Published in final edited form as:

Prostate. 2008 March 1; 68(4): 408–417.

Increased Distributional Variance of Mitochondrial DNA Content Associated With Prostate Cancer Cells as Compared With Normal Prostate Cells

Takatsugu Mizumachi¹, Levan Muskhelishvili², Akihiro Naito¹, Jun Furusawa¹, Chun-Yang Fan³, Eric R. Siegel⁴, Fred F. Kadlubar⁵, Udaya Kumar⁶, and Masahiro Higuchi^{1,*}

¹ Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Central Arkansas Veterans Health Care System, Little Rock, Arkansas

² Division of Toxicologic Pathology Associates, National Center for Toxicological Research, Food and Drug Administration, Jefferson, Arkansas

³ Department of Pathology, University of Arkansas for Medical Sciences, Central Arkansas Veterans Health Care System, Little Rock, Arkansas

⁴ Department of Biostatistics, University of Arkansas for Medical Sciences, Central Arkansas Veterans Health Care System, Little Rock, Arkansas

⁵ Department of Epidemiology, University of Arkansas for Medical Sciences, Central Arkansas Veterans Health Care System, Little Rock, Arkansas

⁶ Department of Urology, University of Arkansas for Medical Sciences, Central Arkansas Veterans Health Care System, Little Rock, Arkansas

Abstract

BACKGROUND—Mitochondria are key organelles for apoptosis, and mitochondrial DNA (mtDNA) content can regulate cancer progression. Increases in mtDNA mutations and deletions have been reported in cancer; however, a detailed investigation of mtDNA content in cancer cells has not yet been conducted.

METHODS—Quantitative real-time PCR and improved extraction method were established to investigate the mtDNA content in a single prostate cell.

RESULTS—The heterogeneity of mtDNA content was demonstrated between the clones of prostate cancer cell line LNCaP and individual cells in each clone. To investigate whether large distributional variance of mtDNA content is associated with cancer initiation and/or progression, we first compared PZ-HPV-7, an HPV-transformed normal prostate epithelial cell line, with CA-HPV-10, transformed from prostate cancer cells derived from the same donor. We found an enhanced distributional variance of mtDNA content in CA-HPV-10. Then, we investigated mtDNA content in individual cells in laser microdissected cancer and adjacent normal cells from prostate cancer tissue specimens using quantitative real-time PCR method. Results showed that the mtDNA content per cell follows a higher skewed distribution in cancer cells as compared in normal cells. We also observed that mtDNA content was increased in seven of nine (78%) of prostate cancers compared to normal prostate tissue.

CONCLUSIONS—These results indicate that prostate carcinogenesis may involve dysregulation of mtDNA content.

*Correspondence to: Masahiro Higuchi, 4301 W. Markham St., Slot 516 Little Rock, AR 72205. E-mail: mhiguchi@uams.edu.

Keywords

mitochondrial DNA; cancer initiation; cancer progression

INTRODUCTION

Prostate cancer is the third leading cause of cancer death in men, and more than 230,000 new cases are diagnosed with prostate cancer annually in the United States [1]. Prostate carcinogenesis is a very complex process reflected in the heterogeneity of its clinical and pathological diagnosis, and in a long latency in the development of life-threatening prostate cancer [2]. However, the primary cause and the molecular events leading to prostate cancer are still poorly understood. Even though prostate cancer has a long natural history, usually spanning a decade or more, the mechanisms leading to cancer progression are still unknown.

The mitochondrion has its own genome, the mitochondrial DNA (mtDNA). Human mtDNA is remarkably small (16,569 bp) compared with nuclear DNA (nDNA; approximately 3×10^9 bp). Each mitochondrion contains between 2 and 10 mtDNA molecules, and the number of mitochondrial genomes in a cell ranges from several hundreds to more than 10,000 copies, depending on the cellular type. The identification of increased or reduced mtDNA content has been reported. Breast [3], renal [4], and liver [5,6] cancers reportedly have reduced mitochondrial content, resulting in reduced mitochondrial activity. On the other hand, head and neck [7], lung [7], thyroid [3], and pancreas [8] cancers have increased mtDNA content. mtDNA plays an important role in apoptosis, and mutation and depletion of mtDNA cause inhibition of apoptosis [9]. Furthermore, several reports indicate that mtDNA plays an important role in cellular sensitivity to cancer therapeutic agents. Depletion of mtDNA causes resistance to cisplatin and adriamycin [10], and D-loop mutations appear to factor into resistance to fluorouracil-based adjuvant chemotherapy in colon cancers [11]. We previously showed that increased mtDNA induces acquired docetaxel resistance in head and neck cancer cells [12]. Because the D-loop region is responsible for controlling mtDNA replication and transcription, a mutation in this region might cause a decrease in copy number that results in repressing overall expression of the mitochondrial genome. Lee et al. [13] showed that 39.3% of hepatocellular cancer cells had mutations in the D-loop region of mtDNA and that the mtDNA copy number was significantly reduced in 70.8% of cancer cells with mutations in this region. Previously, we showed that mtDNA-deficient myelogenous leukemia cells were resistant to apoptosis induced by tumor necrosis factor [14]. We also showed that androgen-independent cells have far less wild-type mtDNA and far more of the deletion-bearing form than androgen-dependent cells; furthermore, mtDNA depletion shifted androgen-dependent cells to androgen-independence [15]. Therefore, the change of mtDNA may be critically important for cancer initiation and progression.

