Extensive Somatic Mitochondrial Mutations in Primary Prostate Cancer Using Laser Capture Microdissection

Junjian Z. Chen, Neriman Gokden, Graham F. Greene, Perkins Mukunyadzi, and Fred F. Kadlubar

Division of Molecular Epidemiology, National Center for Toxicological Research, Food and Drug Administration, Jefferson, Arkansas 72079 [J. Z. C., F. F. K.], and Departments of Pathology [N. G., P. M.] and Urology [G. F. G.], University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205

Abstract

Prostate cancer is the second leading cause of cancer deaths among men in the United States, but the precise molecular events leading to prostate carcinogenesis are not well understood. We isolated histologically defined cell populations from prostate cancer and its preinvasive lesions using laser capture microdissection, and performed genetic analysis on the mitochondrial genome, a sensitive cytoplasmic DNA. An extremely high incidence of somatic mutation (90% of prostatectomy cancer specimens) was found in the control region (the displacement loop) of mitochondrial DNA. The massive induction of lesion-associated mutations suggests active mitochondrial mutagenesis in both prostate cancer and its preinvasive lesions. Inspection of these mutations provides new insights into prostate cancer genetics and reveals unique patterns of somatic mutations in prostatic neoplastic lesions.

Introduction

Prostate cancer is the second leading cause of cancer deaths among men in the United States, but the precise molecular events leading to prostate carcinogenesis are not well understood. Genetic characterization of prostate cancer has centered on the nuclear genome. Complex chromosomal instability has been identified as a major genomic alteration (1), but the cause of the striking case-to-case diversity is still not clear. Infrequent substitution mutations are reported in a few oncogenes and tumor suppressor genes, and are often associated with late events in prostate cancer progression (2). Because prostate cancers are almost exclusively adenocarcinomas arising in the glandular epithelium, conventional genetic analysis has been complicated by tissue heterogeneity and the infiltrating nature of the disease. LCM (1) is a powerful technique for selective isolation of defined cell populations from heterogeneous tissue sections (3). Moreover, this approach provides new opportunities to investigate somatic alterations in specific cell populations of prostate cancer and high-grade PIN, a putative preinvasive lesion (4).

The mitochondrial genome is maternally inherited cytoplasmic DNA that exists in high copy number in each cell. Because of its unique genetics and functional importance in cellular oxidative phosphorylation and apoptotic control, it has drawn increasing attention in cancer research (5). mtDNA is a critical target for cellular oxidative stress and to trace the clonal lineage of tumors. mtDNA mutagenesis in both prostate cancer and its preinvasive lesions using a LCM-based approach. Inspection of these mutations not only provides new insights into prostate cancer genetics but also reveals unique patterns of somatic mutation in prostate cancers.

Materials and Methods

Tumor Specimens. Sixteen prostate tumor specimens were obtained from the surgical pathology files of the University of Arkansas for Medical Sciences (Little Rock, AR) from patients who had undergone radical prostatectomy between 2000 and 2002. Surgical specimens were fixed in 10% buffered formalin and embedded in paraffin for conventional histological examination. Patients were all Caucasians and ranged from 47–77 years of age. The tumor grade varied from well to poorly differentiated according to the Gleason system (11).

Microdissection and DNA Extraction. Serial tissue sections of 5-μm were prepared for each specimen and stained with H&E using a protocol developed at NIH.4 Benign and malignant histology was identified by two human pathologists, and pure target cells were procured by the PixCell II LCM System (Arcturus, Mountain View, CA) for benign epithelial cells, adenocarcinoma cells, and paired PIN lesions when available from each specimen. On average, 500 cells were collected for each cell population using a CapSure HS LCM Cap, and DNA was extracted using a PicoPure DNA Extraction kit (Arcturus) according to the manufacturer’s recommendation with minor modifications.

PCR Amplification of the Control Region. Two overlapping fragments were PCR amplified for sequence analysis of the entire sequence of the control region. Primers MFI5990 (5'-CTTTAACTCCACATTAGGC-3') and MR36 (5'-ACCAAATGCTGGAGGACTC-3') were used to amplify a 602-bp fragment containing the HV1 [MF or MR designates mtDNA forward or reverse primer; the number specifies the position of the primer's 3' terminus according to the revised Cambridge reference sequence; Ref. 12)]. Primers MF34 (5'-ACCCCTATACCCACCTACG-3') and MR583 (5'-GCTTT-GAGGAGGTAAAGCTAC-3') were used to amplify a 558-bp fragment harboring the HV2. One-fifth of extracted DNA (~100 cell equivalents) was subjected to DNA amplification using the Expand High Fidelity PCR system (Roche Molecular Biochemicals, Indianapolis, IN) and a step-down PCR protocol that consisted of 95°C for 2 min, 1 cycle; 94°C for 30 s, 63°C for 1 min, 70°C for 1 min, 3 cycles; 94°C for 30 s, 60°C for 1 min, 70°C for 1 min, 3 cycles; 94°C for 30 s, 57°C for 1 min, 70°C for 1 min, 39 cycles; and a final extension at 70°C for 5 min.

