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Complex-I Alteration and Enhanced Mitochondrial Fusion Are Associated With Prostate Cancer Progression

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Abstract

Mitochondria (mt) encoded respiratory complex-I (RCI) mutations and their pathogenicity remain largely unknown in prostate cancer (PCa). Little is known about the role of mtDNA loss on mt integrity in PCa. We determined mtDNA mutation in human and mice PCa and assessed the impact of mtDNA depletion on mt integrity. We also examined whether the circulating exosomes from PCa patients are transported to mt and carry mtDNA or mt proteins. We have employed next generation sequencing of the whole mt genome in human and Hi-myc PCa. The impact of mtDNA depletion on mt integrity, presence of mtDNA, and protein in sera exosomes was determined. A co-culture of human PCa cells and the circulating exosomes followed by confocal imaging determined co-localization of exosomes and mt. We observed frequent RCI mutations in human and Hi-myc PCa which disrupted corresponding complex protein expression. Depletion of mtDNA in PCa cells influenced mt integrity, increased expression of MFN1, MFN2, PINK1, and decreased expression of MT-TFA. Increased mt fusion and expression of PINK1 and DNMT1 were also evident in the Hi-myc tumors. RCI-mtDNA, MFN2, and IMMT proteins were detected in the circulating exosomes of men with benign prostate hyperplasia (BPH) and progressive PCa. Circulating exosomes and mt co-localized in PCa cells. Our study identified new pathogenic RCI mutations in PCa and defined the impact of mtDNA loss on mt integrity. Presence of mtDNA and mt proteins in the circulating exosomes implicated their usefulness for biomarker development.

Screening for prostate cancer has become highly controversial (Eckersberger et al., 2009; Kramer and Croswell, 2009) and PSA screening has led to the potential over-diagnosis and -

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treatment of low-risk disease (Draisma et al., 2009; Welch and Albertsen, 2009). Definitive treatment with radical prostatectomy or radiation therapy are essentially curative, however, it is estimated that over 1,055 men need to be screened and 37 cancers detected to prevent one prostate cancer death 11 years later (Schröder et al., 2012). There is a need for improved biomarkers for detection of aggressive disease (Cooperberg, 2012), and selection of men who may benefit from active surveillance protocols (Dall'Era et al., 2012; Bangma et al., 2013). Nevertheless, prostate cancer (PCa) is the second leading cause of death among men in the United States (Cazares et al., 2011; Saron et al., 2011; Barlow and Shen, 2013). In 2014, there were estimated 233,000 new cases and 29,480 deaths in the USA (www.nci.gov). Age, ethnicity, family history, obesity and diet, low level of vitamin D intake are risk factors for PCa (Shen and Abate-shen, 2010; Saron et al., 2011; Barlow and Shen, 2013). The molecular basis of PCa progression remains less well defined (Shen and Abateshen, 2010; Cazares et al., 2011; Lonergan and Tindall, 2011; Saron et al., 2011). Development of new PCa marker and understanding of the molecular progression cascade may improve monitoring and surveillance (Barlow and Shen, 2013).

Mitochondrial (mt) energy balance and reprogramming are hallmarks in cancer progression and implicated in biomarker development (He et al., 2010; Archer, 2013). In PCa, mtDNA mutations from various regions of the mt genome have been reported (Kloss-Brandstätter et al., 2010; Chatterjee et al., 2011). An earlier study reported complex-IV (COI) and complex-V (ATP6) mtDNA mutation in human PCa (Petros et al., 2005). Another recent study identified mtDNA mutation in the mt genome of PCa patients encompassing the regions COI, ATP6, rRNA, and tRNA (Kloss-Brandstätter et al., 2010). However, the extent and the role of RCI mutation in PCa development and progression remain largely unknown.

The mtDNA mutation may influence mt fusion and fission, which is important mechanisms for maintaining mt homeostasis and function (Archer, 2013). Many proteins, including MFN1, MFN2, and DNM1L (a.k.a. DRP1) are central regulators of these processes (Archer, 2013; Boland et al., 2013; Qian et al., 2013; Ferreira-da-Silva et al., 2015). Altered expression of these molecules could also be associated with respiratory complex targeting mtDNA mutations. On the other hand, mt biogenesis is a process involving replication of the mt genome and co-ordinated expression of both nuclear and mt encoded molecules and assembly of the oxidative phosphorylation (OXPHOS) complexes (Boland et al., 2013; Zhu et al., 2013; Da silva et al., 2014). The mt biogenesis could also be influenced by frequent mtDNA mutations. Many factors, including PINK1, MT-TFA, and IMMT play a critical role in regulating mt-biogenesis and maintaining mt integrity (Boland et al., 2013; Zhu et al., 2013; Da silva et al., 2014). The role of altered mt biogenesis in association with mtDNA mutations in PCa development and progression remains less well defined.