Several reports showed mtDNA content in cancer and normal tissues using real-time PCR [3, 16,17]. However, the method seems to have several problems to extract mtDNA, since the methods were established to extract and purify nDNA. Some of the results showed that ratio of mtDNA content to nDNA content was below 1.0, which suggests that mtDNA content is lower than nDNA content [3,16]. These results could indicate that mtDNA may have been lost during the purification step. Another possible problem is that some of these studies concentrated on the analysis of bulk tissue samples. However, prostate cancer has infiltrative nature [18], which makes it difficult to isolate pure cancer samples from bulk tissues. Laser microdissection is a strong research tool allowing the rapid isolation of selected cell population or single cells from complex tissues. Combining laser microdissection technique with real-time PCR enables us to assess mtDNA copy number more accurately. Several reports show

that mtDNA copy number can be assessed in tissue specimens using these techniques in endometrial and ovarian cancer [19,20].

Since it has been suggested that there is a large variation in the amount of mtDNA between cells [21], we investigated whether there is a difference in mtDNA content in cancer cells. We analyzed mtDNA copy number at a single cell level and compared mtDNA copy number between cancer and adjacent normal cells in vitro. We also investigated mtDNA copy number at a single cell level, in which single cells were obtained from cancer and adjacent normal tissue from the same tissue specimen using laser microdissection. Our results indicate that there may be an enhancement of distributional variance of mtDNA content associated with cancer initiation and/or progression.

MATERIALS AND METHODS

Cell Culture

LNCaP prostate cancer cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 (American Type Culture Collection) supplemented with 10% FCS (Hyclone, Logan, UT). Epithelial prostate cells were purchased from Cambrex Bio Science Walkersville, Inc. (Chicago, IL). PZ-HPV-7 cells (American Type Culture Collection), which was derived from epithelial cells cultured from normal tissue from the peripheral zone of the prostate and then transformed by the transfection with HPV18 DNA, and CA-HPV-10 cells (American Type Culture Collection), which was derived from cells from a prostatic adenocarcinoma of Gleason Grade 4 + 4 and then transformed by transfection with HPV18 DNA [22] were cultured in keratinocyte-serum free medium supplemented with 5 ng/ml human recombinant EGF and 0.05 mg/ml bovine pituitary extract (Invitrogen, Carlsbad, CA).

Comparison of Recovery Rate of nDNA and mtDNA

We extracted DNA from LNCaP cells using three kinds of methods to compare the recovery rate of nucleic acid. The first one was SDS/proteinase K digestion (direct method) [23]. One microliter of the cells suspension in PBS was added with 19 μ l of digestion buffer containing 0.0015% SDS, 0.2 mg/ml proteinase K, and 4.5 mM EDTA to ensure isolation of DNA. After incubation at 55°C for 3 hr, the samples were boiled at 95°C for 10 min to inactivate proteinase K. The other methods were performed using Qiagen Blood and Cell Culture DNA Midi Kit (Qiagen, Valencia, CA) and ZR Genomic DNA II Kit (Zymo Research, Orange, CA) according to the instructions of the manufacturer.

For the determination of mtDNA and nDNA, the forward primer 5'-GTGAAGGCTCATGGCAAGAA AG-3' and the reverse primer 5'-TGTCACAGTGCAGCTCACTCAGT-3' (complementary to the sequences of the human β -globin gene) were used to amplify a 106-bp product. For the analysis of the mtDNA, the forward primer 5'-TGACCCCTTGCCATAATATGATTT-3' and the reverse primer 5'-TTCGATGTTGAAGCCTGAGACTAG-3' which are complementary to the sequence of the *ND1* gene, were used to amplify a 108-bp PCR product. Primers were obtained from Sigma. In selected primer regions, no mutations are described for prostate cancer [24]. Real-time quantitative PCR were performed using the ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA). The template DNA was added to a final 25 μ l reaction mixture containing 1 $\times$ Premix Ex Taq (TaKaRa), 200 nM each primers, SYBR green I dye. The following quantification cycling protocol was used: 95°C for 10 sec, followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec. Relative recovery rate of nDNA is calculated as follows:

Relative recovery rate of nDNA = Cell number
corresponding to the amount of DNA extracted by
direct method/Input LNCaP cells per reaction $\times$ 100

Relative recovery rate of mtDNA content is calculated considered with relative nDNA recovery rate as follows:

Relative recovery rate of mtDNA = Relative amount
of mtDNA to control $\times$ recovery rate of nDNA

Evaluation of DNA Extraction Recovery by Direct Extraction Method

Prostatic epithelial cell was suspended at a concentration of 1,000 cells/ μ l in PBS. One microliter of cell suspension was lysed by 19 μ l of lysis buffer and the DNA was extracted according to the method mentioned above. The samples were collected in six replicates. For the determination of nDNA, the forward primer 5'-GGTGGTCTCCTCTGACTTCAA-3' and the reverse primer 5'-AGCTTGACAAAGTGGTCGTTGAG-3' (complementary to the sequences of the human glyceraldehyde-3-phosphate dehydrogenase gene) were used to amplify a 90-bp product. One microliter of the sample, corresponding to 50 cells, was applied to real-time quantitative PCR as mentioned above. The 10 folds-serially diluted genomic DNA, ranging from 40 pg to 40 ng, was used for plotting standard curve.

