclear whether diverse mtDNA haplogroups differentially affect the STING system and lead to different downstream regulations of cancer-related genes. The present study investigates (a) if the SNP variants within H versus K haplogroups differentially regulate the expression of...
five cancer genes and seven STING-pathway-related genes,
(b) whether the H mtDNA versus K mtDNA differentially
modulate genes through the STING DNA sensing system,
and (c) what impact lacking mtDNA (Rho0 cells) has on the
expression of certain cancer genes.

2 | MATERIALS AND METHODS

2.1 | Cybrid cell lines generation and
culture conditions

Institutional review board approval was obtained from the
University of California, Irvine (IRB #2003–3131). There
was no significant difference between the ages of the H
subjects (n = 4, 42.5 ± 7.32 years) and K subjects (n = 5,
48.4 ± 3.59, p = 0.463) (Table 1).

Peripheral blood was collected in sodium citrate tubes
and DNA was isolated using the DNA extraction kit
(PUREGENE, Qiagen). Using a series of centrifugation
steps, platelets were isolated, suspended in Tris buffer sa-
line (TBS), and then fused with ARPE-19 cells that were
deficient in mtDNA (Rho0) as described previously.31
Cybrids were cultured until confluent in DMEM-F12 con-
taining 10% dialyzed fetal bovine serum (FBS), 100 unit/ml
penicillin and 100 μg/ml streptomycin, 2.5 μg/ml fun-
gizone, 50 μg/ml gentamycin, and 17.5 mM glucose. All
experiments used cybrid cells at Passage 5.

2.2 | Next-generation whole mtDNA
genome sequencing for cybrid samples

DNA extracted from blood and cybrids was utilized for
next-generation sequencing (NGS) as described previ-
ously.19 Our NGS technology allows for independent se-
quencing of both strands of mtDNA in both directions
and can be used to quantitate the haplogroup-defining
single nucleotide polymorphisms (SNPs), the private
SNPs (which are not haplogroup defining), and the low-
frequency heteroplasmy SNPs observed across the mi-
 tochondrial genome. Identification of pathogenic SNPs
utilized two web resources, HmtVar (www.hmtvar.uniba.
.it) and www.mitomap.org, and only published results
were considered as potential disease associations.

2.3 | Isolation of RNA and
amplification of cDNA

Total RNA was isolated from untreated and STING-KD
cultures (H cybrids, n = 4; K cybrids, n = 5) using the
RNeasy Mini-Extraction kit (Qiagen) as described
previously.31 The cDNA generated from 2 μg of individual
RNA samples with the QuantiTect Reverse Transcription
Kit (Qiagen) was used for qRT-PCR analyses.

2.4 | Quantitative real-time PCR (qRT-
PCR) analyses

The qRT-PCR was performed on individual samples
using QuantiFast SYBR Green PCR Kits (Qiagen) on an
Applied Biosystems ViiA7 qRT-PCR detection system.
Primers (QuantiTect Primer Assay, Qiagen or KiCqStart
Primers, Sigma) were used to analyze 12 different genes
in cancer (BRCA1, ALK, PD1, EGFR, and ERBB2 [HER2]);
and STING (CGAS, TBK1, IRF3, IkBa, NFKB2, TRAF2,
and TNFRSF19; Table 2) pathways. Primers were stand-
ardized with the HPRT1 or HMBS housekeeping genes.
All analyses were performed in triplicate. The fold values
were calculated using the $2^{(−ΔΔCt)}$ formula.

2.5 | Knock-down of STING

For siRNA-mediated knockdown of STING, H and K
cybrids were seeded in 6-well plates at 7 × 10⁵ cells/well.
Thirty pmol final concentrations of STING siRNA
(#128591, ThermoFisher) or scrambled siRNA were di-
luted in OPTI-MEM (Invitrogen) and incubated at room
temperature for 5 min. Transfection reagent Lipofectamine
2000 (Invitrogen) was mixed separately with OPTI-MEM
as per the manufacturer’s protocol and incubated for
5 min at room temperature (RT). The OPTI-MEM/siRNA
and OPTI-MEM/Lipofectamine tubes were combined and
incubation was carried out for 5 min at room temperature
to allow the formation of the siRNA-lipid complex. The

| TABLE 1 | Subject information for H and K cybrids |

| Cybrid | Gender | Age  | Haplogroup |
|--------|--------|------|------------|
| 11–10  | M      | 30   | H4a1a3     |
| 11–35  | F      | 30   | H1         |
| 13–52  | F      | 58   | H1         |
| 13–65  | F      | 52   | H4a1a4b    |
| 13–57  | F      | 45   | K1a4       |
| 13–65  | F      | 38   | K1a1b1a    |
| 13–75  | F      | 56   | K1c2       |
| 13–77  | F      | 57   | K1a1b1a    |
| 13–80  | F      | 46   | K1a1b2a1a  |

