Somatic Mitochondrial DNA Mutations in Prostate Cancer and Normal Appearing Adjacent Glands in Comparison to Age-Matched Prostate Samples without Malignant Histology

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Studies of somatic mitochondrial DNA mutations have become an important aspect of cancer research because these mutations might have functional significance and/or serve as a biosensor for tumor detection. Here we report somatic mitochondrial DNA mutations from three specific tissue types (tumor, adjacent benign, and distant benign) recovered from 24 prostatectomy samples. Needle biopsy tissue from 12 individuals referred for prostate biopsy, yet histologically benign (symptomatic benign), were used as among individual control samples. We also sampled blood (germplasm tissue) from each patient to serve as within individual controls relative to the somatic tissues sampled (malignant, adjacent, and distant benign). Complete mitochondrial genome sequencing was attempted on each sample. In contrast to both control groups [within patient (blood) and among patient (symptomatic benign)], all of the tissue types recovered from the malignant group harbored significantly different mitochondrial DNA (mtDNA) mutations. We conclude that mitochondrial genome mutations are an early indicator of malignant transformation in prostate tissue. These mutations occur well before changes in tissue histopathology, indicative of prostate cancer, are evident to the pathologist. (*J Mol Diagn* 2006, 8:312–319; DOI: 10.2353/jmoldx.2006.050112)

Mitochondria have been implicated in the carcinogenic process partly because of their role in apoptosis and other aspects of tumor biology.^{1–4} Damage accrued by the mitochondrial genome is associated with increased cellular stress and organelle dysfunction.⁵ Several groups have demonstrated mitochondrial genome alterations in many cancers.^{6–8} In some of these cancers, identical single nucleotide polymorphisms were observed in biofluids such as blood, saliva, and urine, indicating the potential utility of mtDNA mutations as biomarkers for disease management.^{9–11}

Studies of mtDNA alterations in prostate cancer (PCa) have indicated a high frequency of mutations in the prostate, probably as a result of increased reactive oxygen species production.^{10,12–15} Although these studies demonstrate the importance of mtDNA alterations in the pathogenesis of PCa, they are limited in terms of genome coverage, because less than 10% of the mitochondrial genome was examined. These studies have also been critiqued on the basis of a lack of appropriate controls and associated analyses.^{16,17} Thus, to unravel the full spectrum of disease-associated mtDNA somatic mutations in PCa, we attempted full mitochondrial genome sequencing of pure malignant and benign prostate epithelial glands obtained by laser capture microdissection (LCM), in comparison to blood, which we determined to be an appropriate control tissue (germline versus somatic—within individual control) from both malignant and symptomatic benign individuals (among individual control). This experimental design enabled us to make the following observations. First, somatic mtDNA mutations are frequent in PCa. Second, in the presence of a tumor, normal appearing benign glands harbored somatic mtDNA mutations as well. Third, mtDNA mutations in the prostate gland seem to demonstrate a progressive pattern of malignant disease. For example, mtDNA analyses of prostate needle biopsies from individuals suspected of having PCa, but who were found to be histologically benign (symptomatic benign), revealed somatic mutations that were mostly confined to the noncoding region

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Table 1. Clinical Data for All 36 Study Participants

