Published in final edited form as:

Bone. 2015 September ; 78: 81–86. doi:10.1016/j.bone.2015.04.046.

Bone Metastasis in Prostate Cancer: Recurring Mitochondrial DNA Mutation Reveals Selective Pressure Exerted by the Bone Microenvironment

Rebecca S. Arnold^{1,2}, Stacey A. Fedewa³, Michael Goodman^{3,4}, Adeboye O. Osunkoya^{1,2,4,5}, Haydn T. Kissick¹, Colm Morrissey⁷, Lawrence D. True⁸, and John A. Petros^{1,2,4,5,6}

¹Department of Urology, Emory University School of Medicine, Atlanta GA 30322

²The Atlanta VA Medical Center, Decatur, GA 30033

³Emory University School of Public Health, Department of Epidemiology, Atlanta GA 30322

⁴Emory University Winship Cancer Institute, Atlanta, GA 30322

⁵Department of Pathology, Emory University School of Medicine, Atlanta GA 30322

⁶Department of Hematology and Medical Oncology, Emory University School of Medicine, Atlanta GA 30322

⁷Department of Urology, University of Washington, Seattle WA 98195

⁸Department of Pathology, University of Washington Medical Center, Seattle WA 98195

Abstract

Background—Cancer progression and metastasis occurs such that cells with acquired mutations enhancing growth and survival (or inhibiting cell death) increase in number, a concept that has been recognized as analogous to Darwinian evolution of species since Peter C. Nowell's description in 1976. Selective forces include those intrinsic to the host (including metastatic site) as well as those resulting from anti-cancer therapies. By examining the mutational status of multiple tumor sites within an individual patient some insight may be gained into those genetic variants that enhance site-specific metastasis. By comparing these data across multiple individuals, recurrent patterns may identify alterations that are fundamental to successful site-specific metastasis.

Methods—We sequenced the mitochondrial genome in 10 prostate cancer patients with bone metastases enrolled in a rapid autopsy program. Patients had late stage disease and received androgen ablation and frequently other systemic therapies. For each of 9 patients, 4 separate

© 2015 Published by Elsevier Inc.

Corresponding Author: John Petros, 1365 Clifton Road Building B, Department of Urology, Emory University School of Medicine, Atlanta GA 30322, Phone: 404-778-4847, Fax: 404-778-5016, jpetros@emory.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

tissues were sequenced: the primary prostate cancer, a soft tissue metastasis, a bone metastasis and an uninvolved normal tissue that served as the non-cancerous control. An additional (10th) patient had no primary prostate available for sequencing but had both metastatic sites (and control DNA) sequenced. We then examined the number and location of somatically acquired mitochondrial DNA (mtDNA) mutations in the primary and two metastatic sites in each individual patient. Finally, we compared patients with each other to determine any common patterns of somatic mutation.

Results—Somatic mutations were significantly more numerous in bone compared to either the primary tumor or soft tissue metastases. A missense mutation at nucleotide position (np) 10398 (A10398G; Thr114Ala) in the respiratory complex I gene ND3 was the most common (7 of 10 patients) and was detected only in bone. Other notable somatic mutations that occurred in more than one patient include a tRNA Arg mutation at np 10436 and a tRNA Thr mutation at np 15928. The tRNA Arg mutation was restricted to bone metastases and occurred in three of 10 patients (30%). Somatic mutation at 15928 was not restricted to bone and also occurred in three patients.

Conclusions—Mitochondrial genomic variation was greater in metastatic sites than the primary tumor and bone metastases had statistically significantly greater numbers of somatic mutations than either the primary or the soft tissue metastases. The genome was not mutated randomly. At least one mutational “hot-spot” was identified at the individual base level (nucleotide position 10398 in bone metastases) indicating a pervasive selective pressure for bone metastatic cells that had acquired the 10398 mtDNA mutation. Two additional recurrent mutations (tRNA Arg and tRNA Thr) support the concept of bone site-specific “survival of the fittest” as revealed by variation in the mitochondrial genome and selective pressure exerted by the metastatic site.

Keywords

mitochondrial DNA; prostate cancer; bone metastases; cancer evolution

1.0 Background

1.1 Clinical prostate cancer and predilection for bone metastasis

In 2014, the National Cancer Institute estimates there will be 233,000 new cases of prostate cancer in the US (<http://www.cancer.gov/cancertopics/types/prostate>), the second leading cause of cancer mortality in US men. Bone, lymph nodes, liver and lung are the most frequent sites of metastases. Bone metastasis is a multistep process and in some instances, the time from dissemination to detectable metastasis can exceed 10 years (1). Crosstalk between the prostate tumor cells and the bone microenvironment occurs through multiple signaling pathways that promote a more aggressive phenotype (2). While bone metastasis is the hallmark of advanced and typically incurable prostate cancer, little is known about the molecular mechanisms responsible for the high rate of bone metastasis in this disease.