Note: H ages = 42.50 ± 7.320 N = 4.
K ages = 48.40 ± 3.59 N = 5.
Difference = −5.9 ± 7.61.
p = 0.4634.
Mean ± SEM.
| Symbol | Gene name | Gene RefSeq number | Functions |
|--------|-----------|--------------------|-----------|
| **Cancer genes** |  |  |  |
| **BRCA1** | Breast cancer type 1 susceptibility protein | NM_007294; NM_007295; NM_007296; NM_007297; NM_007298 | BRCA1 helps repair DNA damage with an important role in the error-free repair of double-strand breaks. Mutations in BRCA1 and BRCA2 are associated with an increased risk for breast cancer. |
| **ALK** | Anaplastic lymphoma kinase | NM_004304; NM_001353765 | ALK is a receptor tyrosine kinase that can be oncogenic by forming a fusion gaining additional gene copies, or from DNA mutations. |
| **PD1** | Programmed cell death protein 1 | NM_005018 | Programmed cell death protein 1, also known as PDCD1 and CD279 (cluster of differentiation 279) is a cell surface receptor most often expressed in T cells and pro-B cells. It has a role in down-regulating the immune system and can also prevent immune cell activity against cancer. Many cancers highly express PD-L1, the ligand of PD-1, thus targeting the PD-1 receptor is of interest for cancer treatment. |
| **EGFR** | Epidermal growth factor receptor | NM_001346897; NM_001346898; NM_001346899; NM_001346900; NM_001346941 | Epidermal growth factor receptor (EGFR) is a transmembrane protein associated with a variety of signal transduction pathways including MAPK, Akt, and JNK pathways. Deficient signaling of the EGFR and other receptor tyrosine kinases in humans is associated with diseases such as Alzheimer's, while over-expression is associated with the development of a wide variety of tumors. |
| **HER2** | Receptor tyrosine-protein kinase erbB-2 | NM_001005862; NM_001289936; NM_001289937; NM_001289938; NM_004448 | HER2 is a member of the human epidermal growth factor receptor (HER/EGFR/ERBB) family. Overexpression is associated with the development and progression of certain breast cancers and is used as a biomarker. It is a common target of therapy in breast cancer patients. |
| **Sting pathway genes** |  |  |  |
| **CGAS** | Cyclic GMP-AMP synthase | NM_138441 | CGAS is a nucleotidyltransferase that serves as a cytosolic DNA sensor, capable of binding microbial as well as self DNA that is present in the cytoplasm, then triggering an immune response. CGAS binds cytosolic DNA directly, leading to activation and synthesis of cGAMP, a second messenger that binds to and activates TMEM173/STING, thereby triggering type-I interferon production. |
| **TBK1** | TANK-binding kinase 1 | NM_013254 | TANK-binding kinase 1 (TBK1) is a serine/threonine-protein kinase that is capable of phosphorylating interferon regulatory factors (IRFs) leading to transcriptional activation of antiviral and pro-inflammatory interferons. Inhibition of TBK1 and related targets have been investigated as therapeutic options for the treatment of inflammatory diseases and cancer. |
| **IRF3** | Interferon regulatory factor 3 | NM_001197122; NM_001197123; NM_001197124; NM_001197125; NM_001197126 | Interferon regulatory factor 3 (IRF3) is involved in the innate immune system response by activating the transcription of interferons alpha and beta as well as other downstream gene targets. |
| **IκBα** | Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha | NM_020529 | Belongs to a family of proteins that inhibit the NF-κB transcription factor, by masking the nuclear localization signals and sequestering NF-κB in the cytoplasm. Additionally, IκBα blocks NF-κB from binding DNA, inhibiting its proper function. Defects with IκBα can lead to chronic overexpression of NF-κB. |
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The final mixture was applied to cybrid cells in culture and allowed to incubate for 48 h before RNA isolation.

2.6 | Statistical analyses

Data were subjected to statistical analyses by an unpaired t-test, GraphPad Prism (Version 5.0, La Jolla, CA). p ≤ 0.05 was considered statistically significant. Error bars in the graphs represent SEM (standard error mean).