Patient	Age	PSA	DRE	Stage	Grade	Focus	Extra-prostatic involvement
2	75	6.3	NR	NR	Benign	N/A	N/A
35	52	4.31	33g	NR	Benign	N/A	N/A
51	71	4.55	63g	NR	Benign	N/A	N/A
105	58	6.79	1	pT3a	7	Multifocal	No
208	61	8.38	1	pT3a	7	Multifocal	Yes
209	67	5.8	1	NR	Benign	N/A	N/A
270	59	4.07	Small/firm	NR	Benign	N/A	N/A
278	62	5.17	2	NR	Benign	N/A	N/A
378	57	4.8	1	pT2cN0MX	6	Unifocal	No
375	64	6.13	2	NR	Benign	N/A	N/A
382	67	7.8	1	pT2cN0MX	6	Unifocal	No
384	59	8.87	Small/firm	pT3a	7	Unifocal	Yes
386	62	Normal	1	pT3a	6	Multifocal	No
416	68	7.8	1 (hard)	pT2cN0MX	7	Unifocal	No
418	59	6.16	1	pT3a	6	Multifocal	Yes
426	61	30	1	pT3bN0MX	9	Multifocal	Yes
450	62	1.4	Small/firm	pT2a	7	Unifocal	No
451	53	9.9	1	pT2b	7	Multifocal	No
452	66	12.19	1	pT3a	6	Multifocal	Yes
455	55	4.69	1	pT3bN0MX	7	Multifocal	Yes
456	59	10.89	1	None given	7	Unknown	No
458	48	4.92	Small/firm	pT2cN0MX	7	Multifocal	Yes
460	57	9.9	1	pT3aN0MX	7	Multifocal	Yes
461	60	7.23	Small/firm	pT2bN0MX	7	Multifocal	Yes
463	59	4.5	Small/firm	pT2bN0MX	6	Multifocal	Yes
464	66	9.29	2	pT3a	7	Multifocal	Yes
466	65	6.15	1	pT2cN0MX	5	Multifocal	No
467	59	4.56	1	pT2c	7	Multifocal	Yes
480	63	0.81	NR	NR	Benign	N/A	N/A
501	59	1.01	NR	pT2c	7	Multifocal	No
503	63	6.53	NR		Benign	N/A	N/A
504	68	7.12	Small/firm	pT2cN0MX	7	Unifocal	No
505	63	15.36	1	pT2cN0MX	7	Unifocal	No
536	63	5.28	47.2cc	NR	Benign	N/A	N/A
560	81	22.5	0	NR	Benign	N/A	N/A
858	45	1.03	30g	NR	Benign	N/A	N/A

NR, not recorded; N/A, not applicable.

(NCR; ntps16024-576); however, on development of an adenocarcinoma, mutation load increased dramatically in the coding region (CR; ntps577-16023). Finally, cloning data suggests that some of these mutations are linked. The potential role these mutations might play in PCa development is discussed.

Materials and Methods

Patients

Patients were asked to participate in this study by their urologist according to the Tri-Council Policy Statement on Ethical Conduct for Research Involving Humans (<http://www.nserc.ca/programs/ethics.htm>). Guided by the pathology reports, malignant prostate core needle biopsy samples were requested from an initial group of patients for a pilot study not reported in detail here. Tissue from a group of patients who had undergone radical prostatectomy as a treatment for advanced PCa ($n = 24$; Table 1) were likewise requested according to the results of the pathology report. A blood sample was obtained from each participant as well. In addition, blood was collected from a subset of maternal relatives to those recruited to the study. A final group of

patients underwent prostate biopsy to exclude the presence of PCa and were found to be free of detectable disease or to be symptomatic benign ($n = 12$; Table 1). The same procedure and sampling was followed for these patients. Requested tissues (prostatectomy and biopsy samples) were cut from formalin-fixed, paraffin-embedded blocks and processed for LCM. LCM was performed by a qualified pathologist who captured malignant glands (M), benign glands adjacent to tumor (AB), and distant benign glands (DB) from a field free of abnormal histology. The DB glands were from a nondiseased lobe or at least 10 cell diameters from the tumor when in the same lobe. By direct observation of the process, approximately three to four cells were harvested per laser pulse, or capture event, and ~2000 captures were recovered from each tissue type.

mtDNA Amplification

DNA was recovered from LCM samples by proteinase K digestion and from blood by means of the UltraClean DNA BloodSpin kit (MO BIO Laboratories, Inc., Carlsbad, CA). Polymerase chain reactions (PCRs) were seeded with 5 μ l of DNA extract. Amplification of the complete mitochondrial genome was attempted using 34 primer sets. In general,

amplicons were limited to an average size of 625 bp to ensure complete amplification of formalin-damaged nucleic acids. Template was amplified using TaKaRa LA *Taq* (Takara Bio Inc.). Reaction conditions were as follows: 1× LA PCR Buffer II (Mg²⁺ plus), 0.4 mmol/L each dNTP mixture, 1× bovine serum albumin (New England Biolabs Inc., Beverly, MA), 0.6 μmol/L each primer, 1.25 U LA *Taq*, 0.5% Ficoll 400, and 1 mmol/L tartrazine (Sigma-Aldrich). Total reaction volume was 25 μl. Cycling parameters were 94°C for 2 minutes, followed by 40 cycles of 94°C for 20 seconds, 30 seconds annealing at optimized primer temperatures, and 72°C for 90 seconds. Cycling was performed with a DNA Engine Tetrad 2 (Bio-Rad, Hercules, CA).