Hi-myc is a relevant transgenic mouse model with appreciable similarities with human disease, thereby making it possible to study the evolution of mtDNA/ndDNA mutation and their influence on PCa development and progression (Iwata et al., 2010; Barlow and Shen, 2013). The mice develop prostate intra-epithelial neoplasia (PIN) by 4–6 weeks and invasive adenocarcinoma (ADC) by 6–9 months (Iwata et al., 2010). The histological features and gene expression signature of the PIN and ADC in the Hi-myc mice closely resemble human PCa (Iwata et al., 2010). Although, distant metastasis is not evident in this model, muscle

invasion of the primary tumors can be examined for identifying crucial genetic alterations leading to PCa progression.

In recent studies, genomic DNA and various proteins were detected in the body fluid exosomes of PCa patients (Soekmadji et al., 2013). However, it is not known whether the circulating exosomes from the primary PCa patients carry wild type or mutant mtDNA and mt targeted proteins, which may influence PCa tumorigenesis. The exosomes are small (50–200 nm) secreted and bioactive vesicles from all cell types in the body fluids with endocytic origin (Yang and Robbins, 2011; Azmi et al., 2013; Kahlert and Kalluri, 2013; Melo et al., 2014; Soekmadji et al., 2013; Kowal et al., 2014; Wendler et al., 2013; Tickner et al., 2014). They carry nucleic acids and proteins and act as a mediator for cell–cell communication and bear immense potential for biomarker development for various malignancies including PCa (Soekmadji et al., 2013). Various molecular markers are used to identify exosomes in the body fluids of PCa patients, including CD63, CD9, TSG101, CD81, TfR, and Alix (Soekmadji et al., 2013).

In the present study, we identified frequent RCI targeted mtDNA mutations in both human and Hi-myc mice. The RCI mutations affected the corresponding protein complex expression. Depletion of mtDNA from PCa cells influenced mt integrity accompanied by enhanced expression of MFN1, MFN2, and PINK1 and decreased expression of MT-TFA. The Hi-myc mice derived PCa bearing RCI mutation exhibited increased mt distribution and mt fusion and increased expression of PINK1 and DNMI1L. Complex-I specific mtDNA and mt proteins MFN2 and IMMT were detected in the circulating exosomes. In addition, co-localization of the circulating exosomes and mt were evident in human PCa cells.

Materials and Methods

Human samples and ethical statement

Archived fresh frozen, paraffin embedded formalin fixed (FFPE) tissues, and serum samples were collected from Eastern Virginia Medical School (EVMS) under an IRB approved protocol. All the patients are de-identified and only relevant clinical information such as age, grade, stage, diagnosis, etc. was collected as per the IRB approved protocol.

Hi-myc mice tissue samples

Normal prostate and prostate cancer tissues were procured from Hi-MYC negative and positive mice, respectively at month 6 (Iwata et al., 2010) under an IACUC approved protocol. At this time, ADC develops in the Hi-MYC positive mice. Fresh tissue samples were frozen immediately for DNA and protein isolation. We also processed fresh tissues for electron microscopy analyses (Dasgupta et al., 2009). Lymphocytes were collected from Hi-MYC negative and positive mice, respectively at month 6.

Cell culture

Authenticated PC-3 and LNCaP cell lines were procured from ATCC and cultured in ATCC recommended medium. All cell lines were periodically checked for Mycoplasma

contamination using a Mycoplasma detection kit (Sigma, St. Louis, MO, # MP-0025; Dasgupta et al., 2013; Oyesanya et al., 2014).

Antibodies and reagents

The MFN1 (#ab57602), MFN2 (#ab56889), MT-TFA (#ab119684), IMMT (#ab110329), DNM1L (#ab56788), RCI subunit NDUFB8, 8KDa (#ab110242, #ab110245), and complex-IV subunit 2 (#ab110258) antibodies were procured from Abcam Inc., Cambridge, MA. β -Actin (#3700) was procured from Cell Signaling. The PINK1 antibody (#LS-B3384), was procured from LSBioscience Inc., Seattle, WA. Anti-mouse (#115-035-003) and -rabbit (#111-035-003) secondary antibodies were obtained from Jackson ImmunoResearch, West Grove, PA.

mtDNA sequencing in human PCa

Genomic DNA was extracted from the primary PCa tissues as described previously (Dasgupta et al., 2010, 2012a, 2012b). The amplification and quality control of the mtDNA population in the patients' sample were carried out before sequencing of the entire mt-genome (16.5 kb) on Illumina's NGS platform. The purified mtDNA was interrogated on Illumina's NGS ultra-deep mtDNA sequencing platform (Illumina HiSeq2000 sequencing, PE100). This human mtDNA NGS deep sequencing platform was capable of sequencing the whole human mtDNA with $>2,000 \times$ average coverage and detecting mtDNA mutations in as low as 1–2% heteroplasmy. Data analysis were performed using NextGENe v.2.2.0 software (DNAnexus). Revised Cambridge Reference Sequence (rCRS) was used as the reference sequence (Dasgupta et al., 2010, 2012a,b; Chatterjee et al., 2011). All the resulted mtDNA sequences were interrogated at different Human Mitochondrial Genome Databases to identify and categorize specific mtDNA sequence variants as described earlier (Dasgupta et al., 2010, 2012a,b; Chatterjee et al., 2011).