1.2. Mitochondrial genetics

Mitochondria are found in all cells and are fundamental to ATP production through oxidative phosphorylation. Mitochondria contain independent DNA (mtDNA) that is circular, intron free, present at high copy numbers, and self-sufficient, encoding two ribosomal RNAs, 22 tRNAs and 13 polypeptides that are part of respiratory complexes (RC)

I, III, IV, and V. MtDNA is maternally inherited and contains unique single-nucleotide polymorphisms (SNPs) that define haplogroups (3). Both germline (SNPs) and somatic mutations in mtDNA have been associated with cancers of the breast, colon, ovary, prostate, lung, brain, kidney, thyroid and pancreas, as well as, such non-neoplastic diseases as Alzheimer's and Parkinson's diseases (4). Heteroplasmy in which a single individual harbors different proportions of wild type and mutant mtDNA at the same base have been reported. Approximately 25% of healthy individuals exhibit point heteroplasmy (5).

1.3. MtDNA as a driver of metastasis

MtDNA mutations have been definitively demonstrated to be the sole determinant of metastatic capacity in experimental model systems. Lewis lung carcinoma cell lines were used to demonstrate that metastatic potential can be driven by specific mtDNA mutations (6). Low metastatic potential cell lines (P29) and high metastatic potential cell lines (A11) differ by two alterations in the mitochondrially encoded ND6 gene (a point mutation and an insertion) that are present in the A11 (high metastatic potential) cells but not the P29 (low metastatic potential) sub-line. Whether these mutations were homoplasmic or had low level heteroplasmy was not reported in this study. Together, these alterations cause decreased respiratory complex I (RCI) activity and increased reactive oxygen species (ROS) generation. When the mtDNA of these two cells lines was reciprocally switched, the low metastatic cell line became metastatic and the high metastatic cell line lost this potential. The respiratory complex defect and the increased ROS also continued to track with the mutations and the increased metastatic potential. When the metastatic cells were pre-treated with oxygen radical scavengers, they lost metastatic potential, showing that metastasis of these cells was regulated by ROS-mediated reversible up-regulation of nuclear genes (cell signaling) but not by ROS-mediated acceleration of genetic instability. Thus, the mtDNA mutations caused increased ROS which in turn induced changes in the expression of nuclear genes that in turn either enabled the capacity for metastasis.

1.4. Cancer as an evolutionary process

Nearly 40 years of research have substantially supported Newell's description of cancer as an evolutionary system (7). A neoplasm can be perceived as a large genetically heterogeneous population of cells. This genetic diversity is the result of the intrinsic genetic instability of the cancer cell and the resultant mutational events can be positive (enhancing survival fitness), negative (diminishing survival fitness) or neutral. Environmental selective pressure is then applied by numerous factors including the host immune system, mechanical pressures within a tumor, vascularization, nutrient availability, tumor microenvironment and any chemotherapy or other tumor treatments applied. The combination of genetic diversity and selective pressure causes selection of the "fittest", but this occurs over the timescale of a human lifetime as opposed to the selection of organisms that occurs over millennia (8). The bone microenvironment is unique and exerts its own selective pressure on the metastatic cancer cell that attempts to colonize it (9). In the course of human evolution the mitochondrial genome mutates at a rate that is ~10 times higher than the nuclear genome. Within an individual patient's tumor the mitochondrial genome mutates even more rapidly than is seen in evolution of species therefore analysis of the mtDNA in individual patient's metastasis is uniquely informative about the evolution of cancer metastases.

1.5 Overall approach to this project

MtDNA from 10 individuals who died from metastatic prostate cancer was sequenced and analyzed. Inherited base changes relative to the revised Cambridge Reference Sequence (rCRS) were determined in histologically benign tissue. Somatic mtDNA mutations found at the primary cancer site and two metastatic sites, bone and soft tissue were compared. Additional analyses focused specifically on amino acid altering mutations and tRNA mutations.

2.0 Materials And Methods

2.1 Preparation of Genomic DNA

All samples were de-identified and IRB approvals obtained. Samples were obtained from University of Washington's Rapid Autopsy Program. Individual clinical details for each patient are in supplemental data Table S1. All tissues included in this analysis were first grossly identified as tumor (or normal) then histologic confirmation was performed. Normal tissues did not contain cancer. Cancer tissues contained at least 75% tumor nuclei. Some tissues (as indicated) were subjected to laser capture microdissection to obtain pure populations of tumor cells. All other tissues contained some stromal cells in addition to tumor cells. In all cases, FFPE tissue samples were used. DNA from multiple microtome slices of normal, prostate, bone and soft metastatic tissue (metastases in sites other than bone) was purified using the EZNA FFPE DNA Kit (Omega Bio-Tek, Norcross GA) according to manufacturer's protocol. DNA from LCM tissue (bone and prostate) was purified using Arcturus Pico Pure DNA Extraction Kit (Fisher Scientific, Pittsburgh, PA) according to the manufacturer's protocol.