3 | RESULTS

3.1 | Sequencing of mtDNA From H and K Cybrids

The entire mtDNA from the H and K cybrids were sequenced using NGS technology. The private SNPs are those that do not define the H or K haplogroups (non-haplogroup defining). The unique SNPs are not listed in www.MitoMap.org or other programs. Table 3A shows the SNPs in the K haplogroup cybrids. There were 5 private SNPS in the mtDNA regions of the K cybrids: Cyb 13–57 with m.9048T>C (rs#386829059, MT-ATP6), Cyb 13–57 with m.10586G>A (rs#28358281, MT-ND4L), Cyb 13–80 with m.10586G>A (rs#28358281, MT-ND4L), Cyb 13–57 with m.155320T>C (no rs#, MT- CYB). The non-coding Control Region of the K cybrids possessed 11 SNPs and 9 of those defined the K haplogroup. The NGS methodology allowed the identification of heteroplasmic SNPs in the K cybrids. The 15 heteroplasmy SNPs showed a range from 3.4% to 40.5% occurrence in five of the cybrids (Table 3A).

Table 3B shows the SNPs in the H haplogroup cybrids. There were 6 private SNPS in the mtDNA regions of the H cybrids: Cyb 13–65 with m.1709G>A (rs#200251800, MT-RNR2), Cyb 13–52 with m.5471G>A (no rs#, MT-ND2), Cyb 11–10 with m.6951G>A (no rs#, MT-CO1), Cyb 13–52 with m.9932G>A (rs##377610479, MT-CO3) Cyb 11–10 with m.12130T>C (no rs#, ND4) and Cyb 13–65 with m.15132T>C (no rs#, MT- CYB). There were 2 SNPs in the non-coding Control Region, both of which are H haplogroup defining. Three of the cybrids (Cyb 11–10, Cyb 13–52, and Cyb 13–65) possessed heteroplasmy that ranged from 4.3% to 30.7%. Cyb 11–35 lacked heteroplasmy in the mtDNA (Table 3B).

Table 3C lists the SNPs present in either H or K haplogroup cybrids that have published data suggesting a link between the SNP and a disease or condition. SNP m.5460G>A has been associated with risk for Alzheimer’s disease and Parkinson’s disease. SNP m.5460G>A has been associated with risk for Alzheimer’s disease and Parkinson’s disease.
| Locus: MT- | SNP | AA Change | Functional Loci: MT- | rs# | Cyb 13–57 | Cyb 13–64 | Cyb 13–75 | Cyb 13–77 | Cyb 13–80 |
|-----------|-----|-----------|---------------------|-----|-----------|-----------|-----------|-----------|-----------|
| HV2/OHR  | 114 C>T | NonCoding | na | K1a1 | K1c | K1a1 | K1a1 |
| HV2/OHR  | 146 T>C | NonCoding | rs370482130 | K1c | K1c | K1c | 4% Htroplsmy |
| HV2/OHR  | 152 T>C | NonCoding | rs117135796 | K1a | K1a | K1a | 2.2% Htroplsmy |
| HV3      | 497 C>T | NonCoding | rs28660704 | K1a | K1a | K1a | K1a |
| HV3/HSP1 | 567 A>C | NonCoding | na | 22.3% Htroplsmy |
| RNR1     | 1189 T>C | rRNA | rs28358571 | K1 | K1 | K1 | K1 |
| RNR1     | 1560 T>C | rRNA | na | 20.2% Htroplsmy |
| RNR2     | 1811 A>G | rRNA | rs28358576 | U2′3′4′7′8′9 | U2′3′4′7′8′9 | U2′3′4′7′8′9 | U2′3′4′7′8′9 |
| RNR2     | 2528 G>A | rRNA | na | 20.5% Htroplsmy |
| ND1      | 3480 A>G | Syn:Lys58 | rs28358584 | U8b′c | U8b′c | U8b′c | U8b′c |
| ND1      | 3483 G>A | Syn:Glu59 | rs367578983 | U8b′c | U8b′c | U8b′c | U8b′c |
| ND1      | 3777 T>C | Syn:Ser157 | rs386828921 | K1a1b2a1 |
| ND2      | 5460 G>A | Ala31Thr | rs3021088 | 3.4% Htroplsmy |
| CO1      | 7278 T>C | Phe459Leu | na | K1a1b2a1 |
| CO2      | 7717 A>G | Syn:Leu44 | na | 5.9% Htroplsmy |
| CO2      | 7729 A>G | Syn:Thr48 | na | K1a1b2a1 |
| CO2      | 7762 G>A | Syn:Gln59 | na | 4.6% Htroplsmy |
| ATP6     | 8902 G>A | Ala126Thr | na | 4.2% Htroplsmy |
| ATP6     | 9006 A>G | Syn:Leu160 | na | K1c2 |
| ATP6     | 9048 T>C | Syn:lle174 | rs386829059 | PVT-b |
| ATP6     | 9055 G>A | Ala177Thr | rs193303045 | U8b | U8b | U8b | U8b |
| CO3      | 9698 T>C | Syn:Leu164 | rs9743 | U8 | U8 | U8 | U8 |
| CO3      | 9800 T>C | Syn:Phe198 | na | K1a1b2a1 |
| ND4L     | 10586 G>A | Syn:Ser39 | rs28358281 | PVT-b |
| ND4L     | 10588 A>G | Syn:Ser39 | rs28358281 | PVT-b |
| ND4      | 10896 A>G | Asn46Ser | na | 7% Htroplsmy |
| ND4      | 10978 A>G | Syn:Leu73 | na | K1a1b1a | K1a1b1a |
| ND4      | 11299 T>C | Syn:Thr180 | rs28358285 | K | K | K | K |
| ND4      | 11447 A>G | Syn:Leu236 | rs2853493 | U | U | U | U |
| ND4      | 11470 A>G | Syn:Lys237 | na | K1a1b1 | K1a1b1 |
### TABLE 3A (Continued)