Isolation of LNCaP Cell Clones

LNCaP cells were plated at low density and five individual colonies (LNCaP clone 11–15) were isolated for analysis of mtDNA. LNCaP cloned cells were cultured in RPMI 1640 supplemented with 10% FCS.

DNA Isolation From Cultured Individual Cells

Individual cells were picked from cell suspension in PBS under inverted microscope and covered with 19 μ l of digestion buffer containing 0.0015% SDS, 0.2 mg/ml proteinase K, and 4.5 mM EDTA to ensure isolation of DNA. After incubation at 55°C for 3 hr, the samples were boiled at 95°C for 10 min to inactivate proteinase K.

Determination of mtDNA Copy Number Using Real-Time Quantitative PCR

For the amplification of a 108-bp DNA fragment from *ND1* region of mtDNA, the forward primer 5'-TGACCCTTGCCATAATATGATTT-3' and the reverse primer 5'-TTCGATGTTGAAGCCTGAGACTAG-3' and dual-labeled probe (6FAM-5'-AGACCAACCGAACCCCTTCGACC-3'-TAMRA) were used. Primers and dual-labeled probes were obtained from Sigma. Real-time quantitative PCR were performed using the ABI PRISM 7700 Sequence Detector. Lysate (2.5 μ l) of LMD samples were added to a final 25 μ l reaction mixture containing 1 $\times$ Premix Ex Taq (TaKaRa), 200 nM each primers, 250 nM dual-labeled probe. The following quantification cycling protocol was used: 95°C for 10 sec, followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec. Recombinant plasmid containing mtDNA fragment in *ND1* gene coding region was used as standard DNA. The real-time PCR reactions were carried out in duplicate.

Prostate Tissue Samples

Samples and matched clinical information were obtained from the Division of Urology, McClellan's Memorial Veteran's Hospital, Little Rock. Ten prostate cancer patients (age range 49–73 years, average 61.7 years) participated in the IRB-approved study (Table III). Nine cancerous and their adjacent normal prostate tissues were collected from radical prostatectomy specimens. The cancers ranged from Gleason grade 3 + 3, 3 + 4, or 4 + 3. According the clinical

information available, none of these individuals had any medical condition or had any treatment known to affect the prostate.

Laser Microdissection and DNA Isolation

Cancer and adjacent normal epithelial cells were collected by laser microdissection. Fresh prostatectomy specimens were frozen at -80°C and sectioned by cryostat to yield 8- μm sections. Each section was fixed in methanol and stained in Mayer's hematoxylin. Before microdissection, cut sections were dehydrated in ethanol. Malignant and normal epithelial regions were selected by an expert pathologist and individual cells were collected by laser microdissection using Leica AS LMD system (Leica Microsystems, Wetzlar, Germany). Figure 3 shows the picture of the microdissected tissue. The dissected cells were then transferred by gravity alone into a microcentrifuge tube cap placed directly underneath the section. The tube cap was filled with 20 μl of digestion buffer containing 0.0015% SDS, 0.2 mg/ml proteinase K, and 4.5 mM EDTA to ensure isolation of DNA. After incubation at 55°C for 3 hr, the samples were boiled at 95°C for 10 min to inactivate proteinase K.

Data Analysis

Data analysis was descriptive in nature; no formal statistical hypothesis-testing was conducted. Single-cell mtDNA copy numbers were displayed visually as dot plots, using a base-10 logarithmic scale for the vertical axis to accommodate the pronounced right-skewing of copy numbers from each clone or sample. Copy-number distributions were also summarized as the average, the CV (coefficient of variation), the median, the range (i.e., the minimum and maximum), and the fold difference in range (i.e., the ratio of maximum to minimum copy number). The average is reported along with the median, despite the right-skewing of the copy-number distribution, because it is an unbiased estimate of the value one would have gotten from determining average copy number on a bulk sample instead of single cells. The CV is the ratio of the standard deviation (SD) to the average, and is generally recognized as being superior to the SD for summarizing variability of data whose right-skewing requires a logarithmic scale for adequate visual display.

RESULTS

Comparison of the Recovery of nDNA and mtDNA by Several Extraction Methods

Most of the extraction methods to investigate the content of DNA were established to evaluate the content of nDNA but not mtDNA. Several reports showed mtDNA content in cancer and normal tissues using real-time PCR [3,16,17], and some of their results showed that the copy-number ratio of mtDNA to nDNA was below 1.0 [3,16], indicating that mtDNA content is lower than nDNA content. We believe that the content of mtDNA in a single cell should be higher, and therefore, these results strongly imply that mtDNA may have been lost in purification procedure. Therefore, we tried to find the best method to extract mtDNA. We extracted DNA from LNCaP cells using three different methods to compare the recovery rate of nucleic acid. To compare the recovery rate of nDNA, the threshold cycle number (Ct) values of the nDNA were determined using serial dilutions of DNA containing 10, 100, 1,000 LNCaP cells using direct extraction method (direct method). As shown in Table I, the average Ct for each of these dilutions was 32.43, 28.32, and 25.34, respectively. The linear correlation between the Ct values and the logarithm of the number of LNCaP cells was 0.998. Using Qiagen (Q method) and Zymo kit (Z method), DNA was extracted from five million and one million LNCaP cells, respectively. The amount of extracted DNA by Q method and Z method, which was calculated by measuring OD260, was 61 and 3.5 μg , respectively. The average Ct value from 2 ng of DNA extracted from 164 LNCaP cells using Q method was calculated as being equal to the Ct of DNA from 132 LNCaP cells extracted using direct method. The relative recovery rate of nDNA using Q method was 81% when compared with the direct method. The

average Ct value of 2 ng of DNA extracted from 571 LNCaP cells using Z method corresponded to that of DNA from 209 LNCaP cells extracted by the direct method. The recovery rate of nDNA using Z method was 37% when compared with the direct method (Table II).