2.2 PCR

Primer (IDT, Coraville, IA) pairs covered the entire mtDNA (supplemental data Tables S2 and S3). PCR reactions contained between 35–50ng DNA, 40 cycles of amplification were used. In some cases, DNA was pretreated with UDG prior to addition of PCR reagents in order to minimize PCR errors associated with formalin fixation (10).

2.3 mtDNA Sequencing

Sequencing was performed using an ABI 3100 capillary sequencer as described (11) with sequencing primers specific for the amplified region of DNA (Table S2). All nucleotide substitutions were compared to the rCRS (<http://www.mitomap.org/bin/view.pl/MITOMAP/MitoSeqs>) (GenBank #: NC_012920). Whole mitochondrial genome sequence was attempted on all samples except LCM. Due to the quality of some of the samples whole genome sequence was not obtained in some samples. Amplified segments range in size from 132bp to 681bp. For LCM samples, only sequencing of the region around position 10398 was performed.

2.4 Data Analysis

Rules for data inclusion (or exclusion) were developed. Data were reproduced at least once or the base observed was inherited and present in multiple tissues. In a few cases, a mutation

or heteroplasmy was observed in multiple independent runs. In such cases, both bases are reported. While it is impossible to assign whether heteroplasmy existed within one cell or different cells, it is possible that sequencing chromatograms showing heteroplasmy reflect the admixture of different cells with distinct mtDNAs. If any base was not sequenced in normal tissue then the data for that position is not presented in any tissue. For clarity, the rare polymorphisms present in the rCRS (263A, 750A, 1438A, 4769A, 8860A, 15326A) were not present unless specifically discussed.

2.5 Statistical Analysis

The proportions of mtDNA sequenced in this study varied by patient and tissue type. For this reason, the number of sequenced mtDNA bases in each sample was calculated by multiplying the total number of mtDNA bases ($n=16,568$) by the proportion of mtDNA bases sequenced. The corresponding calculations restricted to peptide encoding mtDNA were performed using the same approach, but with 11,341 total bases. The statistical analyses compared frequencies of somatic mtDNA mutations in the prostate cancer biopsies to those in soft tissue metastases and bone lesions. The comparisons were carried out using two different methods while adjusting for the number of mtDNA bases (total or peptide encoding) sequenced in each specimen. In the first method (Method 1) we used Poisson models to estimate prevalence ratios (PR) and 95%CI of the number of somatic mutations per mtDNA sequenced for soft tissue and bone specimens compared to prostate cancer biopsy. Poisson models with repeated measures were used because each patient provided three (prostate, soft tissue, and bone) tissue samples. Additional Poisson models also adjusted for presence of heteroplasmy as a dichotomous variable (any/none). Potential over-dispersion of the Poisson model was accounted for using a scaling parameter. In the second method (Method 2) two-by-two tables were constructed for each patient, first comparing number of mtDNA sequences with and without mtDNA mutations in primary tumors vs. soft tissue metastases, and then separately for bone lesions, again using primary prostate cancer as reference. The Cochran–Mantel–Haenszel (CMH) method was used to calculate summary ORs and 95% CI and chi-square test statistics with corresponding p-values across the 10 patient-specific strata. Cochran-Armitage Test for Trend which tests for increasing mutations from prostate to soft tissue to bone) was also calculated. By conducting within-subject analyses both methods allowed taking into consideration patient characteristics such as age. SAS Version 9.3 was used for all statistical analysis.

3.0 Results

3.1 Somatic Mutations in Primary Tumor and Metastases

Table 1 lists the nucleotide position of each somatic mutation for each patient and each tumor site (prostate primary, soft tissue metastasis and bone metastasis). Expanded analysis of each patient that includes each nucleotide change, frequency of the change in GenBank, resultant amino acid alterations and conservation index can be found in supplemental Tables S4–S13. Because an uninvolved tissue was also sequenced for each patient it was possible to determine those mutations that had arisen in the tumors (somatic). The source of the normal control tissue is also listed in Table 1. Some mutations were present in all three malignant tissues analyzed. For example, patient 148 had a G9820A (Gly to Glu) somatic mutation that

was heteroplasmic in the prostate and soft tissue metastasis and homoplasmic mutant in the bone (Table 1 and Table S8). Other mutations were present in the primary prostate and bone metastasis but did not appear in soft tissue metastasis as in the n.p. 9377 mutation in patient 140 (Table 1 and Table S7). Numerous mutations were found only in the bone but not in the primary or soft tissue metastasis (demonstrated in all patients except patient 069 whose only bone mutation was also present in the other two cancer sites) (Table 1). The average proportions of completed mtDNA sequencing for normal tissue, primary cancer, and soft tissue and bone metastases were 97.3%; 88.7 %; 96.6 %; and 53.0% respectively.