| Locus: MT-SNP | AA Change | Functional Loci: MT- | rs# | Cyb 13–57 | Cyb 13–64 | Cyb 13–75 | Cyb 13–77 | Cyb 13–80 |
|--------------|-----------|---------------------|-----|-----------|-----------|-----------|-----------|-----------|
| ND4          | 11485 T>C  | Syn:Gly242          | rs28529320 | K1a4     |           |           |           |           |
| ND4          | 11914 G>A  | Syn:Thr385          | rs2853496  | K1a1     | K1a1     | K1a1     |           |           |
| TL2          | 12308 A>G  | tRNA                | rs2853498  | U         | U         | U         | U         | U         |
| ND5          | 12372 G>A  | Syn:Leu12           |           |           | rs2853499 | U         | U         | U         | U         |
| ND5          | 12685 T>C  | Phe117Leu           |           |           |           | na        |           |           |           |
| ND5          | 12954 T>C  | Syn:Ala206          |           |           |           | K1a1b1a   |           |           |           |
| ND5          | 13289 G>A  | Gly318Asp           | rs28683136 |           |           |           |           |           | 4.5% Htrolsmy |
| ND5          | 13326 T>C  | Syn:Cys330          |           |           |           |           |           |           | K1a1b2   |
| ND5          | 13665 T>C  | Syn:Ile443          |           |           |           |           |           |           | PVT-b   |
| ND5          | 14002 A>G  | Thr556Ala           | rs386829198 | K1c2     |           |           |           |           |           |
| ND5          | 14040 G>A  | Syn:Gln568          | rs57180882 | K1c2     |           |           |           |           |           |
| ND6          | 14167 C>T  | Leu7Phe             | rs193302977 | U8b       | U8b       | U8b       | U8b       | U8b       |
| ND6          | 14249 G>A  | Val34His            |           |           |           |           |           |           | PVT-a   |
| CYB          | 14798 T>C  | Ph18Leu             | rs28357681 | K         | K         | K         | K         | K         |
| CYB          | 15226 A>G  | Syn:Leu160          | rs527236174 |           |           |           |           |           | 6.1% Htrolsmy |
| CYB          | 15525 A>C  | Asn260Thr           |           |           |           |           |           |           | 22.2% Htrolsmy |
| CYB          | 15530 T>C  | Syn:Leu262          |           |           |           |           |           |           | PVT-a   |
| CYB          | 15758 A>G  | Ile338Val           | rs527236193 |           |           |           |           |           | K1a1b2a1 |
| TT           | 15924 A>G  | tRNA                | rs2853510  | K1a1b     | K1a1b     | K1a1b     |           |           |
| HV2/TAS2     | 16093 T>C  | NonCoding           | rs2853511  | K1a       |           |           |           |           |
| HV2/TAS2     | 16129 G>A  | NonCoding           | rs41534744 | 9.9% Htrolsmy |           |           |           |           |
| HV1          | 16224 T>C  | NonCoding           | rs386420031 | K         | K         | K         |           |           |
| HV1          | 16234 C>T  | NonCoding           | rs368259300 | K1a1b1a   | K1a1b1a   |           |           |           |
| HV1          | 16311 T>C  | NonCoding           | rs34799580 | K         | K         | K         |           |           |
| HV1          | 16320 C>T  | NonCoding           | rs62581338 | K1c2      |           |           |           |           |

**Note:** All SNP’s had a Quality (A Phred-scaled quality score assigned by the variant caller) Score of 100 and PASSed all the Filters.