Determination of mtDNA mutation

Somatic mtDNA sequence variants were identified as base pair changes in mtDNA of cancer tissues when compared to an mtDNA sequence of matched normal lymphocytes (Dasgupta et al., 2010, 2012a,b; Chatterjee et al., 2011). Germ line sequence variants were identified as base pair changes present in the normal lymphocytes/adjacent tissues as well as cancer tissues compared to the reference sequences (rCRS). In each case, mtDNA sequences were interrogated at the available Human Mitochondrial Genome Databases for defining each sequence variant appropriately as employed in our studies (Dasgupta et al., 2010, 2012a,b; Chatterjee et al., 2011). We also identified novel germ line mtDNA mutations and polymorphic variants that are reported in normal population using the above databases.

Determination of mtDNA haplotype and deletion

Haplotypes were assigned to each sample using HaploGrep (Kloss-Brandstätter et al., 2011), which searches for patterns of haplogroup specific or haplogroup associated polymorphisms recognized in PhyloTree (van Oven and Kayser, 2009; <http://www.phylotree.org>. doi: 10.1002/humu.20921), a comprehensive phylogenetic tree of human mitochondrial variation. Haplotype assignments from all samples were visualized in the PhyloTree

framework (Build 16 from 19 February 2014). To determine mtDNA deletion and insertion status in the tumor samples, frequency of nucleotide deletions (delA/G) and insertions (insA/C) were counted after normalization with the matched normal samples.

mtDNA sequencing in Hi-myc mice

Genomic DNA was extracted from matched lymphocytes and the prostate ADC tissues obtained from Hi-myc positive mice at month 6 (Iwata et al., 2010). We utilized Illumina's ultra-deep mouse mtDNA sequencing platform for the whole mt-genome (16,299 bp) sequencing (Illumina HiSeq2000 sequencing, PE100). Data analysis were performed using NextGENe v.2.2.0 software (DNAexus). Mouse mitochondrial genome sequence (NC_005089) was used as the reference sequence.

Determination of mtDNA mutation in Hi-myc mice

Somatic mtDNA sequence variants were identified as base pair changes in mtDNA of prostate cancer tissues when compared to an mtDNA sequence of matched normal lymphocytes. Germ line sequence variants were identified as base pair changes present in the normal lymphocytes as well as the prostate cancer tissues compared to the reference sequences (NC_005089).

Immunohistochemistry, confocal imaging, and Western blotting

Immunohistochemistry (IHC) was performed using specific antibody in the paired primary tissue specimens as described earlier (Dasgupta et al., 2013; Oyesanya et al., 2014). All the IHC and histological evaluations were done per pathologist's guidance as described earlier (Dasgupta et al., 2013; Oyesanya et al., 2014). The confocal imaging was done as described previously (Oyesanya et al., 2014). Preparation of the whole cell lysates and Western blotting analysis was performed following protocols as described earlier (Dasgupta et al., 2013; Oyesanya et al., 2014).

Generation of rho⁰ cells

LNCaP and PC-3 cells were cultured in their recommended growth medium supplemented with 100 ng/ml of ethidium bromide, 50 µg/ml of uridine and 1 mmol/L of sodium pyruvate (Delsite et al., 2002). Cells were cultured in this medium for 3 weeks before any further experimentation. The mtDNA depletion status (rho⁰) was confirmed by Western blot analysis using antibodies for Complex-I (subunit-NDU5B8), complex-IV (subunit-II) as described above. These antibodies were randomly selected to determine respective complex expression.

Electron microscopy

Control and rho⁰ cells were cultured in 35-mm culture dishes and fixed with a fixative containing 2% paraformaldehyde, 2% glutaraldehyde, 1 M PIPES, 3 mM CaCl₂ pH 7.2 for 1-h room temperature on a slow rocker (Dasgupta et al., 2009). After a 30-min buffer rinse, cells were post-fixed in 1% osmium tetroxide reduced with 0.8% potassium ferrocyanide, 0.1 M sodium cacodylate, 3 mM CaCl₂ at 4°C for 1 h in the dark. Thin compression-free sections (80 nm) were obtained with a diatome diamond knife. Sections were picked up on

200 mesh copper grids and stained with uranyl acetate followed by lead citrate. Grids were examined on a Hitachi H-7600 TEM operating at 80 Kv. Images were digitally captured with an AMT 1 K × 1 K CCD camera. For reliable counting and structural analysis of mitochondria, images were captured at 6,000 magnification and at least 30 cells were randomly selected from each group. Data were shown as mean ± SE. We measured the percent of cytoplasm occupied by mitochondria, the number of mitochondria and the volume of an average mitochondrion as described earlier (Dasgupta et al., 2009). We measured the average nuclear size and nuclear to cytoplasm ratio as a measure of cell volume (Dasgupta et al., 2009). The normal and cancer tissues from Hi-myc mice were also fixed and processed similarly as described above.

