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RESEARCH ARTICLE

# 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 anti-tumor 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*, *IκBa*, *NFκB*, *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. Rho*0* (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, IRF3*, and *TNFRSF19* genes but increased transcription for *IκBa* and *NFκB* compared to H cybrids; (b) STING KD decreases expression of *EGFR* in both H and K cybrids, and (c) *PD1* expression is negligible in Rho*0* 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.

**KEYWORDS**
cancer genes, mitochondrial DNA haplogroups, simulator of interferon genes (STING)

## 1 | INTRODUCTION

The regulation of immune responses plays a significant role in tumor development and progression.[1] 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.[2] In cancers, tumor-derived DNA activates STING leading to upregulation 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.[3] When STING is activated in conjunction with Cetuximab treatment, the natural killer (NK) cell activation and dendritic cell (DC) maturation levels are increased.[4] The cGAS-STING complex can be activated by cytosolic DNA that has been released from damaged nuclei or mitochondria.[5] Permeabilization of the mitochondrial inner membrane enables the release of mtDNA into the cytosol where the cGAS-STING signaling pathway is activated.[6] In a mouse model, the absence of TFAM, a protein critical for maintaining intact mtDNA, leads to accumulation of cytoplasmic mtDNA, activation of the innate immune STING pathway, and increased cell death along with renal failure.[7] 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.[8–15] 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.[8,9,16] 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 MOTsC are encoded from the *16s* and *12s* rRNA regions of the mtDNA, respectively, and are involved in various pathological processes.[17,18] 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.[10,13,19,20]

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.[21] 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.[21,22] 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, hypercholesteremia, hyperlipidemia, cardiovascular disease, Gaucher disease type 1, Usher Type 3A, and Tay-Sachs disease.[23–27]

Transmitochondrial cybrids (cytoplasmic hybrids) are used to identify the effects of an individual's mtDNA upon cellular homeostasis.[28–31] 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-$\beta_{1\text{-}42}$ (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.[32] 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 upregulated type 1 interferon genes and viral resistance.[33] 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 (Rho*0* 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 saline (TBS), and then fused with ARPE-19 cells that were deficient in mtDNA (Rho*0*) as described previously.[31] Cybrids were cultured until confluent in DMEM-F12 containing 10% dialyzed fetal bovine serum (FBS), 100 unit/ml penicillin and 100 μg/ml streptomycin, 2.5 μg/ml fungizone, 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 previously.[19] Our NGS technology allows for independent sequencing 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 mitochondrial 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.

**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.

### 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 standardized 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 $7 \times 10^5$ cells/well. Thirty pmol final concentrations of STING siRNA (#128591, ThermoFisher) or scrambled siRNA were diluted 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 2** Description of genes analyzed by qRT-PCR

| 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. |

TABLE 2 (Continued)

| Symbol | Gene name | Gene RefSeq number | Functions |
|---|---|---|---|
| *NFκB2* | Nuclear factor NF-kappa-B p100 subunit | NM_001077494; NM_001261403; NM_001288724; NM_002502; NM_001322934 | This gene encodes a subunit of the transcription factor complex nuclear factor-kappa-B (NF-κB). The NF-kappa-B complex is present in almost all cell types and is involved in a wide variety of cellular processes including the immune response to infection. Dysregulation has been associated with cancer, inflammatory and autoimmune diseases. |
| *TRAF2* | TNF receptor-associated factor 2 | NM_021138 | TNF receptor-associated factor 2 (TRAF2) associates with and mediates signal transduction for members of the TNF receptor superfamily by interacting directly with TNF receptors. TRAF2 specifically is required for TNFa-mediated activation of NF-κB. |
| *TNFRSF19* | Tumor necrosis factor receptor superfamily, member 19. Also known as TROY | NM_001204459; NM_018647; NM_148957; NM_001354985 | Receptor has been shown to interact with TRAF family members and is capable of activating JNK signaling pathways. Able to induce apoptosis through a caspase-independent mechanism. |
| Housekeepers | | | |
| *HPRT1* | Hypoxanthine phosphoribosyltransferase 1 | NM_000194 NM_000190; NM_001024382 | Transferase catalyzes the conversion of hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate. Critical to the generation of purine nucleotides through the purine salvage pathway. Endogenous control for QPCR. |
| *HMBS* | Hydroxymethylbilane synthase | NM_001258208; NM_001258209 | Member of the hydroxymethylbilane synthase superfamily. Third enzyme of the heme biosynthetic pathway. Endogenous control for QPCR. |