Sequencing

PCR products were purified and sequenced at Lark Technologies (Houston, TX) using BigDye Terminator chemistry (ABI) and capillary electrophoresis. In general, each amplicon was sequenced in both forward and reverse directions. Complete mitochondrial genome sequencing was attempted on all samples. Sequences were analyzed using the Phred-Phrap-Consed package¹⁸ and aligned to the revised Cambridge reference sequence (rCRS)¹⁹ using Sequencher software (Gene Codes Corp.). Blood-derived mtDNA sequences were compared between 30 of the participants and their maternal relatives to determine whether blood was a reliable source of comparative mitochondrial sequence. Sequence variation between blood and rCRS was recorded as maternal polymorphisms (data not shown), whereas somatic mutations were scored as sequence variation between prostate samples and matched blood.

Cloning

The intramolecular relationships of mutations were determined by cloning and sequencing one separate but specific amplicon from two patients (patients 382 and 452). Corresponding PCR products were cloned into a TOPO TA cloning vector (Invitrogen, Carlsbad, CA), transformed, and colonies selected according to the manufacturer's recommendations. Approximately 25 clones were selected and sequenced in forward and reverse. All three tissue types were included for each patient: AB, DB, and M.

Numt Precaution

As part of a separate study to directly characterize and catalogue all nuclear-embedded mitochondrial pseudogene (numt) sequences that co-amplified with our primers,²⁰ primer sets that co-amplified numts were used to amplify ρ⁰-derived template. ρ⁰ cells were prepared from a human osteosarcoma cell line 143B (ATCC CRL-8303) treated with ethidium bromide to deplete cytoplasmic mitochondrial DNA (kindly provided by E. Shoubridge, Department of Human Genetics, Montreal Neurological Institute, McGill University, Montreal, QC, Canada).²¹ Cells were grown to confluence in high-glucose Dulbecco's modified Eagle's medium with pyruvate, L-glutamine,

uridine (50 μg/ml), and 5% fetal bovine serum. At confluence cultures were harvested, DNA was extracted (QIAmp DNA mini kit), and amplified. Resulting ρ⁰ amplicons were cloned, sequenced, and extensively analyzed and compared against hetero- and homoplasmic sites in sequence data to preclude any inclusion of paralogous nuclear mutations in the final data set.

Statistical Analysis

Comparative statistical analyses of mutations were made with the Student's *t*-test and one-way analysis of variance (ANOVA). A *P* value of less than 0.05 was considered significant. Phylogenetic analyses were performed on the resulting sequences to guard against contamination and sample mix-up.²² Whole mitochondrial sequences were also included from all members of the laboratory who handled samples. Both neighbor-joining²³ and Bayesian²⁴ analyses were conducted using a maximum likelihood estimated best-fit model of evolution.²⁵ Phylogenetic analyses were conducted in PAUP*²⁶ and MrBayes.²⁷

Results

Quality Control

The primer sets used to amplify 12S and 16S rRNA regions co-amplified numts, which demonstrated pseudo heteroplasmy in PCR amplification product. Interestingly, several chromosomes were amplified in these particular reactions, which added to the pseudogene presence through this region, likely increasing the numt copy number to detectable, and contaminating, quantities. Data from these regions were omitted from this study. Phylogenetic analysis showed strong clustering of mtDNA from different tissue types from the same patient, supporting the overall quality of the data and ruling out sample mix-up. Likewise, sample sequences did not cluster with Genesis Genomics Inc. employee sequences supporting the lack of problems with contamination (Figure 1).

mtDNA Analysis of Malignant Prostatectomy Samples

Sequences of all three tissue types from the 24 prostatectomy samples were compared with paired blood sequence as the nondisease germline control. Results indicate that 16 of 24 (66.7%) had mutations in all three designated tissues from the prostate; 22 of 24 (91.7%) had mutations in malignant samples; 19 of 24 (79.2%) expressed alterations in adjacent benign samples, and 22 of 24 (91.7%) displayed changes in distant benign glands. All patients had at least one somatic mutation among the three glandular tissues. The average sequence coverage per genome was 78%, leaving the mutations in the remaining 3.6 kb uncharacterized. The distribution of mutations across the mitochondrial genome in the three tissues in relationship to percent sequence coverage, missing data, and average number of mutations per patient was examined (Figure 2, A–C). In