Exosome preparation from human PCa sera and mtDNA detection in the exosomes

The exosomes were isolated from human sera using commercially available kits and protocols (#EXOQ5A-1, System Bioscience, Mountain View, CA). Total protein was isolated from the exosomes for downstream Western blot analysis. The exosomes were treated with proteinase K before protein isolation (Melo et al., 2014). We have isolated DNA from the circulating sera derived exosomes of patients with BPH (N = 5) and PCa (N = 10; Dasgupta et al., 2010, 2012a,b). The exosomes were treated with RNase and Proteinase K before DNA isolation (Melo et al., 2014). Following primers were used for PCR amplification of the exosomal DNA. MT-ND4 (gene ID: 4538): F- ACTCACAAACACCCTAGGCTC; R-GCTTCGACATGGGCTTTAGG; CD63 (gene ID 967): F-TCCCTTCCATGTCTGAAGAAC, R-TCCCAAAACCTCGACAAAAG; TSG101 (gene ID 7251): F-GATACCCTCCCAATCCCAGT; R-GTCACTGAC-CGCAGAGATGA. The PCR reaction was carried out in 50 µl volumes as follows: 95°C for 5 min × 1 cycle; 95°C-30", 60°C-30", 72°C-1 min × 35 cycles; 72°C-5 min for final extension. The PCR products were run on a 1% agarose gel and imaged using a Bio-Rad Chemidoc XRS imaging station.

MFN2 and IMMT protein detection in the exosomes

Western blotting was performed using 40 µg of total exosome proteins isolated from the circulating sera of the BPH and PCa patients to detect IMMT and MFN2 expression. CD63 was used as an exosome marker (Soekmadji et al., 2013; Kumar et al., 2015).

Exosome labeling, PC-3 transfection, and co-culture

Exosomes (1×10^6) isolated from the sera of one BPH case was labeled with Exo-Green following a specific protocol (System Biosciences, # EXOG200A-1). PC-3 and LNCaP cells were stably transfected with mt-targeted COXIV-DSRed2 plasmid (Clontech, # 632421) in the presence of G418 for fluorescent labeling of mt. The mt-labeled PC-3 and LNCaP cells were then co-cultured with the pre-labeled exosomes for 7 days followed by confocal imaging. The PCa cells were cultured in exosomes-depleted FBS (System Biosciences, # EXO-FBS-50A-1) as recommended.

Statistical analysis

χ^2 , Fisher's exact, or Student's *t*-tests were used when appropriate. All *P*-values were two-sided and all confidence intervals were at the 95% level. Computation for all the analysis was performed using the Statistical Analysis System (SAS).

Results

Haplotype determination of the PCa patients

We have employed next generation sequencing (NGS) of the entire mt genome (16,559 bp) in six primary PCa tumors. Haplotype assignments for each sample (Fig. 1) scored at least 92% or higher on HaploGrep's quality assessment, indicating identification are quite reliable. Normal and tumor samples from each individual were assigned to the same haplotype. Between 41 and 60 single nucleotide polymorphic sequence variants in the mtDNA distinguished and determined the haplotype of each patient. HaploGrep attributed this variation to a combination of mutational hotspots, mutations known from other accessions in PhyloTree, and mutations not previously observed in PhyloTree. The last category includes all examples of heteroplasmy, which are currently not otherwise assessed by HaploGrep.

Respiratory complex-I mutation and nucleotide deletion are early events in PCa tumorigenesis

Through next generation ultradeep sequencing of the whole mt genome (16,569 bp) in six primary PCa tumors, we identified frequent RCI targeted somatic mtDNA mutations (Table I). All the primary PCa patients have exhibited at least one mtDNA mutation compared to their corresponding normal and a total of 12 somatic mtDNA mutations was detected (Table I). Eighty-three percent (10/12) of the mtDNA mutations were heteroplasmic nucleotide transitions and non-synonymous in nature (Table I). Ninety-two percent (11/12) of the mutations were from the coding mtDNA regions (Table I). Of the various coding mtDNA mutations, 67% (8/12) were in respiratory complex-I, 25% (3/12) were in complex-V, and 1 mutation (8%) was in complex-IV (COIII), respectively (Table I). Only one non-coding mutation was detected in the 12SrRNA region (Table I). Thus, RCI encoding mtDNA mutations were frequent in the primary PCa tumors.

The RCI is an integral part of the oxidative phosphorylation system (OXPHOS) (Chatterjee et al., 2011). We observed one deletion mutation at nucleotide position 11038 (MT-ND4, 11038delA) of the mt genome encoding complex-I in one primary PCa (# 5252, Table I). A severely decreased protein expression (*P* = 0.0002) of the RCI was evident in the primary tumor of the patient harboring the 11038delA mutation (Fig. 2A and B). In another patient, we observed a novel insertion mutation (insA) at nucleotide position 12426, spanning ND5 region of the RCI (Table I). Thus, RCI specific mtDNA mutation affected corresponding complex expression in the same individual harboring the mutation.