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). $p \leq 0.05$ 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.13665T>C (no rs#, MT-ND5), Cyb 13–77 with m.14249G>A (no rs#, MT-ND6) and 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 4.39% 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

**TABLE 3A** SNPs found by NGS in K haplogroup cybrids

| 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 | | K1a1 | K1a1 |
| HV2/OHR | 146 T>C | | NonCoding | rs370482130 | | | K1c | | |
| HV2/OHR | 152 T>C | | NonCoding | rs117135796 | | | K1c | | 4% 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 | 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 | 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 | U8b'c |
| ND1 | 3483 G>A | Syn:Glu59 | | rs367578983 | | 4.5% Htroplsmy | | | |
| ND1 | 3777 T>C | Syn:Ser157 | | rs386828921 | | | | | K1a1b2a1 |
| ND2 | 5460 G>A | Ala331Thr | | rs3021088 | | 3.4% Htroplsmy | | | |
| CO1 | 7278 T>C | Phe459Leu | | na | | | | | K1a1b2a1a |
| CO2 | 7717 A>G | Syn:Leu44 | | na | | | | | 5.9% Htroplsmy |
| CO2 | 7729 A>G | Syn:Thr48 | | na | | | | | K1a1b2a |
| 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:Ile174 | | rs386829059 | PVT-b | | | | |
| ATP6 | 9055 G>A | Ala177Thr | | rs193303045 | U8b | U8b | U8b | U8b | U8b |
| CO3 | 9698 T>C | Syn:Leu164 | | rs9743 | U8 | U8 | U8 | U8 | U8 |
| CO3 | 9800 T>C | Syn:Phe198 | | na | | | | | K1a1b2a |
| ND4L | 10550 A>G | Syn:Met27 | | rs28358280 | K | K | K | K | K |
| ND4L | 10586 G>A | 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 | K |
| ND4 | 11467 A>G | Syn:Leu236 | | rs2853493 | U | 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 | U |
| ND5 | 12685 T>C | Phe117Leu | | na | | | 40.5% Htroplsmy | | |
| ND5 | 12954 T>C | Syn:Ala206 | | na | | K1a1b1a | | K1a1b1a | |
| ND5 | 13289 G>A | Gly318Asp | | rs28683136 | | | 4.5% Htroplsmy | | |
| ND5 | 13326 T>C | Syn:Cys330 | | na | | | | | K1a1b2 |
| ND5 | 13665 T>C | Syn:Ile443 | | na | | | | | 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 | Va34His | | na | | | | PVT-a | |
| CYB | 14798 T>C | Ph18Leu | | rs28357681 | K | K | K | K | K |
| CYB | 15226 A>G | Syn:Leu160 | | rs527236174 | | | | | 6.1% Htroplsmy |