3.2 Significantly more somatic mutations in bone metastasis

As discussed in the methods section, the comparisons were made using two methods (Poisson and CMH) normalizing the number of mutations to the number of bases sequenced. These results are shown in Table 2. Corrected for differences in mtDNA sequencing proportions, the average numbers of mtDNA mutations per 10,000 bases sequenced were 1.18, 2.17 and 12.45 for prostate, soft tissue metastases, and bone, respectively. Based on the Poisson model the prevalence of mtDNA mutations in the metastatic soft tissue was 1.83 times higher than in prostate cancer tissue, missing statistical significance with a 95% confidence interval (CI) of 0.72 to 4.67. When comparing frequency of somatic mtDNA mutations in bone to the primary site, the prevalence ratio (PR) was more pronounced and was statistically significantly different from the null value (PR=10.50 95% CI: 6.55–16.84). The results of the Cochran–Mantel–Haenszel (CMH) method were generally consistent with those obtained from the Poisson models. In the CMH analysis, however, the results for both soft tissue and bone metastases reached statistical significance with corresponding odds ratios (OR) of 2.13 (95% CI: 1.14–3.99) and 6.78 (95% CI: 3.67–12.51). The test for trend, which examines increasing mutations from prostate to somatic to bone tissue was statistically significant (Z-Statistic=−3.4175, p-value =0.0006).

3.3 Somatic mutations that occurred in multiple patients

Most notable was the mutation at position 10398 that was found in 7 out of 10 patients and was detected exclusively in bone. Because low level heteroplasmy can exist all sequencing chromatograms were analyzed specifically for evidence of the 10398 mutation in all tissues. No chromatographic evidence of low level heteroplasmy was observed in any tissue other than bone. It should be noted that the limit of detection for capillary sequencing is ~10% heteroplasmy, thus levels below this threshold would not be detected. Another mutation at n.p. 10463 is in the tRNA for Arg and occurred in 3 separate patients, again only in bone. Additionally, at least two patients have mutations at nucleotide positions 152, 4216, 7028, 9899, 10463, 11251, 11719, 14766, 15607, 15928, 16256, 16294, and 16519. Somatic mutations occurred at five nucleotides more than twice. Three are synonymous mutations in *COIII* and *ND4*, the bone-restricted tRNA Arg mutation mentioned above and the remaining recurrent mutation is in the tRNA for Thr (n.p. 15928) occurring in three patients (one soft tissue metastasis and two bone metastases).

3.4 A single missense mutation in 77% of all bone metastases

One mtDNA nucleotide position was frequently mutated in bone samples: A10398G (Thr114Ala) in the ND3 gene of respiratory complex I (RCI). Of the 59 mtDNA mutations in bone, 7 of them were 10398 (Table 1). Assuming the same prevalence (7/59) of 10398 mutation among all patients, the probability of observing this mutation in exactly 7 out of 10 is 0.000027187 and the probability of observing 7 or more events (i.e., the typical right p-value) is 0.000028601. Table 3 lists the presence or absence of the 10398 mutation in each patient at each of the four tissues sequenced (control, primary tumor, soft tissue metastasis and bone metastasis). For the three of the patients 050, 148 and 165, the 10398 mutation was only detected in LCM samples. For six of the seven patients with the mutation, some heteroplasmy was observed. Sequencing coverage for all samples at this site can be found in Supplemental Table 14. A previous study by our group found three additional patients with a somatic mutation at the same nucleotide (A10398G) (12). Taken together, we observed a bone-metastasis 10398 mutation in 77% (10/13) of patients.

4.0 Discussion

4.1 MtDNA mutations alter malignant phenotype

Germline and somatic mtDNA mutations have been reported in a wide variety of cancers. There appear to be two classes of mtDNA mutations in cancer cells: 1) mutations that inhibit oxidative phosphorylation and stimulate tumorigenesis; 2) mutations that help cancer cells adapt to changing environments (3). Severe mutations may be advantageous in the initial phase; however, as the tumor becomes more vascularized or metastatic (and returns to a higher oxygen tension environment) it may be advantageous for the cells to revert back to a more oxidative mtDNA phenotype (3).

4.2 MTDNA mutations in cancer increase ROS that in turn alters multiple signaling pathways favoring growth and metastasis

Mutations in mtDNA are functionally relevant. MtDNA mutations have been shown to increase tumor potential in human tumor cell lines. A mutation at base 8993 (Leu156Arg in ATPase6) increases ROS and enhances tumorigenicity of PC3 cybrid cells (13). In addition this mutation stimulated the cybrid cell growth in a bone stromal environment (14). A mutation at base 6124 (Met74Thr in COI) increased ROS, nitric oxide production, decreased apoptosis, altered nuclear gene transcription and increased tumorigenicity of 143B osteosarcoma cybrid cells (15). MtDNA mutation in cancer cells can be the sole determinant of metastatic potential (6). In that study, reciprocal exchange of mutant mitochondria between tumor mouse tumor cell lines, one with low metastatic cell potential and one with high metastatic potential resulted in the exchange of the metastatic potential (6). The mutations responsible for the increased metastatic potential occurred in ND6 of RCI.

4.3 The 10398 mutation found in this study previously found to be relevant to prostate and breast cancers

The most common somatic mutation in our study was at position 10398. This mutation occurred only in bone, alters an amino acid and is within RCI. An A10398 results in a threonine at amino acid 114 the ND3 subunit of RCI while G10398 codes for alanine.