Other than the somatic mtDNA mutations, we identified six germ line mtDNA mutations, a couple of which were novel (human mitochondrial database) spanning mtDNA coding regions *ND2* and *ATPase6* (Table II). Notably, all the germ line mutations were

homoplasmic and non-synonymous in nature (Table II). In our NGS analysis, other than nucleotide transitions, we also observed nucleotide insertion or deletion in various mtDNA regions. Compared to the insertions (insA and insC), nucleotide deletions (delA and delG) were higher ($P = 0.0042$) in the primary PCa tumor (Fig. 2C). Thus, novel germ line mtDNA mutations and mtDNA loss were present in the primary PCa tumors.

mtDNA depletion induced mt fusion in human PCa cells

The NGS analysis revealed significant nucleotide deletion mutations compared to nucleotide insertion in the primary PCa tissues (Fig. 2C). To understand the impact of mtDNA loss on mt integrity, we depleted mtDNA in PC-3 and LNCaP cells using ethidium bromide. Depletion of mtDNA was confirmed by Western blotting with complex-IV and -I antibodies (Fig. 3A). In mtDNA depleted PCa cells, we observed an increased number of fused mt with enhanced mt volume (Fig. 3B). This was accompanied by elevated expression of MFN1, MFN2, and PINK1 and decreased expression of MT-TFA, critical regulators of mt fusion and biogenesis (Fig. 3C). Thus, mtDNA depletion in established human PCa cultures resulted in enhanced mt fusion and altered expression of mt fusion and biogenesis associated molecules.

Respiratory complex-I mutations are also targeted in Hi-myc PCa progression

We also performed NGS of the entire mt-genome (16,299 bp) in the primary Hi-myc tumors. Invasive prostate tumor tissues at month 6 and matched lymphocytes were procured from Hi-myc positive mice to determine the nature and extent of mtDNA mutations. Similar to human PCa, we identified frequent RCI specific mtDNA mutation in the Hi-myc positive mice derived cancer tissues (Table III). A total of 12 heteroplasmic and non-synonymous somatic mtDNA mutations were detected (Table III). Seventy-five percent (9/12) of the mutations were from the RCI (Table III). The other three mtDNA mutations were distributed in the coding complex-III (CYTB), complex V (ATPase8), and non-coding tRNA^{Arg} (TrnR) regions. Thus, the Hi-myc mice also exhibited a similar pattern of mtDNA mutations as observed in the human counterparts.

Disrupted mt integrity in Hi-myc mice harboring mtDNA mutations

In the next step, we determined mt distribution and volume in Hi-myc positive prostate tumors and normal prostate tissues procured from age matched Hi-myc negative mice. We observed an increased distribution of mt (Fig. 4A and B; $P = 0.005$) and concomitant decrease in mt volume (Fig. 4C; $P = 0.02$) in the cancer tissues compared to the normal. Similar to human PCa, we also observed an enhanced mt fusion in the Hi-myc tumors (Fig. 4D). Compared to the tubular and elongated mt in the normal prostate tissues, the mt in the tumors were rounded in appearance with disrupted cristae and aberrant fusion (Fig. 4D). These phenotypic changes were accompanied by enhanced expression of PINK1 and DNMT1L (Fig. 4E), proteins associated with mt fusion and fission (Archer, 2013; Qian et al., 2013; Ferreira-da-Silva et al., 2015). Thus, increased mt anomaly and an enhanced expression of mt biogenesis and fission associated molecules were evident in representative Hi-myc model of PCa.

Complex-I mtDNA and mt proteins are detectable in the circulating exosomes of PCa

Recent studies detected genomic DNA in the circulating exosomes from PCa (Lázaro-Ibáñez et al., 2014). We have detected RCI mtDNA (MT-ND4) in the sera derived circulating exosomes isolated from 100% (5/5) men with BPH, 100% men with Gleason score 6 (5/5), and Gleason score 9 (5/5) PCa (Fig. 5A). Notably, the circulating exosome populations isolated from the sera of all the above PCa (N = 10) and BPH (N = 5) cases harbored mt fusion and biogenesis associated MFN2 and IMMT proteins (Boland et al., 2013; Zhu et al., 2013; Da Silva et al., 2014; Fig. 5B). Thus, RCI specific mtDNA and mt targeted proteins were detectable in the circulating exosomes of the sera obtained from individuals with benign and malignant PCa tumors.

From the above observations, we hypothesized that the exosomes migrate to the mt and capture mtDNA and mt proteins. We co-cultured PC-3 and LNCaP cells, and the circulating sera exosomes purified from one BPH case for 1 week. Before the co-culture, PC-3 and LNCaP cells were stably transfected with a mt targeted complex-IV-DSReD2 plasmid for fluorescent labeling of mt. Confocal imaging of the exosome treated PC-3 and LNCaP cells confirmed co-localization of mt and exosomes (Fig. 5C and D). These results suggest that the exosomes might have migrated to mt.