| CYB | 15525 A>C | Asn260Thr | | na | 22.2% Htroplsmy | | | | |
| CYB | 15530 T>C | Syn:Leu262 | | na | 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 | | | | K1a |
| HV2/TAS2 | 16129 G>A | | NonCoding | rs41534744 | 9.9% Htroplsmy | | | | |
| 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 | 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 | 73 G>A | | NonCoding | rs3087742 | H | H | H | H |
| HV2/OHR | 195 T>C | | NonCoding | rs2857291 | H4a1a | | | |
| RNR2 | 1709 G>A | | rRNA | rs200251800 | | | | PVT-a |
| RNR2 | 1841 T>C | | rRNA | na | | | 30.7% Htroplsmy | |
| RNR2 | 2706 A>G | | rRNA | rs2854128 | H | H | H | H |
| RNR2 | 3010 G>A | | rRNA | rs3928306 | H | H1 | H1 | |
| ND1 | 3992 C>T | Thr229Met | | rs41402945 | H4 | | | H4 |
| ND1 | 4024 A>G | Thr240Ala | | rs41504646 | H4a | | | H4a |
| ND2 | 4896 T>C | Tyr143His | | na | 4.39% Htroplsmy | | | |
| ND2 | 5004 T>C | Syn:Leu179 | | rs41419549 | H4a | | | H4a |
| ND2 | 5471 G>A | Syn:Thr334 | | na | | | PVT-a | |
| CO1 | 6951 G>A | Val350Met | | na | PVT-a | | | |
| CO1 | 7028 T>C | Syn:Ala375 | | rs2015062 | H | H | H | H |
| CO1 | 7337 G>A | Syn:Ser478 | | rs386829005 | | | | |
| CO2 | 8222 T>C | Syn:Leu213 | | na | | | 7.4% Htroplsmy | |
| CO2 | 8269 G>A | Syn:Term228 | | rs8896 | H4a1a | | | H4a1a |
| ATP6 | 9123 G>A | Syn:Leu199 | | rs28358270 | H4 | | | H4 |
| CO3 | 9507 T>C | Phe101Leu | | na | | | | 6.6% Htroplsmy |
| CO3 | 9932 G>A | Syn:Trp242 | | rs377610479 | | | PVT-a | |
| ND4L | 10750 A>G | Asn94Ser | | rs372297272 | 5.88% Htroplsmy | | | |
| ND4 | 11517 T>C | Leu253Pro | | na | | | | 11.5% Htroplsmy |
| ND4 | 11719 G>A | Syn:Gly320 | | rs2853495 | H | H | H | H |
| ND4 | 12130 T>C | Syn:Phe457 | | na | PVT-a | | | |
| ND5 | 12642 A>G | Syn:Glu102 | | na | | | | H4a1a4b |
| ND5 | 13889 G>A | Cys518Tyr | | na | H4a1a3 | | | |
| ND6 | 14365 C>T | Syn:Met73 | | rs2853815 | H4a1 | | | |
| ND6 | 14569 G>A | Syn:Ser35 | | rs386420019 | | | | H4a1a4 |
| ND6 | 14582 A>G | Val31Ala | | rs41354845 | 8.61% Htroplsmy | | | 10.2% Htroplsmy |
| CYB | 14861 G>A | Ala39Thr | | rs2853505 | 4.96% Htroplsmy | | | |
| CYB | 15132 T>C | Met129Thr | | na | | | | PVT-b |
| CYB | 15884 G>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.