Functional relevance of a 10398 mutation is suggested by the unprecedented frequency of occurrence in bone metastasis but cannot be proven by this observation alone. Others, however, have tested the effect of this mutation on cancer cell growth. Cybrid cell lines were constructed from blood samples from an African American breast cancer patient (10398G) and a healthy control (10398A). The cybrid cell line with the A10398G polymorphism demonstrated cell cycle delay, increased RCI activity, increased ROS, decreased mitochondrial membrane potential, increased apoptosis resistance and increased tumorigenicity and metastases in mice (16). There have been multiple reports that variants at position 10398 are associated with cancer. Canter demonstrated that the 10398 polymorphism influences breast cancer susceptibility in African American women (17). Other reports link A10398G to breast cancer risk in Caucasians (18–19). Czarnecka demonstrated A10398G as an inherited predisposition factor for breast cancer in Polish women (18). A study of European Americans observed an increased risk for A10398G and reported an interaction between the variants A12308G and A10398G affecting women's risk of breast cancer (19). Finally, an association between the 10398 SNP and African American men's prostate cancer risk was reported in a letter in *Cancer Research* (20). However, others found that variation at n.p. 10398 was not significantly associated with breast, colorectal or pancreatic cancer (21, 22, 23), a result possibly due to tumor type or sample size.

4.4 Our finding of the 10398 mutation verified in multiple experiments

In some patients presented in this study at least 8 different sequencing reactions were performed to verify that the 10398 mutation was present. In addition, DNA samples from bone were treated with Uracil-DNA Glycosylase (UDG) in order to minimize PCR errors associated with formalin fixation (10) and the 10398 mutation was still present. Further, it is unlikely that mutations to position 10398 were a result of chemotherapy as no 10398 mutation was found in any other tissue besides bone and chemotherapeutic drugs are not tissue specific.

4.5 Heteroplasmy

One of the unique attributes of the mitochondrial genome is heteroplasmy. Because the genome is present in many copies in each cell (typically hundreds) it is possible to have any percentage of the genomes mutant and any percentage wild type. We observed a large number of heteroplasmic bases in the inherited genome of several patients (e.g. patients # 006, 069, 165), a finding that has been previously observed in other studies (5). While this probably represents true inherited heteroplasmy at these bases it is also possible that the "normal" tissues had some acquired base changes. For example, the normal (non-cancer) tissue sequenced for patient #069 was lung which is chronically exposed to high oxygen tension, a possible mutagenic influence. Similarly the normal tissue for patient #165 was skin which is chronically exposed to UV irradiation, also known to be mutagenic. Heteroplasmy was also observed at the 10398 in the majority of the bone metastases sampled. The potential significance of this finding may be that while the mutation increases the chances of survival in the bone microenvironment a complete homoplasmic alteration may be too deleterious to provide the survival advantage. An analogous situation exists in inherited mitochondrial disorders where heteroplasmy is common since a homoplasmic mutation would be lethal to the host. In that situation the mutation induces the disease but

the wild type base assures survival of the tissue and the individual. For cancer, the mutant base may provide the growth advantage (for example through increasing ROS production) and the wild type base may provide normal metabolism for sustaining energy production.

4.6 Somatic mutations are increased in metastasis compared to the primary tumor

More somatic mtDNA mutations occurred in metastatic prostate cancer compared to prostate with the majority occurring in bone. Given that the majority of sequence obtained for bone was from whole bone containing the metastasis, these mutations may be found either in the metastatic cancer cells, the bone stromal cells or both. Several mutations were confirmed to be from the cancer when LCM material was tested. It is likely that the number of somatic mutations in bone is underreported because of partial mtDNA sequencing in the bone tissue due to limited material available for sequencing. However, our statistical models corrected for variations in the number of mtDNA sequenced, which help mitigate this underreporting. In several patients, rare somatic mutations occurring at the primary were also found in a metastasis. One possible explanation for these results is that these are metastasis-enhancing mutations acquired in the primary tumor. Tumor heterogeneity demonstrates that not all cancer cells within a given tumor are identical (24). In addition, this may also explain some of the observed differences in somatic mtDNA mutations between an individual's different primary and metastatic tumor sites. Tumor heterogeneity may also explain heteroplasmies although this also may be a result of progression of cellular mitochondria from wild type to mutant base.