Discussion

Through NGS of the entire mt-genome, we have identified frequent RCI mtDNA mutations in primary PCa patients. A novel deletion mutation spanning the ND4 region of the RCI significantly abolished the corresponding complex expression indicating the pathogenicity of the sequence variant. The RCI is an integral part of the OXPHOS system composed of five such complexes (I–V), some encoded by the nucleus and some by the mt (Chatterjee et al., 2011). Complex-I regulates mt apoptosis and its loss may prevent cell death and promote progression (Gasparre et al., 2008; Mayr et al., 2008). Previous reports of RCI targeting mtDNA mutations in PCa (Jerónimo et al., 2001; Kloss-Brandstätter et al., 2010; Arnold et al., 2015) did not report on the corresponding protein expression in PCa. Interestingly, a study from the Sidransky laboratory (Jerónimo et al., 2001) reported a delA mutation at the nucleotide position 11032 spanning ND4 region, which is very closely located (6 nucleotides upstream) to the region that we have identified in the present study (11038delA). Furthermore, the 11038delA mutation was detected in thyroid cancer and renal oncocytomas (Gasparre et al., 2008; Mayr et al., 2008). In renal oncocytomas, the 11038delA mutation was associated with the loss of RCI activity and assembly (Mayr et al., 2008). Thus, 11038delA mutation appeared to be pathogenic for various malignancies. In addition to the somatic mutations, we also observed a novel germ line delA mutation (4611delA, ND2) in RCI. Of note, the RCI mtDNA mutations were also predominant in the transgenic model of PCa. Thus, our findings support the notion that somatic RCI mutations are pathogenic (Mayr et al., 2008) and may have a causative role in PCa development and progression.

In a recent study, A10398G, a polymorphic mtDNA sequence variant was found predominantly in PCa patients with bone metastasis (Arnold et al., 2015). A cybrid cell line generated from a breast cancer patient harboring A10398G variant was shown to induce an abnormal mt function, increased tumorigenic growth, and metastasis in mice (Arnold et al.,

2015). Other studies also linked A10398G polymorphism with breast cancer susceptibility and increased risk (Arnold et al., 2015). In our study, we observed A10398G as a germ line sequence variant in 2 PCa patients (#5437 and #5359). Notably, the six patients we have sequenced in this study were early staged (Gleason Grades 6 and 7). Thus, the RCI targeting mtDNA mutations appeared to be the early events which were necessary for driving PCa tumorigenesis along with other nuclear encoded genetic alterations.

Recent studies identified mtDNA loss in primary PCa and its association with poor prognosis implicating a contributing role of mtDNA loss in PCa (Cook et al., 2012; Koochekpour et al., 2013). Moreover, mtDNA loss in PCa was shown to activate Ras, AKT, and ERK signaling pathways which were associated with aggressive disease outcome in vitro and in vivo (Cook et al., 2012). However, the events linking mtDNA loss with mt integrity and function and their effects on PCa progression are unknown. Our finding that the mtDNA loss in primary PCa and mtDNA depleted cells increased MFN1, MFN2, and PINK1 expression and decreased expression of MT-TFA may provide evidence linking mtDNA loss and PCa progression. These alterations could lead to perturbed mt function and their biogenesis, thereby aiding in PCa progression. MFN2 was over-expressed and appeared to be oncogenic in lung adenocarcinoma (Lou et al., 2015) and depletion of MFN2 reduced cellular proliferation, cell cycle progression, and invasiveness (Lou et al., 2015). Cigarette smoke increased expression of MFN1 and MFN2 and altered mt structure and function in bronchial epithelial cells (Hoffmann et al., 2013). Increased PINK1 expression and activation promote PI3K/AKT and FOXO signaling pathways (O'Flanagan and O'Neill, 2014). PINK1 also alters mt function and has been proposed as an attractive therapeutic target in human cancers (Lee et al., 2013; O'Flanagan and O'Neill, 2014). In a recent study, frequent truncating mutations of MT-TFA, an mt transcription factor resulted in mtDNA depletion and apoptotic resistance in colon cancer (Guo et al., 2011). Thus, as evident in our study, progressive loss of mtDNA during PCa evolution could be associated with aberrant MT-TFA function or vice versa leading to apoptotic resistance and sustained PCa growth.

Similar to human tumors, upregulation of PINK1 and DNM1L (a.k.a.DRP1) in Hi-myc mice increased mt proliferation and fusion. DNM1L is a mt fission associated protein (Qian et al., 2013) and depletion of DNM1L increased apoptosis of colon cancer cells (Inoue-Yamauchi and Oda, 2012). In Glioblastoma, depletion of DNM1L induced apoptosis of malignant brain tumor initiating cells and suppressed tumor growth (Xie et al., 2015). Moreover, DNM1L activation correlated with the poor prognosis in glioblastoma (Xie et al., 2015). Over-expression of DNM1L was also observed in oncocytic thyroid tumors and regulates cancer cell migration (Ferreira-da-Silva et al., 2015). DNM1L appears to be an attractive therapeutic target in various malignancies (Qian et al., 2013). Thus, Hi-myc PCa development could be associated with the robust expression of DNM1L accompanied with abnormal mt integrity and function.