Figure 1. Neighbor-joining phylogenetic tree of mtDNA sequences from patient samples (blood-B, M, AB, DB, stroma) and individuals who worked in the laboratory where samples were analyzed. Note the strong clustering of mtDNA sequences from different tissue types from the same patient and the lack of clustering of sequences from laboratory members with any patient sequences. Inadequate sequence data from samples P452AB, P466DB, P35stroma, P466AB, P466DB, and P463AB might have accounted for the lack of clustering of these samples with others from the same patient.

Figure 2. The distribution of mutations per patient in the 13 OXPHOS genes, HV1 and HV2 overlaid with line graphs showing the percentage of contributing patients and missing data per region. Note the similarity in the distribution of mutations in HV1 and HV2 in all sample groups (M, AB, DB, and SB) and the paucity of mutations in the CR in SB group compared to the malignant subgroups.

general the likelihood of mutations occurring in a gene is related to its size. Overall, 273 somatic mutations were observed in this sample set, with a heteroplasmic-homoplasmic ratio of 2.29. The synonymous to nonsynonymous mutation rate is calculated to be 1.48-fold greater than that generally observed in humans.²⁸ Nonsynonymous mutations and mutations in transfer RNAs, which might interfere with mitochondrial functions, were quite frequent (Tables 2 and 3).

mtDNA Analysis of Within Patient and Symptomatic Benign Control Tissue

We demonstrated the use of peripheral blood as a source of germline mtDNA sequence by comparison of mitochondrial genome sequences from the blood of 30 of our study participants and their maternal relatives. Sequence comparison between relatives and the patients showed little if any

differences between maternal genomes, precluding age-associated mutational events (data not shown).

Because analysis of the malignant samples revealed mutations in histologically benign perilesional prostate tissue, we questioned the disease-specificity of these somatic mtDNA mutations in PCa. To address this issue in the absence of completely asymptomatic donor samples, control material from 12 age-matched symptomatic benign patients who had undergone biopsy for suspicion of PCa, but did not have malignant histology, were analyzed. These individuals (average age, 64 years) were selected on the criteria of low prostate-specific antigen scores (average prostate-specific antigen score of 6.04) and/or an absence of malignant histology based on the pathology reports (Table 1). Full genome sequencing of amplified mtDNA from pure epithelial cells obtained by LCM was completed. The distribution of mutations across the mitochondrial genome in this group is illustrated in

Table 2. Mutations in Various Tissues of the Malignant and Symptomatic Benign Samples

	DB	AB	M	SB
NCR	46	31	29	12
CR	64	64	53	6
tRNA	6	11	5	1
Total	116	106	87	19
Total heteroplasmy	88	86	58	18
Total homoplasmy	28	20	29	1
Synonymous total	40	41	24	4
Heteroplasmic	31	37	19	3
Homoplasmic	9	4	5	1
Nonsynonymous total	24	23	29	2
Heteroplasmic	22	19	20	2
Homoplasmic	2	4	9	0

Figure 2D. Five patients had no mutations with the remaining seven (58.3%) having between one and five alterations, mainly in the NCR. With sequence coverage of 72%, we detected 16 somatic mutations (12 in NCR, 3 in CR, and 1 in L strand origin of replication) (Table 2). Overall, this symptomatic benign group had fewer mutations, most of which occurred at known polymorphic loci in the NCR (<http://www.mitomap.org>).

Comparison of Mutations in Malignant and Symptomatic Benign Groups

To test for any differences in mutation load between the various sample categories, a Student's *t*-test was performed. No significant difference was observed in the mutation load between the prostatectomy groups (Table 4). A one-way ANOVA was completed (not shown) with all four tissue categories for both CR and NCR. Results are similar