**TABLE 3C** Diseases associated with SNPs identified in the H and K cybrids

| Loci: MT- | SNP | AA Change | Functional Loci: MT- | Cybrids with SNP | Disease[a] |
|---|---|---|---|---|---|
| ND2 | 5460 G>A | Ala331Thr | | 13–64 (K) | Alzheimer's Disease/Parkinson's Disease |
| ATP6 | 8902 G>A | Ala126Thr | | 13–64 (K) | Schizophrenia |
| HV2/TAS2 | 16129 G>A | | NonCoding | 13–57 (K) | Erwing's sarcoma, predisposition to breast cancer, nasopharyngeal carcinoma, Cyclical vomiting |
| ND6 | 14582 A>G | Val31Ala | | 11–10 (H), 13–65 (H) | Leber's hereditary optic neuropathy Bilateral optic atrophy, amblyopia, Goldman bilateral central scotomas |
| CYB | 15132 T>C | Met129Thr | | 13–65 (H) | Hypertrophic cardiomyopathy |

[a]Information obtained from www.MitoMap.org and www.HmtVar.uniba.it

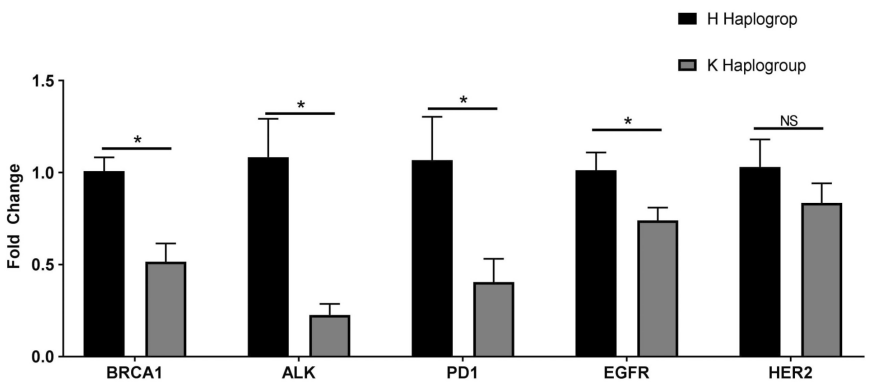


**FIGURE 1** Haplogroup K cybrids exhibit decreased expression levels of *BRCA1*, *ALK*, *PD1*, and *EGFR* cancer-associated genes compared to H cybrids. *$p \leq 0.05$; NS, not significant.

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.

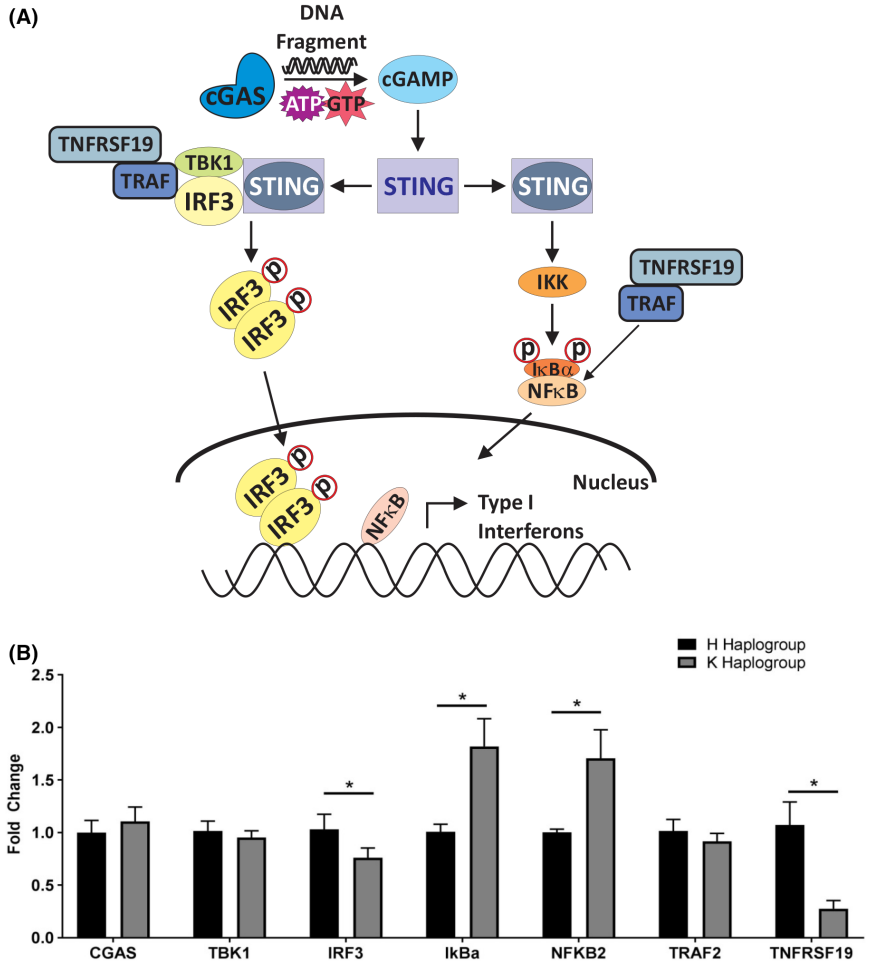


**FIGURE 2** K haplogroup cybrids differentially express genes involved in the STING DNA sensing pathway: (A) Schematic representation of the STING pathway including genes examined. (B) The K cybrids had higher expression of *IkBa* (1.940-fold ±0.302, $p = 0.047$) and *NFκB2* (1.451-fold ±0.119, $p = 0.026$) 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. *$p \leq 0.05$.