Next, to evaluate the recovery rate of mtDNA, the comparative Ct method for relative quantification was used. The efficiency of mtDNA and nDNA were approximately equal. The $-\Delta\Delta\text{Ct}$ (mtDNA to nDNA) are shown in Table II. Taken together, the recovery rate of mtDNA of DNA extracted by Q method and Z method were 31% and 2%, respectively (Table II).

Evaluation of DNA Extraction Recovery by Direct Extraction Method

The genomic DNA was extracted from 1,000 of prostatic epithelial cells by the direct method. The DNA contents in 50 cells (1 μl in 20 μl of lysates) were measured by real-time quantitative PCR. The average of the Ct in six replicate was 30.4 ± 0.2 (CV; 0.7%), which corresponds to 0.8 ± 0.2 ng of genomic DNA, indicating that the recovery of our direct extraction method have small distributional variance.

Significant Distributional Variance of mtDNA Content in Individual LNCaP Cells

Since it has been suggested that there are large variations in the amount of mtDNA between cells [21], it is necessary to measure mtDNA content in single cells to ascertain mtDNA alterations. To determine the amount of mtDNA from individual LNCaP cells, DNA was extracted using the direct method. Real-time PCR was then used to evaluate mtDNA content in twelve individual LNCaP cells. The copy numbers thus obtained (Fig. 1A) show considerable right-skewing, with an average (CV) of 10,530 (63.1%) versus a median (quartiles) of 8,683 (7,476–9,592). The highest and lowest copy numbers of mtDNA per cell respectively were 25,880 and 3,336, demonstrating a 7.8-fold difference in the range of mtDNA content among individual LNCaP cells.

MtDNA Content in Single Cloned LNCaP Cells

To investigate whether the difference in mtDNA content was originated from clonal differences and/or whether single cells in each clones had difference in mtDNA content, we performed subcloning of LNCaP and examined the difference in mtDNA content of single cells. Real-time PCR was used to evaluate mtDNA content in individual LNCaP clones, with results shown in Figure 1B. Cells of LNCaP clone 11 had the highest average (47,086) and median (36,566) mtDNA content, whereas cells of LNCaP clone 14 had the lowest average (7,195) and median (7,400) mtDNA content. There was thus a 6.5-fold range in average mtDNA content, and likewise a 4.9-fold range in median copy number per cell, among the five LNCaP clones examined. Within LNCaP clones, the range (fold difference) in copy numbers per cell were as high as 16,297–98,408 (6.0-fold) for clone 11, and no lower than 3,789–9,576 (2.5-fold) for clone 14. These results indicate that there was considerable variability in mtDNA content both within clones and between clones of LNCaP.

MtDNA Content in Individual Immortalized Prostate Epithelial and Cancer Cells

PZ-HPV-7 is HPV-transformed prostate epithelial and CA-HPV-10 is HPV-transformed prostate cancer cells from the same patient, and their difference was investigated to compare the characteristics of normal cells and cancer cells [22]. To investigate whether cancer cells show higher variability of mtDNA content than normal cells, we compared mtDNA content in PZ-HPV-7 and CA-HPV-10. We examined mtDNA content in 16 normal prostate epithelial PZ-HPV-7 and 17 prostate cancer CA-HPV-10 by real-time PCR. As shown in Figure 2, the mtDNA content was distributed more widely in cancer cells compared with normal cells. The range (fold difference; CV) in copy-number values was 1,554–32,285 (20.8-fold; 80%) for CA-HPV-10 and 12,521–39,522 (3.2-fold; 37%) for PZ-HPV-7. However, cancer cells showed

generally lower mtDNA content, with an average (median) of 10,847 (8,789) in CA-HPV-10 and 23,216 (21,375) in PZ-HPV-7.