Table 4. Statistical Comparison of Mutation Load in Various Tissue Categories

Tissue type	df	<i>t</i> Stats	<i>P</i> value
M versus AB	23	0.42	0.676
M versus DB	23	0.55	0.586
AB versus DB	23	0.07	0.942
CR-SB versus M	29.24	3.90	< 0.05
CR-SB versus AB	26.30	2.88	< 0.05
CR-SB versus DB	28.93	4.43	< 0.05
NCR-SB versus M	19.68	0.08	0.939
NCR-SB versus AB	21.99	1.48	0.884
NCR-SB versus DB	25.75	1.32	0.199

to those in Table 1. Moreover, a gene-to-gene comparison did not reveal any differences in the amount of mutations in all three groups (data not shown), suggesting that, in the presence of a tumor, sampling the prostate gland in the vicinity of the tumor for mtDNA analysis does not require acquisition of histologically malignant cells. In contrast, there was a significant difference in mutation frequencies when the CR of each prostatectomy group was compared to the CR of the control group (independent *t*-test, unequal variances) (Table 4). Mutation load in the NCR did not demonstrate any statistically significant difference between the prostatectomy and the control groups (Table 4).

Cloning Data Reveals Linked Mutations in Single mtDNA Molecules

Analysis by Chen and colleagues¹² indicates that several mutations occur in the same mtDNA molecule in PCa. To investigate this possibility in our series, we cloned and sequenced amplicons from two individuals (patients 382 and 452) who demonstrated several mutations. Cloning data from patient 382 revealed a relatively high frequency of heteroplasmic mutation at 15,527 (nonsynonymous) on

Table 3. Non-synonymous Mutations in Various Complexes and tRNA in the 24 Prostatectomy Group

Complex I								
ND1 (3307 to 4262)	3308	3394	3398	4216	4217			
ND2 (4470 to 5511)	4569	4591	4716	4722	4864	4917	5371	5424
ND3 (10059 to 10404)	10,143							
ND4 (10760 to 12137)	11,069	11,177	11,217	11,420	11,852	11,907	12,012	
ND5 (12337 to 14148)	12,959	13,105	13,294	13,484	13,634	13,759	13,789	13,805
	13,880	13,933	14,044					
ND6 (14149 to 14673)	14,178							
Complex III								
CYTb (14747 to 15887)	14,903	15,162	15,302	15,323	15,324	15,384	15,527	
Complex IV								
COI (5904 to 7445)	6009	6037	6219	6307	6382	6579	6691	7059
	7146	7159	7309	7389	7407			
COII (7586 to 8269)	8027	8133						
COIII (9207 to 9990)	9405	9477	9502	9564	9574	9628		
Complex V								
ATPase6 (8527 to 9207)	8616	8701	8893	8903				
tRNA								
A	5593	5650	5655	5663				
N	5677							
Y	5882							
D	7521							
R	10,439	10,455						
P	15,946	15,995	15,996	15,998	15,999	16,000		

20 of 27 (74%) clones. After cloning and sequencing amplicons from patient 452, six linked mutations (6989, 7055, 7146, 7256, 7389, and 7521) on 11 of 25 (44%) clones were observed. Mutations at 7146 and 7389 are nonsynonymous changes. In addition to these sites, the presence of one or two variant point mutations in each clone was common.

Discussion

In an initial pilot study, we sequenced and analyzed only the NCR of 34 malignant PCa biopsies and matched blood, confirming the previous observations by Jeronimo and colleagues¹⁰ and Chen and colleagues¹³ that mtDNA mutations are very frequent in the NCR in PCa. Jeronimo and colleagues¹⁰ reported 20 mutations in 3 of 16 (19%) samples whereas Chen and colleagues¹³ observed mutations at 34 nucleotide positions in 14 of 16 (88%) patients. Excluding mtDNA microsatellite instability in the homopolymer region (C-stretch), we identified substitution mutations in the NCR at 34 nucleotide positions in 21 of 29 (72%) prostate biopsy samples, consistent with the observation by Chen and colleagues.¹³ In our more comprehensive study involving the entire mitochondrial genome, we did not observe any statistically significant difference in mutation load in the NCR between malignant and symptomatic benign groups. Because the NCR is a mutational hotspot, it is possible that this region bears much of the initial mutational insults, and the CR becomes increasingly involved as disease progresses. Also many of the mutations in the NCR region occurred at polymorphic loci, attesting to the possible constraints imposed by the structure of the NCR on the nature and occurrence of mtDNA mutations in this region.¹³