### 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 (Rho*0*-ARPE19-Control cells) and STING knockdown (Rho*0*-ARPE19-STING-KD cells) were analyzed for expression levels of cancer-related genes (Figure 4). The Rho*0*-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 Rho*0*-ARPE19-Control cells. The Rho*0*-ARPE19-STING-KD cells showed statistically significantly decreased *EGFR* expression levels (0.504-fold ±0.056, $p = 0.009$) compared to Rho*0*-ARPE19-Control cells, indicating that the involvement of the STING DNA sensor system was independent of the presence of mtDNA. Finally, Rho*0*-ARPE19-STING-KD cells

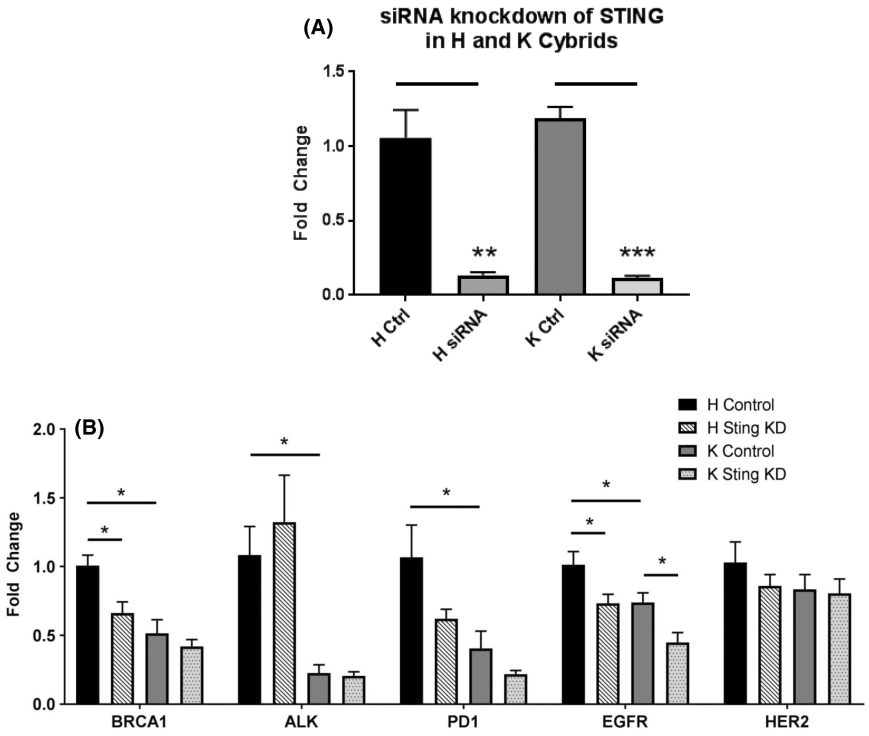


**FIGURE 3** Panel (A) Transfection resulted in significantly decreased expression of STING in H cybrids ($p = 0.0028$) and K cybrids ($p < 0.0001$) compared to the Control cybrids. Panel (B). The H-STING-KD cybrids showed significantly lower *BRCA1* expression levels ($p = 0.02$) and *EGFR* ($p = 0.02$) compared to H-Control cybrids. The K-STING-KD cybrids had significantly lower levels of *EGFR* ($p = 0.0481$) compared to the K-Control cybrids. Expression levels of *ALK, PD1*, and *HER2* were not changed after STING-KD. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. Ctrl, Control; siRNA, small interfering RNA.