**Abbreviations:** na, not available; PVT-a, found in other Haplo K or H; PVT-b, not found in other Haplo K or H.
### Table 3B  SNPs found by NGS in H haplogroup cybrids

| Loci: MT- | SNP  | AA change | Functional Loci: MT- | rs#       | Cyb 11–10 | Cyb 11–35 | Cyb 13–52 | Cyb 13–65 |
|----------|------|-----------|----------------------|-----------|-----------|-----------|-----------|-----------|
| HV2      | 73G>A| NonCoding | rs3087742             | H         | H         | H         | H         | H         |
| HV2/OHR  | 195T>C| NonCoding | rs2857291             | H4a1a     |           |           |           |           |
| RNR2     | 1709G>A| rRNA     | rs200251800           |           | PVT-a     |           |           |           |
| RNR2     | 1841T>C| rRNA     | na                    |           | 30.7% Htroplsmy |           |           |           |
| RNR2     | 2706A>G| rRNA     | rs2854128             | H         | H         | H         | H         | H         |
| RNR2     | 3010G>A| rRNA     | rs3928306             | H         | HI        | H1        | H1        |           |
| ND1      | 3992C>T| Thr229Met | rs41402945            | H4        |           |           |           |           |
| ND1      | 4024A>G| Thr240Ala | rs41504646            | H4a       |           |           |           |           |
| ND2      | 4896T>C| Tyr143His | na                    | 4.39% Htroplsmy |           |           |           |           |
| ND2      | 5004T>C| Syn:Lue179 | rs41419549            | H4a       |           |           |           |           |
| ND2      | 5471G>A| Syn:Thr334 | na                    | PVT-a     |           |           |           |           |
| ND2      | 7028T>C| Syn:Ala375 | rs2015062             | H         | H         | H         | H         | H         |
| ND1      | 7337G>A| Syn:Ser478 | rs386829005           |           | 7.4% Htroplsmy |           |           |           |
| ND2      | 8222T>C| Syn:Leu213 | na                    |           |           |           |           |           |
| ND2      | 8269G>A| Syn:Term228 | rs8896                | H4a1a     |           |           |           |           |
| ND2      | 9123G>A| Syn:Leu199 | rs28358270            | H4        |           |           |           |           |
| ND2      | 9507T>C| Phe101Leu | na                    | 6.6% Htroplsmy |           |           |           |           |
| ND2      | 9932G>A| Syn:Trp242 | rs377610479           | PVT-a     |           |           |           |           |
| ND2      | 10750A>G| Asn94Ser | rs372297272            | 5.88% Htroplsmy |           |           |           |           |
| ND4      | 11157T>C| Leu253Pro | na                    | 11.5% Htroplsmy |           |           |           |           |
| ND4      | 11719G>A| Syn:Gly320 | rs2853495             | H         | H         | H         | H         | H         |
| ND4      | 12130T>C| Syn:Phe457 | na                    | PVT-a     |           |           |           |           |
| ND5      | 12642A>G| Syn:Glu102 | na                    | H4a1a4b   |           |           |           |           |
| ND5      | 13889G>A| Cys518Tyr | na                    | H4a1a3    |           |           |           |           |
| ND6      | 14365C>T| Syn:Met73 | rs2853815             | H4a1      |           |           |           |           |
| ND6      | 14569G>A| Syn:Ser35 | rs386420019           | H4a1a4    |           |           |           |           |
| ND6      | 14582A>G| Val51Ala | rs41534845            | 8.61% Htroplsmy |           |           |           |           |
| ND6      | 14861G>A| Ala39Thr | rs2853505             | 4.96% Htroplsmy |           |           |           |           |
| CYB      | 14861G>A| Ala39Thr | rs2853505             | PVT-b     |           |           |           |           |
| CYB      | 15132T>C| Met129Thr | na                    | PVT-b     |           |           |           |           |
| CYB      | 15884G>A| Ala381Thr | rs527236195           | H4a1a4b   |           |           |           |           |

Note: All SNP’s had a Quality (A Phred-scaled quality score assigned by the variant caller) Score of 100 and PASSed all the Filters.