Our extensive study encompassing the entire genome from known malignant tissue revealed surprising results. Mutations were not restricted to histologically malignant tissue but occurred in adjacent and distant benign-looking glands as well. This suggests that molecular alterations that may signal the presence of a tumor, or contribute to their development, take place long before morphological indications of malignant transformation are visible. Alternatively the presence of a tumor may exert a so-called field effect leading to molecular alterations in histologically normal appearing tissue adjacent to the tumor. Indeed, quantitative analyses of nuclear chromatin and blood vessel architecture indicate changes in normal appearing prostate tissues adjacent to tumors.^{29,30} This field effect has recently been demonstrated in gene expression profiling of PCa.^{31,32} In these studies, gene expression patterns in adjacent benign glands were observed to be more closely related to that of the tumor than donor samples. This suggests that in the presence of a tumor the use of benign tissue as normal control, based on histological criteria alone, is inappropriate. Donor tissue from individuals free of tumors will allow disease-specific genome alterations to be identified.

Another interesting finding from our analysis is the observed difference in the distribution pattern and load of somatic mutations between the symptomatic benign control

and malignant groups. The finding that mutations were indeed very few, occurred mainly in the NCR in the control group, and became frequent in the CR in PCa, suggesting the possible functional importance of mtDNA alterations in PCa. It should be noted that the control group comprised age-matched individuals who were symptomatic for PCa yet had no malignant disease at biopsy. This strongly demonstrates the disease (not age) association of these mutations in the pathogenesis of PCa. The increasing number of mutations in the CR within malignant prostate tissue suggests disease progression when compared to the same rate for benign symptomatic prostate tissues. This indicates that monitoring the somatic mutation pattern in prostate mtDNA may have clinical utility.

Of particular interest is the accelerated somatic mutation rate in these tissues indicating loss of mitochondrial genome stability perhaps related to nuclear-to-mitochondrial pathway disruption.^{33,34} Moreover, robust mtDNA repair mechanisms, with nuclear origins, may not be able to repair the increasing frequency of lesions.⁵ Damage to the highly efficient electron transport components and the resulting loss of ATP seems like a metabolic blunder; however, this mutation process might play a role in malignant cell survival.³⁵ Indeed as proposed by Singh,¹ a "mitocheckpoint" may control the repair of damaged mitochondria. However, in persistent mtDNA damage such as accelerated mitochondrial mutations, "mitocheckpoint" may fail in its reparative process, leading to genomic instability and increased cell survival. Such a process may augment the carcinogenic process.¹ In addition, mitochondrial dysfunction allows greater cell survival after exposure to some chemotherapeutic agents.² Interestingly, a selective component for mitochondrial haplotypes has been demonstrated in human populations, attesting to an evolutionary active molecule.³⁶ The gauntlet of selection likely involves many mutational combinations or perhaps even suites of lesions, driven principally by nonsynonymous alterations in the CR. At some point in the rapid evolutionary process, a particular metabolic threshold is exceeded. Perhaps tumor behavior and tumor aggression relate to the way and degree to which this threshold is breached. Cloning data from patient 452 suggests such a possibility. A series of negative core needle biopsies in this individual were replaced by an aggressive, metastatic PCa diagnosis, based on a 3-month follow-up biopsy series. Linked mutations, both synonymous and nonsynonymous increased in frequency in association with disease progression. This finding suggests that some neutral changes may hitchhike with nonsynonymous mutations, a well-characterized nuclear event.³⁷ The increase in the nonsynonymous mutation rate in our data (Table 2) further supports the notions of progression and hitchhiking. This implies that the use of specific single nucleotide polymorphisms to diagnose some neoplasias and malignancies may be impractical and an oversimplification of a cumulative mutation process driven by nonspecific reactive oxygen species damage.

Could the rich information provided by mtDNA alterations in PCa be harnessed to improve the clinical management of patients? mtDNA alterations in the prostate gland might be a very useful biosensor for early cancer detection and monitoring, given the uniqueness of this molecule and the hy-

permutagenesis of mtDNA in PCa (this study).¹² The mtDNA alterations in the prostate are likely very early events in PCa development because they are observed in pre-malignant histological benign-looking glands. The high copy number of mtDNA coupled with the accelerated mutations in PCa, and in some instances, the homoplasmic nature of the mutations should confer a detection advantage over nuclear genome alterations in prostate tissues and prostatic biofluids. Methods such as microarray sequencing (Human Mitochip) and allele-specific PCR will allow high-throughput facile detection of mutations in biofluids.^{38,39}

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