Exosomes are emerging as a promising biomarker tool for cancer detection and appeared to be involved in human tumorigenesis (Yang and Robbins, 2011; Azmi et al., 2013; Kahlert and Kalluri, 2013; Soekmadji et al., 2013; Kowal et al., 2014; Melo et al., 2014; Tickner et al., 2014; Wendler et al., 2013). Genomic DNA (gDNA) encoding various growth regulatory molecules, including p53, MLH1 was found in the body fluid exosomes of PCa (Lázaro-

Ibáñez et al., 2014). In a recent study, RCI mtDNA (MT-ND1) was detected in the exosomes isolated from cultured astrocytes and glioblastoma cells (Guescini et al., 2010), suggesting that exosome may transport necessary genetic information (gDNA/mtDNA) to other cells for influencing tumor growth (Melo et al., 2014). Other than DNA, some known exosome marker proteins such as CD9, CD63 and CD81 were detected in PCa (Kumar et al., 2015). However, to our knowledge, the presence of respiratory complex specific mtDNA or mt targeted proteins have not yet been reported in the circulating exosomes of PCa patients. Surprisingly, we have detected RCI specific mtDNA (MT-ND4) in the circulating exosomes of primary PCa in the region where we have detected frequent mtDNA mutations. It is likely that the mtDNA (MT-ND4) detected in the exosomes was due to the capture of the mtDNA by the exosomes in the mt as we have observed their co-localization in PCa cells. However, we cannot rule out the possibility that the mtDNA copies were captured by the exosomes outside the mitochondria as mtDNA may leak from the mitochondria to the cytosol due to the loss of membrane potential or damage. The mt proteins might have been captured similarly in the circulating exosomes of the progressive PCa patients. Mechanistically, deleterious RCI mutations and simultaneous mtDNA loss may lead to perturbed complex activity and inhibition of apoptosis in one hand and loss of mt integrity on the other. Eventually, transport of these altered messages in the form of nucleic acids and proteins through the circulating exosomes to the neighboring cells may aid in PCa progression as observed in a recent study on breast cancer (Melo et al., 2014).

In summary, we have detected pathogenic RCI targeted mtDNA mutations and altered mt integrity following mtDNA depletion in PCa. The circulating exosomes from PCa sera carried RCI-mtDNA and mt integrity associated proteins and co-localized with the mt in the PCa cells. A comprehensive categorization of the pathogenic mtDNA mutation from RCI and their detection in the circulating exosomes could be an invaluable tool for early PCa detection, monitoring and surveillance.

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Fig. 1. Phylogenetic representation of benign mitochondrial genomes from six prostate cancer patients. Haplotype taxonomy has been simplified for visualization purposes. Hierarchical relationships of haplotypes and nucleotide changes along branches were aggregated from PhyloTree (van Oven and Kayser, 2009), so positions considered uninformative by PhyloTree (309.1C[C], 315.1C, AC indels at 515–522, 16182C, 16183C, 16193.1C[C] and 16519) were also excluded here. The prefix @ indicates a reversion at that position and heteroplasmic sites are labeled in turquoise.

Fig. 2.

Loss of complex-I expression and mtDNA in PCa. (A) Compared to the matched normal tissue, complex-I protein expression was significantly low ($P = 0.0002$) in the patient (# 5252, Table I) harboring the 11038delA mutation. Two representative areas of normal and corresponding tumor tissues were shown. Magnification $\times 200$. (B) Complex-I expression was also undetectable by Western blotting in the patient # 5252 harboring the 11038delA mutation. Mitochondrial COX-IV was used as a loading control. (C) Nucleotide deletions were significantly higher ($P = 0.0042$) in the tumors compared to nucleotide insertions.

Fig. 3.

Impact of mtDNA depletion on mt integrity in PCa cells. (A) Diminished expression of complex-I (CI) and IV (CIV) following mtDNA depleted (Rho) PC-3 and LNCaP cells compared to the control wild-type (WT) cells. (B) Mitochondria appeared enlarged and fused (arrows) in mtDNA depleted (Rho) PC-3 and LNCaP cells compared to the control wild-type (WT) cells. (C) Enhanced expression of MFN1, MFN2, and PINK1 and decreased expression of MT-TFA in mtDNA depleted (Rho) PC-3 and LNCaP cells compared to the control wild-type (WT) cells. β -Actin was used as a loading control.

Fig. 4.

Mitochondrial distribution and integrity in the Hi-myc PCa tumors. (A,B) Distribution of mitochondria (arrowheads) was higher ($P = 0.005$) in Hi-myc positive mice derived primary prostate tumor tissues compared to normal prostate tissues procured from age matched Hi-myc negative mice. Three representative areas were shown from normal and tumor tissues. (C) Average mitochondria volume was lower ($P = 0.02$) in the Hi-myc positive mice derived primary prostate tumor tissues compared to the normal prostate tissues procured from age matched Hi-myc negative mice. (D) Compared to the elongated tubular mitochondria in the normal prostate tissues, the mitochondria were fused and appeared rounded with disrupted cristae structure in the prostate tumor tissues (arrow). (E) Enhanced expression of PINK1 and DNML1 in Hi-myc positive mice derived primary prostate tumors (T1 and T2) compared to normal prostate tissues procured from age matched Hi-myc negative mice. Beta-actin was used as a loading control.