Abbreviations: na, not available; PVT-a, found in other Haplo K or H; PVT-b, not found in other Haplo K or H.
m.8902G>A is associated with Schizophrenia risk. SNP m.16129G>A is associated with risk for Ewing’s sarcoma, predisposition to breast cancer, nasopharyngeal carcinoma, and cyclical vomiting. SNP m.14582A>G is associated risk with Leber’s hereditary optic neuropathy, bilateral optic atrophy, amblyopia, and Goldman bilateral central scotomas. Finally, SNP m.15132T>C is associated with hypertrophic cardiomyopathy risk. None of the above SNPs with disease association risks are shared between all the cybrids tested. Only SNP m.14582A>G was shared between two cybrids (Cyb 11–10 H and Cyb 13–65 H).

### 3.2 K haplogroup cybrids exhibit decreased gene expression of key cancer target genes

The RNA expression levels of five cancer genes were measured by qRT-PCR in K and H cybrids (Figure 1). The cybrids with mitochondria from the K haplogroup subjects had significantly lower expression levels of four cancer-related genes (BRCA1, 0.516-fold ±0.099, p = 0.007; ALK, 0.226-fold ±0.059, p = 0.003; PD1, 0.405-fold ±0.0126, p = 0.03 and EGFR, 0.739-fold ±0.069, p = 0.0493) compared to H cybrids. The transcription levels for HER2 were similar in H and K cybrids (p = 0.3).

### 3.3 K haplogroup cybrids differentially express genes involved in the STING DNA sensing pathway

The STING complex is comprised of numerous subunits (all of which are encoded by the nuclear DNA) that become activated by DNA fragments, leading to phosphorylation and upregulation of type I interferons (Figure 2A). Seven genes involved in the STING signaling pathway were analyzed by qRT-PCR to determine if the H and K cybrids might have different expression levels for STING-related subunits (Figure 2B). The K cybrids had higher expression of IkBa (1.940-fold ±0.302, p = 0.047) and NFKB2 (1.451-fold ±0.119, p = 0.026) as well as lower expression of IRF3 (0.679-fold ±0.051, p = 0.009) and TNFRSF19 (0.275-fold ±0.079, p = 0.0073) compared to H cybrids. There were no differences in the gene expression levels for CGAS, TBK1, or TRAF2 between the H and K cybrids.
3.4 Knockdown of STING decreases expression levels of BRCA1 and EGFR cancer genes

The STING complex is the intracellular sensor system for DNA fragments. The H (n = 4) and K (n = 5) cybrids underwent STING-KD by transfection for 48 h and expression levels of the STING gene were measured by qRT-PCR (Figure 3A). STING expression was 0.138-fold (p = 0.0028) in H-STING-KD cybrids and 0.095-fold (p < 0.0001) in K-STING-KD cybrids, demonstrating effective STING knockdown. To determine if STING played a role in the differential expression of the cancer-related genes, the transcription levels in H-STING-KD and K-STING-KD cybrids were measured (Figure 3B). The H-STING-KD cybrids showed significantly lower BRCA1 expression levels (0.347-fold ±0.111%, p = 0.02) compared to H-Control cybrids, while the K-STING-KD cybrids showed no significant decrease compared to the K-Control cybrids (p = 0.41). Interestingly, EGFR levels were decreased in both the STING-KD H cybrids (0.71-fold ±0.10, p = 0.02) and the STING-KD K cybrids (0.28-fold ±0.118 lower, p = 0.0481) compared to the respective Control cybrids. The levels for ALK, PD1, and HER2 were not changed in the H-STING-KD and K-STING-KD cybrids as compared to their respective Control cybrids.

3.5 Influence of STING on cancer gene expression requires mitochondria for BRCA1 and ALK1

To determine whether the presence of mitochondria was required for the expression of the five cancer genes, ARPE-19 cells lacking mitochondria (Rho0-ARPE19-Control cells) and STING knockdown (Rho0-ARPE19-STING-KD cells) were analyzed for expression levels of cancer-related genes (Figure 4). The Rho0-ARPE19-STING-KD cells showed a statistically significant increase in expression levels of BRCA1 (1.253-fold ±0.039, p = 0.011) and ALK1 (2.423-fold ±0.47, p = 0.041) as compared to Rho0-ARPE19-Control cells. The Rho0-ARPE19-STING-KD cells showed statistically significantly decreased EGFR expression levels (0.504-fold ±0.056, p = 0.009) compared to Rho0-ARPE19-Control cells, indicating that the involvement of the STING DNA sensor system was independent of the presence of mtDNA. Finally, Rho0-ARPE19-STING-KD cells...
showed no significant change in HER2 expression levels ($p = 0.44$) as compared to Rho0-ARPE19-Control cells. The expression of PD1 was absent in Rho0 cells with or without STING knockdown.