4.7 The finding of the exact same missense mutation in 77% of different patients' bone metastases far exceeds any somatic mutation previously reported in prostate cancer suggesting functional importance

In an effort to put the extremely high frequency of the 10398 mutation bone-specific mtDNA mutation into proper perspective we performed two analyses. First, we compared how frequently the exact same DNA base change/amino acid alteration occurred in primary prostate cancer throughout the genome by analyzing the data presented in a recent report of whole exome sequencing of 112 prostate tumors (25). There was only one gene (*SPOP*) that was commonly mutated (12% of patients had mutations within a 20bp region of the gene) and in only 3 of 112 tumors completely sequenced was the mutation at exactly the same base. Other than *SPOP* there were 16 other mutations that occurred in multiple patients, but none of these mutations occurred in more than 2 patients. Thus, the fact that 10398 was found in 10/13 (77%) bone metastasis argues against this being a purely random event and suggests that the mutation uniquely allows or enables prostate cancer bone metastasis. The second method used to put the frequency of this mutation in perspective is an analysis of how frequently other individual bases in the mitochondrial genome are mutated in our patient cohort. When normalized to the length of the gene (i.e. the number of bases available for mutation) bone metastases demonstrate an excess frequency of mutation in RCI and tRNAs. This also argues against the random generation of mutations since not all areas of the mitochondrial genome are equally mutated. While not able to prove either functionality of the mutation or a cause and effect relationship, these data are consistent with the hypothesis that mtDNA mutations allow or enable prostate cancer to successfully metastasize to the bone.

4.8 Implications of bone metastases having increased total number of mtDNA mutations and recurrent single common mutation

As the data clearly shows there is a statistically significant increase in the number of all somatic mtDNA mutations and the number of somatic missense mtDNA mutations in the bone compared to either the primary or the soft tissue metastasis. Most of these are only found in a single patient's bone metastasis (e.g. the n.p. 10750 mutation in patient 214, the n.p. 4216 mutation in patient 380, etc). It thus appears that the bone metastatic microenvironment permits a great diversity of mutations to survive. While it is possible that some or all of these singleton mutations represent a positive adaptive response to the selective pressure of the bone it is more likely that they are neutral. It does seem likely that they are not significantly deleterious to survival in bone or they would probably have been eliminated. By the same reasoning, the 10398 mutation because it occurred in so many different patients' bone metastases, is likely to confer a growth or survival advantage. It is also possible that the tRNA mutations (n.p. 10463 and 15928) that occurred in three separate patients' metastases are specifically selected for but their lower frequency makes this less certain. Thus, it appears that the bone microenvironment both tolerates neutral genetic diversity and selects for particularly advantageous base changes.

4.9 Next steps

Given the prevalence of the 10398 mutation in prostate cancer bone metastases the next steps in understanding the relevance of this alteration would include both clinical and laboratory analyses. In clinical samples one could use deep-sequencing techniques to determine whether low level heteroplasmy of the mutation occurs in primary cancers. In addition, plasma or serum derived circulating tumor DNA could be analyzed to determine if the mutation could be detected in circulating pre-metastatic tumor cells. The endpoint of these investigations could be to either determine those primary tumors destined to be highly metastatic or to develop a prognostic biomarker. In the laboratory it would be important to study the effect of the mutation on metastasis in mouse models. Because of the previous work demonstrating that mtDNA mutations can be the sole determinant of murine metastasis it is possible that these laboratory studies could identify key molecular pathways (such as increased ROS, or activation of specific oncogenic pathways) that could suggest targets for future therapeutic interventions.

4.10 Summary and conclusions

For the first time, evidence is presented that somatic mtDNA mutations occur in cancer cells in the prostate, in soft tissue and in bone metastases. Interestingly, we observed a single base mutation at nucleotide position 10398 which was detected only in bone in 10 out of 13 patients who died of prostate cancer. An inherited mutation at 10398 has been implicated in multiple types of cancers including breast, prostate and lung. The data indicate the need for further studies of this alteration in ND3 and RCI and suggest that this mutation may uniquely enable prostate cancer to metastasize to bone, indicating a pervasive selective pressure for bone metastatic cells that had acquired the 10398 mtDNA mutation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to thank 1) the patients and their families who participated in the University of Washington Prostate Cancer Donor Program, without them research of this nature would not be possible; 2) Mr. Larry Williams (The Breckenridge Group) for his support of this research; 3) Evans County Cares Foundation. This work is supported by NIH Grant nos. CA098912 (Chung/Petros) a VA MERIT award (Petros). The rapid autopsy material is the result of work supported by the National Cancer Institute Pacific Northwest Prostate Cancer Specialized Program of Research Excellence (SPORE; P50 CA 97186-11)[Morrissey, True], a PO1 grant (PO1CA085859) and the Richard M. LUCAS Foundation.