Fig. 5.

Detection of mtDNA and mt proteins in the circulating exosomes. (A) Amplification of MT-ND4 specific DNA in the circulating exosomes of men with benign prostate hyperplasia and primary prostate cancer (Gleason scores 6 and 9). DNA from normal human prostate tissues was used as positive control in the PCR amplification. CD63 and TSG101 markers were used as negative controls. M: Molecular size marker in base pairs (bp) to indicate the appropriate size of the tested genes. (B) Detection of MFN2 and IMMT expression in the circulating exosomes of men with benign prostate hyperplasia and primary prostate cancer (Gleason score 6 and Gleason score 9). CD63 was used as an exosome marker. (C,D) Co-localization (arrows) of the exosomes (green) and fluorescently labeled mitochondria (red) in the PC-3 and LNCaP cells. Magnification $\times 600$.

TABLE I

Somatic mtDNA mutations in the primary human PCa

Patients ID	mtDNA region	rCRS ^a	N ^b	T ^c	Nature of change	Codon/amino acid change	Gene affected	Affected complex ^d
5428	3468	A	A	A>AC	Heteroplasmic	54K>KN	ND1	CI
	5208	A	A	A>AC	Heteroplasmic	247T>TP	ND2	CI
	9191	T	T	T>CT	Heteroplasmic	222L>PL	ATPase6	CV
	9193	C	C	C>AC	Heteroplasmic	223H>NH	ATPase6	CV
5359	9195	C	C	C>AC	Heteroplasmic	223H>QH	ATPase6	CV
	11126	G	G	G>AG	Heteroplasmic	123E>KE	ND4	CI
	1210	T	T	T>CT	Heteroplasmic	—	12SrRNA	—
5465	3468	A	A	A>AC	Heteroplasmic	54K-KN	ND1	CI
	3447	A	A	A>AC	Heteroplasmic	47Q>QH	ND1	CI
	9582	C	C	C>CA	Heteroplasmic	126P>PA	COIII	CIV
	12426	C	C	insA	Frame shift	—	ND5	CI
5252	11038	A	A	delA	Frame shift	—	ND4	CI

^aRevised Cambridge reference sequence (rCRS).

^bNormal.

^cTumor.

^dC: respiratory complex.

TABLE II

Germ line mtDNA mutations in primary human PCa

mtDNA region	rCRS ^a	N ^b	T ^c	Nature of mutation	Codon/amino acid change	Gene affected	Affected complex ^d
4611	A	delA	delA	Frame shift		ND2	CI
8623	A	A>G	A>AG	Homoplasmic/Heteroplasmic	33T>A; 33T>TA	ATPase6	CV
8860	A	G	G	Homoplasmic	112T>A	ATPase6	CV
5913	G	A	A	Homoplasmic	4D>N	COI	CIV
3337	G	A	A	Homoplasmic	11V>M	ND1	CI
10398	A	G	G	Homoplasmic	114T>A	ND3	CI

^aRevised Cambridge reference sequence (rCRS)

^bNormal

^cTumor.

^dC: respiratory complex.

TABLE III

MtDNA mutation spectrum in Hi-myc mice derived primary tumor

mtDNA region	Reference sequence ^a	N ^b	T ^c	Nature of change	Codon/amino acid change	Gene affected	Affected complex ^d
3045	A	A	A>AG	Heteroplasmic	99N>ND	<i>ND1</i>	CI
3498	A	A	A>AG	Heteroplasmic	250N>ND	<i>ND1</i>	CI
3917	A	A	A>AG	Heteroplasmic	2N>ND	<i>ND2</i>	CI
4642	A	A	A>AG	Heteroplasmic	2431I>IM	<i>ND2</i>	CI
4895	A	A	A>AG	Heteroplasmic	328T>TA	<i>ND2</i>	CI
7857	A	A	A>AG	Heteroplasmic	31Q>QR	<i>ATPase8</i>	CV
9827	A	A	A>AG	Heteroplasmic	—	TrnR	—
10321	T	T	A>AG	Heteroplasmic	52S>SF	<i>ND4</i>	CI
11983	A	A	A>AG	Heteroplasmic	81K>KR	<i>ND5</i>	CI
12748	A	A	A>AG	Heteroplasmic	336K>KR	<i>ND5</i>	CI
13432	A	A	A>AG	Heteroplasmic	564K>KR	<i>ND5</i>	CI
14163	A	A	A>AG	Heteroplasmic	7T>TA	<i>CYTb</i>	CIII

^aMouse mitochondrial genome sequence (NC_005089) was used as the reference sequence.

^bNormal.

^cTumor.

^dC: respiratory complex.