Mitochondrial Genome Deletion Aids in the Identification of False- and True-Negative Prostate Needle Core Biopsy Specimens

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Abstract

We report the usefulness of a 3.4-kb mitochondrial genome deletion (3.4mtΔ) for molecular definition of benign, malignant, and proximal to malignant (PTM) prostate needle biopsy specimens. The 3.4mtΔ was identified through long-extension polymerase chain reaction (PCR) analysis of frozen prostate cancer samples. A quantitative PCR assay was developed to measure the levels of the 3.4mtΔ in clinical samples. For normalization, amplifications of a nuclear target and total mitochondrial DNA were included. Cycle threshold data from these targets were used to calculate a score for each biopsy sample. In a pilot study of 38 benign, 29 malignant, and 41 PTM biopsy specimens, the difference between benign and malignant core biopsy specimens was well differentiated (P < .0001), with PTM indistinguishable from malignant samples (P = .833). Results of a larger study were identical. In comparison with histopathologic examination for benign and malignant samples, the sensitivity and specificity were 80% and 71%, respectively, and the area under a receiver operating characteristic (ROC) curve was 0.83 for the deletion. In a blinded external validation study, the sensitivity and specificity were 83% and 79%, and the area under the ROC curve was 0.87. The 3.4mtΔ may be useful in defining malignant, benign, and PTM prostate tissues.

The unique maternal inheritance pattern of mitochondrial DNA (mtDNA), its small genome size, lack of recombination during gametogenesis, and multiple copy number per cell, in comparison with nuclear DNA, identify this molecule as important and economical to characterize at a population level. It is well known that mitochondria are important participants in cellular function. Not only does the machinery of this organelle produce up to 90% of required cellular energy, it also has a critical function in mediated cell death through the apoptotic pathway.1-3 Moreover, mitochondria modulate appearance, copy number, and location during the cell cycle.4 These characteristics suggest that mitochondria may undergo detectable modifications associated with malignant progression. In particular, mitochondria have been implicated in the carcinogenic process, partly because of their role in apoptosis and other aspects of tumor biology.5 Damage accrued by the mitochondrial genome (mtgenome) is associated with increased cellular stress and organelle dysfunction.6 Indeed, numerous studies have identified somatic mutations in this modest genome.7 Several groups have demonstrated mtgenome alterations in many cancers.8,9 Studies of mtDNA alterations in prostate cancer (PCa) indicate the presence of mutations in the prostate, probably as a result of increased reactive oxygen species production in the glandular epithelial tissues.10,11

Not only does the modest mtgenome code for 13 proteins, which participate in the electron transport chain as well as 22 transfer RNAs (tRNAs) required for transcription of these genes and 2 ribosomes (12S rRNA and 16S rRNA), but it is also saturated with direct repeats that flank deletions, a common mutation genre of this genome, in addition to point mutations. Many mitochondrial myopathies are characterized by
mtgenome deletions. Specifically, much work has been done on a 4,977-base-pair (bp) or “common deletion.” This portion of the mtgenome has been deleted in many pathologic conditions, including cancer. It is important to note that deletions in the mtgenome have been reported in PCa and might mediate androgen independence, although one study could not identify mtDNA deletions in these tumors.

An irregular result from a digital rectal examination (DRE) and/or elevated prostate-specific antigen (PSA) level in the blood generally triggers referral to the urologist for a prostate biopsy procedure. In most cases, the results are negative for malignancy, which is clinically problematic because some of the patients continue to have abnormal PSA and DRE findings. Indeed, about 10% and 5% will have PCa on second and third repeated biopsy procedures. An obvious concern with the use of histopathologic examination for identifying malignant disease is that this method relies on cell morphologic features rather than molecular indications. Because neoplasia and the potential cellular transformation to malignancy are based on alterations in gene function, changes in cell appearance occur relatively late in this process. In contrast, mtgenome mutations may occur early in the transformation process. Thus, a biomarker that can predict a missed tumor in prostate biopsy specimens will be a useful complement in evaluation of prostate biopsy samples. We describe the discovery and characterization of a 3.4-kb mtgenome deletion (3.4mtΔ) that has high statistical association with PCa. It is important to note that this 3.4mtΔ demonstrates a field carcinization, which may have usefulness as an adjunct in evaluation of prostate biopsy specimens.

Materials and Methods

Patients and Sample Criteria

Ethics

All samples were obtained in accordance with the ethical guidelines of the Thunder Bay Regional Hospital Ethics Board (Thunder Bay, Canada) and the Trafalgar Ethics Board (Oakville, Canada). Both boards operate in accordance with the Tri-Council Policy Statement on Ethical Conduct for Research Involving Humans.