Variation of mtDNA Content in Cells From Prostate Cancer Specimen

Next, we investigated whether what we observed in the previous section is also true in cancer cells from cancer specimens. Ten individual cancer cells (Fig. 3C,D) and their adjacent normal cells (Fig. 3C,D) were dissected from each specimen using laser microdissection and mtDNA content was analyzed by quantitative real-time PCR (Fig. 4). Table III shows distribution summaries of single-cell mtDNA content of the prostate cancers and their adjacent normal tissues in the same specimen derived from nine different patients. In eight of the nine patients, the CV of mtDNA content was higher in cancer cells than in adjacent normal cells, and in all of the nine patients, the fold difference in copy-number range was higher in cancer cells than in adjacent normal cells. In two cases, the increase in fold difference for cancer versus adjacent normal single cells was by more than an order of magnitude. In cancer cells from Patient C, the highest and lowest copy number of mtDNA per cell was 14,927 and 104, respectively, representing a 143.0-fold difference in range, whereas in normal cells from Patient C, the highest and lowest copy number of mtDNA per cell was 229 and 29, respectively, representing only a 7.0-fold difference in range. Likewise, in cancer cells from Patient H, the highest and lowest copy number of mtDNA per cell was 8,727 and 71, respectively (a 123.3-fold difference in range), whereas in adjacent normal cells from this patient, the highest and lowest copy number of mtDNA per cell was 337 and 92, respectively, representing only a 3.7-fold difference in range. Figure 4 shows that, in all samples except Patient I, the increased fold difference in copy-number range for cancer versus normal was appreciable; indicating along with the CV results that mtDNA content had a higher distributional variance in cancer cells compared with adjacent normal cells. Moreover, the magnitude of differences seen in these results show that mtDNA content often varied greatly in cancer cells as compared with their adjunct normal cells. We also found that average mtDNA content (Table III) was increased in seven of nine (78%) of prostate cancer cells compared with their adjacent normal cells taken from the same specimen. Overall, average mtDNA content in prostate cancer tissues is increased 4.0-fold compared with their normal adjacent prostate tissue. This may be caused by the enhancement of the distributional variance of single-cell mtDNA copy number in cancer cells compared to normal cells.

DISCUSSION

Our study provides the first evidence that mtDNA content in cells follows a highly skewed distribution when collected at the single-cell level. We also showed that mtDNA content showed enhanced distributional variance in prostate cancers compared to their normal prostate cells in vivo and in vitro.

To evaluate mtDNA content, we improved on several methods. First, we extracted and measured DNA content from tissue specimen or cultured cells without extensive purification. One of the reasons we deleted any purification step is that the effect of the impurities from a single cell on PCR did not affect PCR efficiency [23]. This method enabled us to eliminate any further purification steps which could lose mtDNA content. Our results showed that mtDNA recovery rates in the Qiagen and Zymo methods, both of which involve purification steps, were 31% and 2%, respectively, compared with the direct method. Wang et al. [19,20] extracted DNA from laser microdissected tissue samples by the same method as ours, and their results showed that average mtDNA content per cell is 768 in normal endometrium, 2,012 in endometrial cancer, 929 in normal ovary, and 3,548 in ovarian cancer. These results suggest that laser microdissection with elimination of purification step is more appropriate to extract to measure mtDNA content. Gomez-Zaera et al. [16] showed that no statistically significant

differences were observed when patients' cancer samples were compared with their adjacent normal tissues. However, their samples were obtained from whole specimens that included cancer samples, stroma, microvasculature, and inflammatory cells, any of which might have been included in the samples because prostate cancer tissue tends to be infiltrative. Instead, in our study, DNA was extracted from a single cell dissected using laser microdissection, and we excluded contaminating normal cells from our single-cell "cancer" preparations, and vice versa. This may explain the inability of others [16] to detect any differences between normal and cancer cells.

We also report here the diversity of mtDNA copy number in single cancer cells. We examined mtDNA copy number in cloned LNCaP cells and demonstrated that the high variability in mtDNA copy number occurs both within clones and between clones. To investigate whether this high variability represents an enhancement of the variance of the mtDNA copy-number distribution that is associated with cancer initiation and/or progression, we used CA-HPV-10 and PZ-HPV-7, HPV-transformed prostate cancer and normal prostate cells, respectively. We showed that mtDNA content was distributed more widely in CA-HPV-10 than in PZ-HPV-7. These results suggest that variance enhancement of mtDNA content occurs with cancer development. Then, we examined tissue samples. We compared cancer cells and adjacent normal cells from nine patients with Gleason Grade 3 + 3, 3 + 4, or 4 + 3. Unfortunately, we could not obtain cancer specimens below Gleason Grade 3 + 3 because they are rare. We also could not perform the comparison between cancer cells and adjacent normal cells from the specimens whose Gleason Grade were more than 4 + 4, since all of the specimens with Gleason Grade more than eight had no adjacent normal cells. We demonstrated the enhanced distributional variance at the single cell level and the increase in average mtDNA content in cancer cells in tissue specimens. It is very likely that increased mtDNA content is caused, at least in part, by the enhancement of variability in mtDNA copy number at the single-cell level. It has been reported that mtDNA content is increased in head and neck [3], lung [7], thyroid [3], pancreas [8], endometrial [20], and ovarian cancers [19] compared with their normal cells. In contrast, breast [3], renal [4], and liver cancers [5,6] reportedly have reduced mtDNA content.

The mechanism involved in enhanced variability of mtDNA content in cancer cells remains unknown. The most likely explanation is that the regulation of mtDNA content is dysregulated in cancer cells. Several nuclear-encoded proteins can regulate mtDNA content, and polymerase γ regulates mtDNA replication [25]. A recent report showed that p53 associates with and regulates polymerase γ [26]. P53 mutations are very common in cancer cells, so it is likely that p53 mutations can disrupt regulation of mtDNA content and may induce variance enhancement of mtDNA content. It is clear that mutations of p53 are rare in primary prostate cancer [27] but it is possible that mutation of the genes that regulate mtDNA content will affect mtDNA content in prostate cancer. It has also been indicated that cancer cells have mutation in D-loop region in mtDNA [28], which is the site for mtDNA replication and transcription, and this could lead to disorders of mtDNA replication [20]. We also showed that stresses such as androgen ablation reduced mtDNA content [15]. Therefore, combination of nDNA mutation, mutation of mtDNA and stresses may be affecting the dysregulation of mtDNA content.