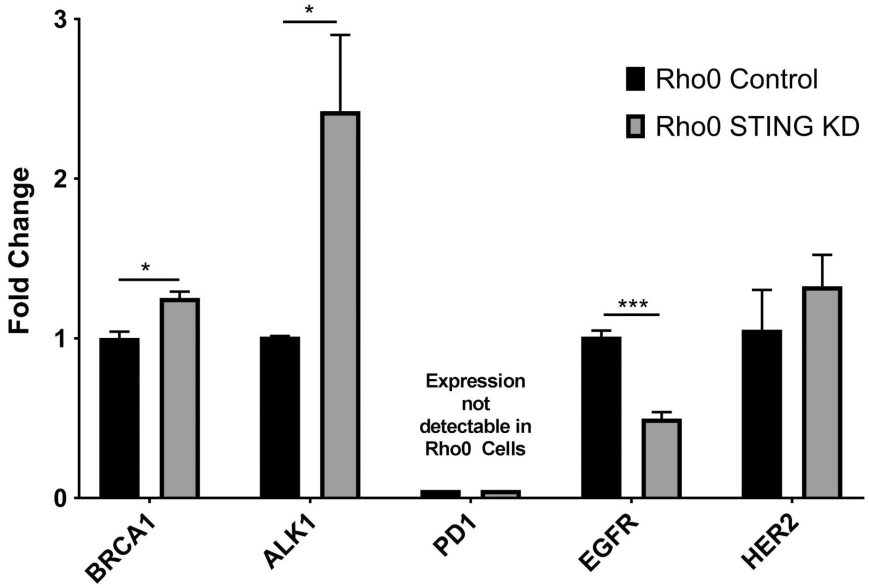


**FIGURE 4** Gene expression levels for cancer genes in Rho*0*-ARPE19-Control cells versus Rho*0*-ARPE19-STING-KD cells. Knockdown of STING results in higher levels of *BRCA1* and *ALK1* and lower gene of *EGFR* compared to the Rho*0*-ARPE19-Control cells. *PD1* was not expressed in the Rho*0*-ARPE19-Control or Rho*0*-ARPE19-STING-KD cells.

showed no significant change in *HER2* expression levels ($p = 0.44$) as compared to Rho*0*-ARPE19-Control cells. The expression of PD1 was absent in Rho*0* 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

**TABLE 4** List of anti-cancer drugs and associated gene targets

| Target | Drugs | Cancer |
|---|---|---|
| EGFR | Cetuximab (Erbitux)<br>Erlotinib (Tarceva)<br>Gefitinib (Iressa)<br>Lapatinib (Tykerb) | Non-small cell lung cancer, pancreatic cancer, breast cancer, colon cancer |
| ALK | Crizotinib (Xalkori),<br>Ceritinib (Zykadia),<br>Alectinib (Alecensa) | Non-small cell lung cancers |
| PD1 | Pembrolizumab(Keytruda), Nivolumab (Opdivo) | Melanomas, non-small cell lung cancer, renal, bladder, head and neck cancers, Hodgkin lymphoma |

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 cybrids, 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 heteroplasmy 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.[34] 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.[19] 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 Rho*0*-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 Rho*0*-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 Rho*0*-STING-KD-ARPE19 cells compared to the Rho*0*-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 Rho*0*-ARPE19-STING-KD cells and Rho*0*-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.[35] 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.[33,36] 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