Pilot Study

We used 38 benign biopsy specimens from 22 patients (average age, 66 years), 41 malignant biopsy specimens from 24 patients (average age, 68 years; mostly Gleason 6/7), and 29 proximal to malignant (PTM) biopsy specimens from 22 patients (average age, 71 years). All patients with malignant biopsy specimens had a follow-up prostatectomy to confirm diagnosis of PCa, and the PTM samples were negative biopsy specimens from this cohort. Malignant and PTM core needle biopsy samples were selected after a review of a pathology report associated with a radical prostatectomy. Benign samples were selected based on a single negative biopsy result.

Confirmation Study

For this study, we used 98 benign biopsy specimens from 22 patients (average age, 62 years), 75 malignant biopsy specimens from 65 patients (average age, 67 years), and 123 PTM biopsy specimens from 96 patients (average age, 67 years). Samples were selected based on biopsy pathology reports in an effort to mimic typical clinical samples. To ensure benign tissue status, patients were required to have at least 2 successive negative biopsy procedures, with biopsy specimens from the first procedure used for analyses. Malignant samples were screened by a qualified pathologist (N.E.), and most were Gleason score 6 or 7. PTM samples were defined as histopathologically normal biopsy specimens adjacent to a malignant needle core specimen.

DNA Extraction and Target Amplification

Nucleic acids from frozen (50 mg) and formalin-fixed, paraffin-embedded (FFPE) prostate needle biopsy (20-µm sections) samples were extracted using a QIAamp DNA Mini Kit (Qiagen, Mississauga, Canada). Negative extraction control samples were processed in parallel with biopsy tissues and monitored for amplification. Extracts were quantified by using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Before amplification, samples were diluted to 2 ng/µL and distributed to master template 96-well plates. Templates from the 96-well plates were amplified using iQ Sybr Green Supermix (Bio-Rad Laboratories, Hercules, CA).

Reaction conditions were as follows: 1x iQ Sybr Green Supermix, 0.25 µmol/L of forward primer, 0.25 µmol/L of reverse primer, and 20 ng of template DNA in a 25-µL reaction volume. Cycling parameters were 95°C for 3 minutes, followed by 45 cycles of 95°C for 30 seconds, 30 seconds annealing at optimized primer temperatures (61.5°C for total mtDNA and tumor necrosis factor [TNF], 66°C for deletion-specific primer), and extension at 72°C for 30 seconds. Each extension was followed by a plate reading. A 10-minute final extension at 72°C was performed before the melting curve (50°C to 105°C, reading every 1°C and holding for 3 seconds). Cycling was performed on a DNA Engine Opticon 2 Real-Time PCR (polymerase chain reaction) Detection System (Bio-Rad Laboratories). One negative PCR control sample was included on every amplification plate for each primer set, as were 6 standards for normalization purposes. These standards were serial dilutions of the target amplicon generated with conventional PCR, purified, quantified, and diluted such
that the 6 standards were at the following concentrations: 3.85 ng, 0.385 ng, 38.5 pg, 3.85 pg, 0.385 pg, and 38.5 fg. A minimum of 4 standards was used in the analysis of any given plate.

Long-range PCR was performed in a 50-µL reaction volume containing 50 ng of template, 0.4 mmol/L each of deoxynucleoside triphosphates, 0.5 U/µL of LA Taq polymerase, 0.2 µmol/L of primers and 1× LA buffer. The cycling conditions were 94°C for 1 minute followed by 30 cycles consisting of 94°C for 10 seconds and 68°C for 15 minutes, followed by a final extension at 72°C for 10 minutes.

Statistical Analysis

The cycle threshold (Ct) of the 3.4mtΔ was compared with the Ct of 2 additional targets designed to capture the total amount of mtDNA (12SrRNA gene) and the amount of nuclear DNA (single-copy TNF). Cycle thresholds of all 3 targets were used in a simple formula [(Ct_{del} – Ct_{tot})/Cttot], which provides a score for each sample. This score is hereafter referred to as the residual mtDNA score or RM score. The mean and SD were calculated for each tissue classification, as were mean and 99% confidence intervals (CIs). A 1-way analysis of variance was used to test for statistical significance between groups.