We have previously showed that mtDNA reduction make the cells resistant to TNF- and serum starvation-induced apoptosis [14]. Amuthan et al. [29] demonstrated that mtDNA-depleted murine skeletal myoblasts C2C12 cells showed invasive phenotypes and over-expression of the tumor-specific markers cathepsin L and transforming growth factor- β , indicating that the loss of mtDNA could contribute to tumor progression and metastasis. We also showed that mtDNA reduction induces androgen-independent phenotype from androgen-dependent prostate cancer [15]. These results imply that the advantage of reduced mtDNA for survival from apoptotic signaling in cancer cells. In contrast, we also showed the advantage of increased

mtDNA content against docetaxel-induced cell death in human head and neck cancer cells [12]. What follows is the possible explanation why dysregulation of mtDNA might show advantage for cancer cells survival: Cancer cells which lack the regulation of mtDNA content have enhanced distributional variance of mtDNA content. Some of cells with reduced mtDNA or increased mtDNA can easily survive under certain conditions which might generally induce cell death. This event may be closely associated with cancer initiation and/or progression. Further analysis is underway to prove this hypothesis.

In conclusion, our data indicate that, in cancer cells as compared with their adjacent normal cells, the distribution of mtDNA content had higher variance. There was an increase of mtDNA copy number during prostate cancer development. These changes of mtDNA may have significant implications in prostate cancer development and may be a critically important factor for cancer progression.

Acknowledgements

We would like to express our gratitude to Dr. Thomas J. Kelly for his advice during preparation of the manuscript. We also thank Ms. Teresa T. Evans for her technical help.

Grant sponsor: Taiho Pharmaceutical Co. Ltd; Grant sponsor: Tobacco Settlement at State of Arkansas; Grant sponsor: National Cancer Institute; Grant number: CA100846.

References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ. Cancer statistics, 2006. *CA Cancer J Clin* 2006;56(2):106–130. [PubMed: 16514137]
2. Chen JZ, Kadlubar FF. Mitochondrial mutagenesis and oxidative stress in human prostate cancer I^{Q2}. *Environ Sci Health C Environ Carcinog Ecotoxicol Rev* 2004;22(1):1–12.
3. Mambo E, Chatterjee A, Xing M, Tallini G, Haugen BR, Yeung S-CJ, Sukumar S, Sidransky D. Tumor-specific changes in mtDNA content in human cancer. *Int J Cancer* 2005;116(6):920–924. [PubMed: 15856456]
4. Heddi A, Faure-Vigny H, Wallace DC, Stepien G. Coordinate expression of nuclear and mitochondrial genes involved in energy production in carcinoma and oncocytoma. *Biochim Biophys Acta* 1996;1316(3):203–209. [PubMed: 8781539]
5. Pedersen PL. Tumor mitochondria and the bioenergetics of cancer cells. *Prog Exp Tumor Res* 1978;22:190–274. [PubMed: 149996]
6. Luciakova K, Kuzela S. Increased steady-state levels of several mitochondrial and nuclear gene transcripts in rat hepatoma with a low content of mitochondria. *Eur J Biochem* 1992;205(3):1187–1193. [PubMed: 1374334]
7. Fliss MS, Usadel H, Caballero OL, Wu L, Buta MR, Eleff SM, Jen J, Sidransky D. Facile detection of mitochondrial DNA mutations in tumors and bodily fluids. *Science* 2000;287(5460):2017–2019. [PubMed: 10720328]
8. Jones JB, Song JJ, Hempen PM, Parmigiani G, Hruban RH, Kern SE. Detection of mitochondrial DNA mutations in pancreatic cancer offers a “mass”-ive advantage over detection of nuclear DNA mutations. *Cancer Res* 2001;61(4):1299–1304. [PubMed: 11245424]
9. Carew JS, Huang P. Mitochondrial defects in cancer. *Mol Cancer* 2002;1:9. [PubMed: 12513701]
10. Qian W, Nishikawa M, Haque AM, Hirose M, Mashimo M, Sato E, Inoue M. Mitochondrial density determines the cellular sensitivity to cisplatin-induced cell death. *Am J Physiol Cell Physiol* 2005;289(6):C1466–C1475. [PubMed: 16107504]
11. Lievre A, Chapusot C, Bouvier A-M, Zinzindohoue F, Piard F, Roignot P, Arnould L, Beaune P, Faivre J, Laurent-Puig P. Clinical value of mitochondrial mutations in colorectal cancer. *J Clin Oncol* 2005;23(15):3517–3525. [PubMed: 15908662]
12. Mizumachi T, Suzuki S, Naito A, Carcel-Trullols J, Evans TT, Spring PM, Oridate N, Furuta Y, Fukuda S, Higuchi M. Increased mitochondrial DNA induces acquired docetaxel resistance in head and neck cancer cells. *Oncogene*. in press