References

1. Morrissey C, Vessella RL. The role of tumor microenvironment in prostate cancer bone metastasis. *J Cell Biochem.* 2007; 101(4):873–86. [PubMed: 17387734]
2. Kingsley LA, Fournier PG, Chirgwin JM, Guise TA. Molecular biology of bone metastasis. *Mol Cancer Ther.* 2007; 6(10):2609–17. [PubMed: 17938257]
3. Brandon M, Baldi P, Wallace DC. Mitochondrial mutations in cancer. *Oncogene.* 2006; 25(34):4647–62. [PubMed: 16892079]
4. Chatterjee A, Mambo E, Sidransky D. Mitochondrial DNA mutations in human cancer. *Oncogene.* 2006; 25(34):4663–74. [PubMed: 16892080]
5. Ramos A, Santos C, Mateiu L, del Mar Gonzalez M, Alvarez L, Azevedo L, et al. Frequency and Pattern of Heteroplasmy in the Complete Human Mitochondrial Genome. *PLoS One.* 2013; 8(10):e74636.10.1371/journal.pone.0074636.eCollection2013 [PubMed: 24098342]
6. Ishikawa K, Takenaga K, Akimoto M, Koshikawa N, Yamaguchi A, Imanishi H, et al. ROS-generating mitochondrial DNA mutations can regulate tumor metastasis. *Science.* 2008; 320(5876):661–4. [PubMed: 18388260]
7. Nowell PC. The clonal evolution of tumor cell populations. *Science.* 1976; 194(4260):23–28. [PubMed: 959840]
8. Merlo LMF, Pepper JW, Reid BJ, Maley CC. Cancer as an evolutionary and ecological process. *Nature Reviews Cancer.* 2006; 6(12):924–935.
9. Gupta GP, Massague J. Cancer metastasis: building a framework. *Cell.* 2006; 127(4):679–695. [PubMed: 17110329]
10. Do H, Dobrovic A. Dramatic reduction of sequence artefacts from DNA isolated from formalin-fixed cancer biopsies by treatment with uracil-DNA glycosylase. *Oncotarget.* 2012; 3(5):546–58. [PubMed: 22643842]
11. Scott TA, Arnold R, Petros JA. Mitochondrial Cytochrome c Oxidase subunit 1 Sequence Variation in Prostate Cancer. *Scientifica.* 2012; 2012:701810. [PubMed: 24124627]
12. Arnold R, Osunkoya A, Chung L, True L, Petros J. Mitochondrial DNA Mutations increase with metastasis in prostate cancer. *J Urol.* 2010; 183 Supplement(4):e87–e88.
13. Petros JA, Baumann AK, Ruiz-Pesini E, Amin MB, Sun CQ, Hall J, et al. mtDNA mutations increase tumorigenicity in prostate cancer. *Proc Natl Acad Sci U S A.* 2005; 102(3):719–24. [PubMed: 15647368]
14. Arnold RS, Sun CQ, Richards JC, Grigoriev G, Coleman IM, Nelson PS, et al. Mitochondrial DNA mutations stimulates prostate cancer growth in bone stromal environment. *Prostate.* 2009; 69(1):1–11. [PubMed: 18850577]
15. Arnold RS, Sun Q, Sun CQ, Richards JC, O'Hearn S, Osunkoya AO, et al. An inherited heteroplasmic mutation in mitochondrial gene COI in a patient with prostate cancer alters reactive oxygen, reactive nitrogen, and proliferation. *Biomed Res Int.* 2013; 2013:239257.10.1155/2013/239257 [PubMed: 23509693]

16. Kulawiec M, Owens KM, Singh KK. mtDNA G10398A variant in African-American women with breast cancer provides resistance to apoptosis and promotes metastasis in mice. *J Hum Genet.* 2009; 54(11):647–54. [PubMed: 19763141]
17. Canter JA, Kallianpur AR, Parl FF, Milikan RC. Mitochondrial DNA G10398A polymorphism and invasive breast cancer in African-American women. *Cancer Res.* 2005; 65(17):8028–33. [PubMed: 16140977]
18. Czarnecka AM, Krawczyk T, Zdrosny M, Lubinski J, Arnold RS, Kukwa W, et al. Mitochondrial NADH-dehydrogenase subunit 3 (ND3) polymorphism (A10398G) and sporadic breast cancer in Poland. *Breast Cancer Res Treat.* 2010; 121(2):511–8. [PubMed: 19266278]
19. Covarrubias D, Bai RK, Wong LJ, Leal SM. Mitochondrial DNA variant interactions modify breast cancer risk. *J Hum Genet.* 2008; 53(10):924–8. [PubMed: 18709563]
20. Mims MP, Hayes TG, Zheng S, Leal SM, Frolov A, Ittmann MM, et al. Mitochondrial DNA G10398A Polymorphism and Invasive Breast Cancer in African-American Women. *Cancer Res.* 2006; 66:1880. [PubMed: 16452251]
21. Setiawan VW, Chu LH, John EM, Ding YC, Ingles SA, Bernstein L, et al. Mitochondrial DNA G10398A variant is not associated with breast cancer in African-American women. *Cancer Genet Cytogenet.* 2008; 181(1):16–9. [PubMed: 18262047]
22. Webb E, Broderick P, Chandler I, Lubbe S, Penegar S, Tomlinson IP, et al. Comprehensive analysis of common mitochondrial DNA variants and colorectal cancer risk. *Br J Cancer.* 2008; 99(12):2088–93. [PubMed: 19050702]
23. Wang L, Bamlet WR, de Andrade M, Boardman LA, Cunningham JM, Thibodeau SN, et al. Mitochondrial genetic polymorphisms and pancreatic cancer risk. *Cancer Epidemiol Biomarkers Prev.* 2007; 16(7):1455–9. [PubMed: 17627010]
24. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med.* 2012; 366(10):883–92. [PubMed: 22397650]
25. Barbieri CE, Baca SC, Lawrence MS, Demichelis F, Blattner M, Theurillat JP, et al. Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer. *Nat Genet.* 2012; 44(6):685–9. [PubMed: 22610119]