Results

Full-length mtgenome amplification of frozen malignant prostate tissues identified a 3,379-bp deletion (deletion junction 10744:14124) in PCa. Long-range PCR was performed in a 50-µL reaction containing 50 ng of template, 0.4 mmol/L each of deoxynucleoside triphosphates, 0.5 U/µL of LA Taq polymerase, 0.2 µmol/L of primers and 1× LA buffer. The cycling conditions were 94°C for 1 minute followed by 30 cycles consisting of 94°C for 10 seconds and 68°C for 15 minutes, followed by a final extension at 72°C for 10 minutes.

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benign prostatic conditions indeed cluster with normal benign samples and are distinctly separate from malignant samples. This demonstrates that the 3.4 mtΔ is associated with PCa and, thus, might be of value in PCa detection. In addition, preliminary analysis of 8 samples of prostatic intraepithelial neoplasia demonstrated that 5 of 8 were malignant.

Initial analysis of prostate cancer samples for the 3.4-kb mitochondrial genome deletion (3.4 mtΔ). A, Locations of primers used for screening and quantitative polymerase chain reaction (PCR) assay development. B, Initial screening of 33 prostate cancer samples with primer pair 1 and 3 shows the presence of the 3.4 mtΔ in 30 samples. b, blood; -ve, PCR negative control; blk, blank well. C, Analysis of frozen malignant prostate (M) and proximal to malignant (PTM) (P) samples with primer set 2 and 3 reveals the presence of the 3.4 mtΔ in PTM samples as well. D and E, Screening of benign, malignant (M), and PTM (P) formalin-fixed, paraffin-embedded biopsy samples demonstrates the virtual absence of the 3.4 mtΔ (d) in benign samples. Amplification of total (t) and TNF (n) targets were included in these runs. This is probably a failed PCR reaction. F, Restriction analysis of the 272-base-pair PCR product with AluI (Al) and BamHI (Ba) authenticate its mitochondrial origin. G, Amplification of template from ρ0 (Z) and prostate (P) indicates absence of amplification of nuclear mitochondrial DNA targets.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Location</th>
<th>Primer Sequence (5’-3’)</th>
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</thead>
<tbody>
<tr>
<td>Total</td>
<td>Forward 708-728</td>
<td>cgttccagtgaatcaccctc</td>
</tr>
<tr>
<td></td>
<td>Reverse 923-945</td>
<td>cacttttaccggctttatt</td>
</tr>
<tr>
<td>TNF</td>
<td>Forward 906-925</td>
<td>cctgcccaatccctttatt</td>
</tr>
<tr>
<td></td>
<td>Reverse 1016-1036</td>
<td>ggtttcgaagtggtggtcttg</td>
</tr>
<tr>
<td>3.4-kb Deletion</td>
<td>Forward 10729-10743/14125-14139</td>
<td>tagactacgataaacttaacctacactccta</td>
</tr>
<tr>
<td></td>
<td>Reverse 14361-14379</td>
<td>gaggtagattgtgctgt</td>
</tr>
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</table>
| LXPCR, long-extension polymerase chain reaction; TNF, tumor necrosis factor.
PCa, suggesting that this deletion demonstrates a cancer field effect. This observation is of interest because it may be of complementary value in the evaluation of false-negative biopsy results. To evaluate this aspect further, a pilot study was conducted using biopsy specimens from 46 patients. The RM scores were statistically significant between benign and malignant ($P < .0001$) and benign and proximal ($P < .003$) samples. Consistent with the field cancerization phenomenon observed in the frozen samples, the PTM samples closely resembled the malignant samples, with no statistical significant resolution between their RM scores ($P < .833$). The mean and 99% CI for the RM scores for benign, malignant, and proximal samples are shown in Figure 3B.

Statistical parameters from the preceding data were used in a power calculation to determine the number of samples required for a blinded confirmation study to assess the robustness and reproducibility of the association of the 3.4mtΔ with PCa. The accuracy of the RM scores in predicting benign and malignant samples with reference to histopathologic examination (the “gold standard”) was determined using an RM score cutoff of 450 and also computation of a receiver operating characteristic (ROC) curve. The sensitivity and specificity were 80% and 71%, respectively, and the area under the ROC curve was fairly good, at 0.77 for the RM scores and 0.83 for the deletion Ct.

Again, there was a statistically significant difference in the RM scores between benign and malignant samples ($P < .0001$). Unlike the pilot study, the RM scores of the proximal samples from the blinded confirmation study were significantly different from malignant ($P < .001$) and benign ($P < .006$) groups. Figure 4B shows the mean RM scores for benign, malignant, and proximal samples.