4 | DISCUSSION

Pathological conditions (e.g. viral and bacterial infections) are often associated with DNA fragmentation and STING activation that modulate the immune responses. However, our findings suggest that STING activation may also be important for retrograde (mitochondria to nucleus) signaling. We also suggest that the different mtDNA haplogroups and STING expression can modulate expression levels of some cancer genes. Drugs targeting specific molecules key for disease progression have revolutionized successful outcomes for cancer therapies (Table 4). Since mtDNA interacts with the STING complex, one could speculate that fragments of H mtDNA versus K mtDNA may activate the STING pathway differently and cause altered expression of key STING pathway genes. Due to the role of STING in regulating inflammation, and the association of inflammation with cancer, alterations in the
STING pathway could influence the downstream regulation of cancer-related genes. Our findings that expression levels of some STING pathway genes and cancer-related genes are different in H cybrids versus K cybrids support these possibilities and deserve further investigation.

In this study, all cybrids share the same nuclear genome of the parent ARPE-19 cell line, and culture conditions, so differences between the H and K cybrids would then be attributable to the influence of the mtDNA SNP variants. Using NGS of the entire mtDNA genome for each cybrid, we discovered that the only SNPs shared by all cybrids of a particular haplogroup were those that were haplogroup defining. We identified unique SNPs (not reported in mtDNA databases) present in individual cybrids, and while we cannot discount the potential influence of these SNPs, they are not shared by all cybrids of a given haplogroup. While we cannot rule out the possibility that private SNPs or heteroplasmay SNPs present in individual samples could contribute to the observed phenotype, it is also possible that the combination of SNP variants defining the H versus K haplogroups are influencing the retrograde signaling (mitochondria to nucleus) that modulates gene expression patterns.

We utilized ARPE-19 transmitochondrial cybrid cells generated from H and K mtDNA haplogroup patients. ARPE-19-based cybrids were used because chemotherapy agents are known to cause retinopathy specifically involving RPE cells. Our previous study using ARPE-19 cybrids showed cisplatin negatively affected RPE cell health and altered cancer gene expression patterns and that different mtDNA haplogroups influenced these effects. The present study focuses on possible mechanisms by examining the influence of different mtDNA haplogroups on the expression levels of cancer and STING genes, and the influence of STING knockdown on cancer gene expression. We report herein that untreated control H cybrids had significantly higher expression of four genes (EGFR, BRCA1, ALK, and PD1) as compared to the untreated K cybrids. After STING-KD for both H and K cybrids, the expression levels of EGFR were decreased further (28.1% and 29.1%, respectively) indicating that EGFR transcription can be partially modulated through the STING DNA sensing system.

In addition, after STING knockdown in Rho0-ARPE-19 cells (lacking mitochondria), the expression levels of EGFR were also lower. Our findings suggest that (a) EGFR gene expression may be modulated by the mtDNA haplogroup of an individual (e.g., H vs. K), (b) EGFR expression levels can be influenced by the STING DNA sensing system, and (c) the STING effect on EGFR expression is mitochondria independent (decreased in Rho0-ARPE19-STING-KD cells) and mtDNA haplogroup independent (decreased equally in both H-STING-KD and K-STING-KD cybrids). Therefore, the STING sensing system may be a previously unrecognized pathway to target EGFR modulation.

In our in vitro studies, the BRCA1 levels were significantly downregulated in the K haplogroup cybrids compared to the H haplogroup cybrids, suggesting its expression can be modulated differentially by mtDNA variants. In STING-KD H cybrids, the expression levels for BRCA1 were significantly downregulated while its levels remained unchanged in the STING-KD K cybrids compared to controls. This finding suggests that BRCA1 levels regulated via the STING system can be modulated by the mtDNA haplogroup profile. The BRCA1 expression levels increased in the Rho0-STING-KD-ARPE19 cells compared to the Rho0-Control-ARPE19 cells suggesting that the STING system is involved irrespective of whether mitochondria are present.