Highlights

- Whole mitochondrial genome sequencing of primary cancer and bone metastases
- Single recurring mutation in 77% of patients' bone metastases is unprecedented
- Bone metastasis exemplifies cancer as evolutionary process
- Mitochondrial DNA (mtDNA) mutations can be sole drive of metastasis
- MtDNA mutations confer growth or survival advantage in bone microenvironment

Table 1

Somatic Mutations.

Patient	Normal Tissue	Inherited SNPs	Prostate Somatic Mutations	Soft Tissue Metastatic Site	Soft Tissue Somatic Mutations	Bone Metastatic Site	Bone Somatic Mutations
214	liver	28	10688, 12994	lymph	1464, 10688, 12994	R. Sacrum	10398, 10463, 10750, 11812, 14233
380	kidney	28	3720, 16250	liver	16250	T11	150, 152, 4216, 8697, 9899, 10398, 10400, 10463, 13368, 15452, 15607
050	liver	6	11435	lymph	4769	T8	73, 152, 3441, 9591, 9899, 10398, 10463, 10589, 11251, 11719, 12633, 14766, 15884, 15928, 16294, 16519
140	kidney	5	9377, 11719	liver	none	Rib	2389, 2706, 7028, 9377, 10398, 13203
148	skin	4	9820, 11351, 13723	liver	9820, 11351, 13723	L3	9820, 10211, 10398, 13723
108	skin	6	NA	liver	none	L. Sacrum	5902, 9899, 10463, 11251, 11719, 15904, 15928
149	liver	27	none	lymph	none	T10	10203, 10238, 10398, 12501, 16129
165	skin	11	12308, 12372	lung	12308, 12372	T9	10398, 13830
006	liver	11	none	lymph	11120, 14793, 16172, 16192, 16256, 16320, 16519	R. Sacrum	217, 11197
069	lung	26	195, 7028	bladder	195, 4216, 4561, 7028, 9477, 9899, 11251, 11719, 13617, 14750, 14766, 15607, 15928, 16224, 16294, 16311	L3	7028

Individual patient somatic mtDNA mutations are indicated as is the tissue site from which the mitochondrial DNA was obtained. For mtDNA sequence from noncancerous "normal" tissue, only the number of inherited SNPs detected is listed. For a full list of mtDNA inherited base changes from rCRS see supplemental tables S4–13.

Table 2

Statistical Analysis of frequency of somatic mtDNA mutations in the primary tumor compared to those in soft tissue metastases and bone lesions.

	Any mtDNA			Peptide Encoding mtDNA		
	Prostate	Soft Tissue	Bone	Prostate	Soft Tissue	Bone
Mean No. Corrected Mutations	1.74	3.48	10.94	0.555	1.27	3.11
Mean No. mtDNA sequenced	14,692	16,006	8,784.35	10,928.94	11,038.20	6,947.50
Mean Corrected mutations per 10,000 mtDNA sequenced	1.18	2.17	12.45	0.51	1.15	4.48
Model 1 Mutation PR and 95%CI ¹	1.00	1.83 (0.72–4.67)	10.50 (6.55–16.84)	1.00	2.01 (0.58–6.94)	8.79 (4.54–21.14)
Model 2 Mutation PR and 95%CI ²	1.00	1.56 (0.58–4.22)	10.21 (3.96–26.34)	1.00	1.71 (0.42–6.94)	8.99 (1.39–58.03)
CMH OR and 95%CI ³	1.00	2.13 (1.14–3.99)	6.78 (3.67–12.51)	1.00	2.13 (0.74–6.19)	7.19 (2.45–21.14)

¹Prevalence Ratio (PR) was calculated using Poisson method. Model accounts for repeated measure across patient.

²PR was calculated using Poisson method. Model accounts for repeated measure across patient and presence of heteroplasmy.

³Cochoran Mantel Haenszel odds ratio (CMH OR) was calculated based on two-by-two tables for each patient, first comparing number of mtDNA sequences with and without mtDNA mutations in primary tumors vs. soft tissue metastases, and then separately for bone lesions, again using primary prostate cancer as reference.

Table 3

Presence of 10398 Mutation Detected

Patient	Non-Cancerous	Prostate	Soft Tissue	Bone
214	No	No	No	Yes
380	No	No	No	Yes
050	No	No	No	Yes
140	No	No	No	Yes
148	No	No	No	Yes
108	No	No	No	No
149	No	No	No	Yes
165	No	No	No	Yes
006	No	No	No	No
069	No	No	No	No [*]

* Patient had heteroplasmy at this location in all tissues indicating inherited heteroplasmy.