**Figure 3A**, The 3.4-kb mitochondrial genome deletion is associated with prostate cancer. The mean residual mitochondrial DNA (RM) scores and 95% confidence intervals of benign prostatic conditions (Atr, atrophy; Ben, benign without other lesions; Hyp, hyperplasia; Inf, inflammation; Mult, multiple prostatic lesions; Nsp, nonspecific benign changes) and prostate cancer (Mal) are plotted. Above each plot is the number of samples analyzed. **B**, The deletion demonstrates a field cancerization effect. Plots of the mean RM scores and 99% confidence interval (CI) of the pilot studies for benign, malignant, and proximal samples are shown. Note how closely the RM scores of the proximal samples resemble the malignant samples. It is also noteworthy that the 99% CI of the benign samples is extremely vast, which is likely because these samples were from single benign biopsy procedures, some of which might already be demonstrating the field cancerization effect.

**Table 2**, Behavior of the 3.4mtΔ in PIN Samples

<table>
<thead>
<tr>
<th>Patient ID No.</th>
<th>Age (y)</th>
<th>Histopathologic Diagnosis</th>
<th>Prediction by 3.4mtΔ</th>
</tr>
</thead>
<tbody>
<tr>
<td>833</td>
<td>68</td>
<td>1 or 2 foci of LPIN; &lt;5%</td>
<td>Malignant</td>
</tr>
<tr>
<td>1152</td>
<td>78</td>
<td>Features of PIN</td>
<td>Malignant</td>
</tr>
<tr>
<td>292</td>
<td>75</td>
<td>Focal LPIN</td>
<td>Malignant</td>
</tr>
<tr>
<td>789</td>
<td>73</td>
<td>Focal PIN</td>
<td>Malignant</td>
</tr>
<tr>
<td>787</td>
<td>75</td>
<td>Tiny focal area of HPIN; &lt;5%</td>
<td>Benign</td>
</tr>
<tr>
<td>1093</td>
<td>79</td>
<td>Focal PIN</td>
<td>Benign</td>
</tr>
<tr>
<td>646</td>
<td>65</td>
<td>PIN</td>
<td>Benign</td>
</tr>
<tr>
<td>1053</td>
<td>60</td>
<td>HPIN</td>
<td>Malignant</td>
</tr>
</tbody>
</table>

HPIN, high-grade PIN; LPIN, low-grade PIN; PIN, prostatic intraepithelial neoplasia; 3.4mtΔ, 3.4-kb mitochondrial genome deletion.
scores and 99% CI for the benign, malignant, and proximal biopsy specimens.

For half of the patients (n = 48) who contributed proximal biopsy specimens for the confirmation study, we also obtained the corresponding malignant biopsy specimen for comparative analysis. In this patient category, the predictive accuracy of the RM scores of the proximal biopsy specimens in correctly calling the presence of a malignant focus (ie, demonstrating field cancerization) was 67% (32/48). When 2 or more proximal biopsy specimens from the same patient were considered, the predictive power was 78% (14/18), suggesting that increasing the number of histologically normal biopsy specimens for analysis will increase the detection rate of the 3.4mtΔ.
A subset of nucleic acids extracted from serial prostate needle biopsy specimens, included in the confirmation study, was submitted to the Biochemical Science Division of the National Institute of Standards and Technology (NIST) for analytic cross-validation under the National Cancer Institute Early Detection Research Network program. The standard operating procedures developed and used at Genesis Genomics (GGI) were followed. All samples had known Cts (from work done at GGI) and were blinded to NIST scientists. These samples consisted of 46 benign, 41 PTM, and 25 malignant cores. NIST results demonstrated a sensitivity of 83% and a specificity of 79%, very close to the results obtained at GGI. Likewise, the area under the ROC curve was 0.87 Figure 5A, in comparison with 0.83 for GGI data. Figure 5B depicts the mean Cts and 95% CI for the NIST study.
**Figure 4** A. A receiver operating characteristic curve for the deletion cycle thresholds and residual mitochondrial DNA (RM) scores computed with reference to histopathology for benign and malignant samples indicates good performance of the deletion alone and the RM scores in discriminating benign from malignant biopsy specimens. Area under the curve: black, 3.4-kb mitochondrial genome deletion (3.4mtΔ) cycle thresholds, 0.83; gray, RM scores, 0.77. B. The 3.4mtΔ is reproduced in a larger study. The RM scores and 99% confidence interval (CI) for benign, malignant, and proximal samples for the confirmation studies are plotted.