13. Lee H-C, Li S-H, Lin J-C, Wu C-C, Yeh D-C, Wei Y-H. Somatic mutations in the D-loop and decrease in the copy number of mitochondrial DNA in human hepatocellular carcinoma. *Mutat Res* 2004;547(1–2):71–78. [PubMed: 15013701]
14. Higuchi M, Aggarwal BB, Yeh ET. Activation of CPP32-like protease in tumor necrosis factor-induced apoptosis is dependent on mitochondrial function. *J Clin Invest* 1997;99(7):1751–1758. [PubMed: 9120020]
15. Higuchi M, Kudo T, Suzuki S, Evans TT, Sasaki R, Wada Y, Shirakawa T, Sawyer JR, Gotoh A. Mitochondrial DNA determines androgen dependence in prostate cancer cell lines. *Oncogene* 2006;25(10):1437–1445. [PubMed: 16278679]
16. Gomez-Zaera M, Abril J, Gonzalez L, Aguilo F, Condom E, Nadal M, Nunes V. Identification of somatic and germline mitochondrial DNA sequence variants in prostate cancer patients. *Mutat Res* 2006;595(1–2):42–51. [PubMed: 16472830]
17. Rogounovitch TI, Saenko VA, Shimizu-Yoshida Y, Abrosimov AY, Lushnikov EF, Roumiantsev PO, Ohtsuru A, Namba H, Tsyb AF, Yamashita S. Large deletions in mitochondrial DNA in radiation-associated human thyroid tumors. *Cancer Res* 2002;62(23):7031–7041. [PubMed: 12460924]
18. Rubin MA. Use of laser capture microdissection, cDNA microarrays, and tissue microarrays in advancing our understanding of prostate cancer. *J Pathol* 2001;195(1):80–86. [PubMed: 11568894]
19. Wang Y, Liu VWS, Xue WC, Cheung ANY, Ngan HYS. Association of decreased mitochondrial DNA content with ovarian cancer progression. *Br J Cancer* 2006;95(8):1087–1091. [PubMed: 17047655]
20. Wang Y, Liu VWS, Xue W-C, Tsang PCK, Cheung ANY, Ngan HYS. The increase of mitochondrial DNA content in endometrial adenocarcinoma cells: A quantitative study using laser-captured microdissected tissues. *Gynecol Oncol* 2005;98(1):104–110. [PubMed: 15921730]
21. Ashley N, Harris D, Poulton J. Detection of mitochondrial DNA depletion in living human cells using PicoGreen staining. *Exp Cell Res* 2005;303(2):432–446. [PubMed: 15652355]
22. Weijerman PC, Konig JJ, Wong ST, Niesters HG, Peehl DM. Lipofection-mediated immortalization of human prostatic epithelial cells of normal and malignant origin using human papillomavirus type 18 DNA. *Cancer Res* 1994;54(21):5579–5583. [PubMed: 7923200]
23. Khrapko K, Bodyak N, Thilly WG, van Orsouw NJ, Zhang X, Coller HA, Perls TT, Upton M, Vijg J, Wei JY. Cell-by-cell scanning of whole mitochondrial genomes in aged human heart reveals a significant fraction of myocytes with clonally expanded deletions. *Nucleic Acids Res* 1999;27(11):2434–2441. [PubMed: 10325435]
24. Brandon MC, Lott MT, Nguyen KC, Spolim S, Navathe SB, Baldi P, Wallace DC. MITOMAP: A human mitochondrial genome database—2004 update. *Nucleic Acids Res* 2005;33(Database issue):D611–D613. [PubMed: 15608272]
25. Hubscher U, Kuenzle CC, Spadari S. Functional roles of DNA polymerases beta and gamma. *Proc Natl Acad Sci USA* 1979;76(5):2316–2320. [PubMed: 287074]
26. Achanta G, Sasaki R, Feng L, Carew JS, Lu W, Pelicano H, Keating MJ, Huang P. Novel role of p53 in maintaining mitochondrial genetic stability through interaction with DNA Pol gamma. *EMBO J* 2005;24(19):3482–3492. [PubMed: 16163384]
27. Dong JT. Prevalent mutations in prostate cancer. *J Cell Biochem* 2006;97(3):433–447. [PubMed: 16267836]
28. Carew JS, Huang P. Mitochondrial defects in cancer. *Mol Cancer* 2002;1:1–12.
29. Amuthan G, Biswas G, Zhang SY, Klein-Szanto A, Vijayasarathy C, Avadhani NG. Mitochondria-to-nucleus stress signaling induces phenotypic changes, tumor progression and cell invasion. *EMBO J* 2001;20(8):1910–1920. [PubMed: 11296224]

Fig. 1.

MtDNA content in individual LNCaP and LNCaP clone cells. **A:** Twelve individual LNCaP cells were used to extract DNA using the direct method and followed by real-time PCR for mtDNA as described in Materials and Methods Section. A scatter plot is shown for mtDNA content in individual LNCaP cells. The average of mtDNA content in individual LNCaP cells was $10,530 \pm 6,641$. **B:** Six (clone 14), seven (clones 11, 12), eight (clone 15), and nine (clone 13) individual LNCaP clone cells were measured mtDNA content using real-time PCR. A scatter plot is shown for mtDNA content in individual LNCaP clone cells. The average of mtDNA content in individual LNCaP clone cells was $47,086 \pm 28,588$ (clone 11), $11,452 \pm 6,736$ (clone 12), $25,736 \pm 12,002$ (clone 13), $7,195 \pm 2,245$ (clone 14), and $27,995 \pm 13,005$ (clone 15).