Next, we examined the influence of mtDNA haplogroup and STING on the expression of ALK, PD1, and HER2, which are all targets of interest in cancer. The ALK and PD1 were expressed at much lower levels in the K cybrids as compared to the H cybrids, demonstrating that the mtDNA variant pattern influences the expression levels of both genes. In contrast to the EGFR and BRCA1 expression levels, STING-KD H cybrids and STING-KD K cybrids showed no change in transcription levels for either PD1 or ALK. Interestingly, PD1 expression was completely absent in Rho0-ARPE19-STING-KD cells and Rho0-ARPE19-Control cells, demonstrating that not only could the haplogroup influence the PD1 expression, but that mtDNA was needed for its expression. Finally, HER2 expression levels were not influenced by haplogroup or modulation of STING for the H or K cybrids.
K cybrids exhibit differential expression of four key genes (IRF3, IkBa, NFκβ2, and TNFRSF19) associated with the STING pathway compared to the H cybrids. The K cybrids had decreased gene expression levels of IRF3. The K cybrids also demonstrated increased expression of NFκβ2, an important gene associated with the inflammatory pathway of STING. Additionally, untreated K cybrids exhibited increased expression of IkBa, an inhibitor of NFκβ, and decreased levels of TNFRSF19, which can activate JNK and cause cell death by a caspase-independent pathway. There was no difference in the expression of other key STING pathway-related genes, such as CGAS, TBK1, and TRAF2. Most of the literature on the STING complex has focused on its activation in response to viral and bacterial infections, demonstrating that exogenous DNA can induce cellular inflammatory responses. However, there is a growing body of work suggesting that endogenous sources of DNA from the mitochondria can signal through the STING pathway and that this can be important in diseases such as cancer. Our cybrid system demonstrates that in non-stressed cells, the endogenous mtDNA can modulate the nuclear genome in its expression of STING pathway elements and cancer genes. These findings support our hypothesis that the mtDNA uses the STING DNA sensing system in non-pathogenic retrograde signaling between the mitochondria and nuclear genomes.

The K cybrids have lower transcription levels of BRCA1, ALK, PD1, and EGFR compared to the H cybrids (Figure 5A,B) and when STING knockdown occurs, only the EGFR levels are decreased while the transcription

**FIGURE 5** Schematic summary of H versus K haplogroups after STING knockdown. The H cybrids show higher expression levels of BRCA1, ALK, PD1, and EGFR (Panel A) compared to the K cybrids (Panel B). After STING knockdown, the expression levels for BRCA1 and EGFR are decreased in STING-KD H cybrids (Panel C) while the STING-KD K cybrids show lower levels for only EGFR (Panel D). Since the H and K cybrids have identical nuclear genomes, these results demonstrate that cells with H mtDNA variants can differentially modulate the expression levels of four cancer genes. Moreover, activation of the STING pathway is involved with expression levels of BRCA1 and EGFR.
levels of the other genes are unchanged (Figure 5D). When the H cybrids undergo STING knockdown, then BRCA1 and EGFR expression levels are significantly decreased (Figure 5C). Since all cybrids have identical nuclei but mitochondria from either H or K haplogroup individuals, our results demonstrate that (a) the mtDNA variants can influence gene expression levels of elements of the STING pathway (IRF3, IkBa, NFKB2 and TNFRSF19) and four critical cancer-related genes (BRCA1, ALK, PD1, and EGFR); (b) the STING system is involved in the expression of BRCA1 and EGFR genes, but not ALK, PD1 or HER2; (c) our Rh00-ARPE19 cell experiments demonstrate that even when mitochondria are lacking, the STING system is involved in the expression of BRCA1, ALK, and EGFR genes; (d) expression of PD1 requires the presence of mtDNA within the cell. Further experiments will utilize high throughput RNA sequencing in order to explore additional pathways potentially regulated by mtDNA haplogroups or STING.

AUTHOR CONTRIBUTIONS

K. Schneider was involved with conceiving and designing the research, performing the experiments, acquiring the data, analyzing the experiments, and interpreting the data. M. Chwa and S. R. Atilano were involved in performing the experiments and interpreting the data. M. C. Kenney was involved in conceiving and designing the research and interpreting the data. The above authors as well as S. Nashine, N. Udar, D. S. Boyer, S. M. Jazwinski, M. V. Miceli, A. B. Nesburn, and B. D. Kuppermann were all involved in providing laboratory materials along with drafting and revising the manuscript.

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CONFLICT OF INTEREST

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