**Figure 5** The receiver operating characteristic curve (A; area under the curve, 0.87) and mean cycle thresholds and 95% confidence interval (CI) (B) of the external validation study at the National Institute of Standards and Technology/Early Detection Research Network demonstrate that the 3.4-kb mitochondrial genome deletion is reproducible in identifying prostate cancer.
Discussion

Previously, our group studied the accelerated mutation rate of the mtgenome within the malignant prostate. Briefly, malignant, adjacent, and distant benign samples were used. These histologic tissue types were captured from 24 prostatectomy samples by using laser capture microdissection. Complete mtgenome sequencing was attempted on all samples. In comparison with results from benign samples, representing people with moderately high PSA values and negative biopsy reports, results demonstrated an accelerated but equal mtgenome somatic mutation rate in all tissues recovered from a malignant prostate gland. This finding suggests that underlying molecular alterations are apparent in normal-appearing tissue, before visual morphology, indicative of malignant tissue, before visual morphology, indicative of malignant transformation. We continued this work by screening for large-scale mtgenome deletions in PCa. The mtDNA deletions are known to be associated with many cancers. The objective was to locate a simple deletion biomarker that can discriminate benign from malignant prostate tissue.

The pilot and confirmation studies demonstrated that the 3.4mtΔ is a highly significant biomarker for PCa (P < .0001) in comparison with histopathologic examination. To determine if the malignant transformation process was underway and discernible at the molecular level within “normal” prostate tissue, visually normal glandular tissue in proximity to a malignant lesion (PTM) was characterized for the 3.4mtΔ. Malignant and PTM biopsy samples for the pilot study were procured from cases after consulting the associated prostatectomy pathology report. This facilitated tight control over the selection of PTM samples. Moreover, the size and extent of the malignant lesion were known. Finally, benign samples were chosen on the basis of a single negative biopsy result.

Criteria for the confirmation studies were slightly different because the samples were taken from several urologic clinics. This was intended to model the use of biopsy samples procured directly from clinics; therefore, it was impossible to determine the exact location of negative tissue in respect to tumor because patients were recruited before treatment was finalized. Only the general anatomic location of the biopsy was known. Finally, to ensure the negative status of benign samples, the first of at least 2 successive benign biopsy procedures was used.

Irrespective of these parameter variations between the pilot and confirmation studies, both results supported a prostate field cancerization. This is reflected in the clustering of the PTM samples. If there is a field cancerization, the PTM samples should cluster with the malignant samples or intermediately between the malignant and benign samples. Interestingly, the mean of the well-defined location of the proximal samples selected in the pilot study cannot be statistically resolved from the mean of the malignant grouping. In contrast, the PTM samples of the confirmation study resolved into an intermediate position between the benign and malignant categories. The 99% CI of this grouping does not overlap with that of the malignant samples. Furthermore, the confirmation study demonstrated a tighter cluster of the benign samples, in comparison with the pilot study, which might be due to the larger benign sample size used in the confirmation study, resulting in a smaller CI. In addition, the strict criteria used to define benign samples for the confirmation study should exclude biopsy specimens that may be found malignant on a second procedure. Furthermore, in both studies, the 99% CIs of the PTM overlapped slightly with the benign interval, suggesting that some of these samples are actually benign and uninfluenced by the cancerization field effect. This might be a function of the size of this field or may indicate the directional spread of a tumor. These aspects, however, require further study. It is important to note that the marker seems unaffected by common benign prostate conditions.

Field cancerization was first described in 1953 by Slaughter and colleagues (reviewed by Dakubo et al18). More recently, molecular field cancerization has been described for several cancers, including gastric, breast, head and neck, colorectal, acute promyelocytic and chronic myeloid leukemia, larynx, pancreas, bladder, and lung cancers.18 In PCa, field cancerization has been described for genomic instability and gene expression profiles. With regard to the 3.4mtΔ, additional studies are required to determine the extent and significance of this field cancerization in PCa. This has important implications for “clean” tumor margins based on histopathologic examination because it is well known that molecular markers can more accurately determine actual disease-free margins. Finally, is this effect present before malignant transformation, and if so, can it be used for risk assessment and early detection of PCa? These issues are the subject of continuing studies.

A useful biomarker in the management of PCa will be one that not only detects the disease but also behaves as a biosensor of premalignant and suspicious lesions indicative of disease progression. As well, a biomarker that predicts tumor behavior (indolent vs aggressive tumors) will tremendously aid patient management. Future studies of this biomarker, using laser-capture microdissection, will address the preceding questions. Whereas the present molecular data do not tremendously advance the management of PCa, they have some usefulness in early detection of the disease. First, a positive result will prompt early rather than late rebiopsy. Second, a positive anatomic site will indicate an area where a lesion is possibly present and, therefore, requires special attention on rebiopsy.

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* These authors work for and own stock options in Genesis Genomics.

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