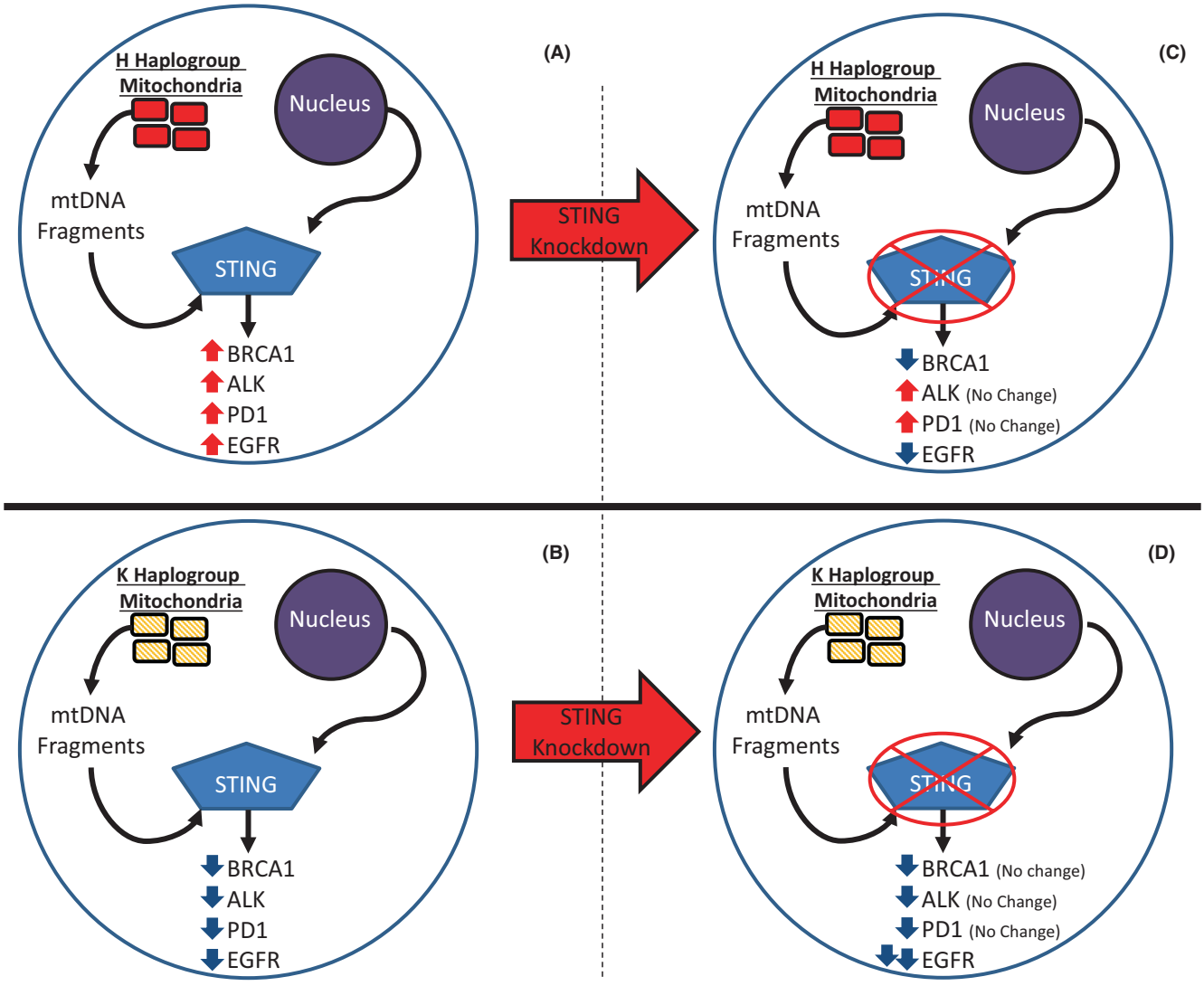


**FIGURE 5** Schematic summary of H versus K haplogroups after STING knockdown. The H cybrids show higher expression levels of *BRCA1, ALK, PD1*, and *EGFR* (Panel A) compared to the K cybrids (Panel B). After STING knockdown, the expression levels for *BRCA1* and *EGFR* are decreased in STING-KD H cybrids (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 Rho*0*-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

MCK. Discovery Eye Foundation (DEF) is a 501(c)3 that has supported her mitochondrial research. She serves as a Board Member for DEF. The terms of this arrangement have been reviewed and approved by the University of California, Irvine in accordance with its conflict-of-interest policies. MCK. Collaborations with Allegro, Ophthalmics.

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