Fig. 2. MtDNA content in individual immortalized prostate epithelial (PZ-HPV-7) and cancer (CA-HPV-10) cells. DNA was quantified from 16 (PZ-HPV-7) or 17 (CA-HPV-10) individual cells using the direct method and followed by real-time PCR for mtDNA as described in Materials and Methods Section. A scatter plot is shown for mtDNA content in individual PZ-HPV-7 and CA-HPV-10 cells. The average of mtDNA content in individual PZ-HPV-7 and CA-HPV-10 cells was $23,216 \pm 8,503$ and $10,847 \pm 8,633$, respectively.

Fig. 3. Laser microdissection. Prostate cancer (**A,B**) and adjacent normal (**C,D**) cells were selected (**A,C** arrow) and dissected (**B,D** arrow). Magnification: 400×.

Fig. 4.

MtDNA content in individual prostate cancer (C) and adjacent normal (N) cells. A scatter plot is shown for mtDNA content in individual cells. The average of mtDNA content in individual normal and cancer cells was 429 ± 353 and $1,288 \pm 1,723$ (Patient A), 291 ± 282 and 455 ± 531 (Patient B), 117 ± 62 and $3,078 \pm 4,721$ (Patient C), 541 ± 598 and 693 ± 997 (Patient D), 396 ± 589 and $1,035 \pm 1,325$ (Patient E), $1,337 \pm 260$ and $829 \pm 1,020$ (Patient F), 153 ± 153 and $881 \pm 1,491$ (Patient G), 141 ± 74 and $1,722 \pm 2,987$ (Patient H), and 424 ± 426 and 260 ± 325 (Patient I), respectively.

TABLE IAverage Ct Value for β -Globin at Serial Diluted Input Amounts

Input Amount of LNCaP cells per reaction	Average Ct nDNA
10	32.43
100	28.32
1,000	25.34

Relative Quantification Using the Comparative Ct Method

TABLE II

Method	Input LNCaP cells per reaction	Average C_T nDNA	Cell number corresponding to the amount of DNA extracted by direct method	Recovery rate of nDNA (%)	Average C_T mtDNA	Average ΔC_T mtDNA-nDNA	- $\Delta\Delta C_T$ - (ΔC_T - ΔC_{Tn} Control)	Relative amount of mtDNA to control	Recovery rate of mtDNA (%)
Direct	1,000	25.34	1,000	100	16.05	-9.29	0	1	100
Q	164 (2 ng)	28.27	132	81 ^a	20.37	-7.9	-1.39	0.38	31 ^c
Z	571 (2 ng)	27.56	209	37 ^b	22.43	-5.13	-4.16	0.06	2 ^d

^a Genomic DNA (61 µg) was extracted from five million LNCaP cells using Qiagen Blood and Cell Culture DNA Midi Kit (Qiagen) (Q method). Average Ct value from 2 ng of DNA extracted from 164 LNCaP cells using Q method is equal to that of DNA from 132 LNCaP cells extracted by direct method. Relative recovery rate of nDNA is calculated as follows; Relative recovery rate of nDNA = Cell number correspond to the amount of DNA extracted by direct method/Input LNCaP cells per reaction × 100.

^b Enomic DNA (3.5 µg) was extracted from one million LNCaP cells using ZR Genomic DNA II Kit (Zymo Research) (Z method). Average Ct value of 2 ng of DNA extracted from 571 LNCaP cells using Z method is equal to that of DNA from 209 LNCaP cells extracted by standard method. Relative recovery rate of nDNA is calculated as follows; Relative recovery rate of nDNA = Cell number corresponding to the amount of DNA extracted by direct method/Input LNCaP cells per reaction × 100.

^c Relative recovery rate of mtDNA is calculated considered with relative nDNA recovery rate as follows; Relative recovery rate of mtDNA = Relative amount of mtDNA to control × recovery rate of nDNA.

^d Relative recovery rate of mtDNA is calculated considered with relative nDNA recovery rate as follows; Relative recovery rate of mtDNA = Relative amount of mtDNA to control × recovery rate of nDNA.

TABLE III
Mitochondrial DNA Copy Number in Prostate Cancer and Adjacent Normal Cells

mtDNA copy number: Distribution summaries by subject and sample type						
Subject ID	Age	Gleason Grade	Average (CV)		Range (fold difference)	
			Normal	Cancer	Normal	Cancer
A	63	3 + 3	429 (82.5%)	1,288 (133.8%)	107–1,358 (12.7)	90–5,200 (58.0)
B	54	3 + 4	291 (97.1%)	455 (116.8%)	59–771 (13.1)	59–1,809 (30.5)
C	73	3 + 3	117 (53.4%)	3,078 (153.4%)	29–229 (7.0)	104–14,927 (143.0)
D	71	3 + 4	541 (110.6%)	693 (143.8%)	128–2,097 (16.4)	123–3,393 (27.6)
E	52	3 + 3	396 (148.6%)	1,035 (128.0%)	90–1,997 (22.1)	72–4,049 (56.0)
F	64	3 + 3	1,337 (19.5%)	829 (123.0%)	1,048–1,926 (1.8)	292–3,525 (12.1)
G	63	3 + 4	153 (99.8%)	881 (169.3%)	79–564 (7.1)	72–4,682 (64.9)
H	49	4 + 3	141 (52.8%)	1,722 (173.4%)	92–337 (3.7)	71–8,727 (123.3)
I	66	3 + 3	424 (100.5%)	260 (125.3%)	66–1,140 (17.2)	61–1,091 (17.9)