Sequencing of mtDNA. PCR products were fractionated with a 1.2% agarose gel followed by purification with the QIAEXII gel extraction kit (Qiagen, Valencia, CA). Cycle sequencing was performed using Thermosequenase (USB, Cleveland, OH) and IRDye 800 terminator mix kit (Li-cor, Lincoln, NE). Sequences were resolved on a long polyacrylamide gel (66 cm, 3.7%) mounted on a NEN Global IR2 sequencer (Li-cor) according to the manufacturer’s recommendations. The two DNA fragments were each sequenced in both directions using nested primers MR25 (5'-AGAGCTCCGGTGAGTTG-3') and MF16004 (5'-TTTAGCCCCAAAAGCTAAGATTTC-3') for the HV1-containing segment, and MF49 (5'-AGGGAGGTCTCCATGCA-3') along with MR572 (5'-GCTTTGAGGGAAGGTAACTAC-3') for the HV2-containing segment. DNA sequences were edited and aligned for inter- and intraindividual variation using e-Seq and AlignIR software (Li-cor). The numbering of sequence variation was based on the revised Cambridge reference sequence (12). The mtDNA sequence from matched benign cells served as an internal control for the determination of somatic mutation in diseased cells.

Data Analysis. The independence of substitution mutation and mtDNA instability was tested using a continuity-corrected χ² test (13). Expected cell counts were also estimated and examined. Sequence divergence was calculated using the Neighbor-Joining method and confirmed by the minimum parsimony analysis in MEGA 2.1.

Results and Discussion

LCM-based Approach for mtDNA Analysis. To overcome tissue heterogeneity and the infiltrating nature of prostate tumors, we used LCM to obtain pure tumor cells, coexisting PIN lesions, and distant benign epithelial cells from archived prostatectomy specimens for each of the 16 cases. On the basis of careful estimates, each dissection contained >95% of the desired cells. The use of surrounding benign epithelial cells as an internal control not only allowed the unambiguous determination of genetic alterations associated with diseased cells, but also served as a critical step for quality control of potential experimental errors. Our primary analysis was focused on the main regulatory region of the mitochondrial genome, a hypervariable, 1.1-kb noncoding region (the D-loop). Two overlapping fragments were each amplified by PCR from 100-cell equivalents of DNA, purified, and sequenced using an infrared dye-based sequencing system capable of the reliable identification of rare mutations (>15% in frequency). The accurate procurement of target cells coupled with the relative ease of PCR amplification of mtDNA from archived tissues allowed the precise genetic characterization of heterogeneous prostate lesions based on their histology and pathology.

Somatic Mutations in the Control Region. Fourteen of 16 cases of prostate cancer (90%) were found with somatic mtDNA mutations in tumor and/or PIN lesions when a total of 29 diseased foci were analyzed for sequence variation. Somatic alterations were observed at 34 different nucleotide positions in the D-loop region, among which 30 had substitution mutations and 4 had small deletions or insertions (Table 1). Among the substitution mutations, 80% of them were transitions and more than half were homoplasmic or near homoplasmic in analyzed cell populations. Some of the mutations appeared to be locally distributed because of their absence in a second cancer focus when analyzed in several cases. Five of the changes were detected in >1 patient including a transition of A73G detected in 3 unrelated cases. Multiple mutations were detected frequently in the same focus with the highest incidence being 10 near-homoplasmic substitutions observed in case 4 (Fig. 1A). Surprisingly, multiple heteroplasmic mutations were also detected in several cases and appeared to link on the same DNA molecules when analyzed using allele-specific amplification of mutant species. The massive induction of disease-associated genetic alterations suggests active mtDNA mutagenesis in prostate cancer and its preinvasive lesions, likely caused by increased cellular oxidative damage, and/or altered repair machinary in the tumor tissue. No association was apparent between the incidence of somatic mutations and the age or tumor grade of the patient.

The somatic mutations cannot be explained by experimental error or by contamination of nuclear mtDNA pseudogenes (14). This is because all of the substitutions detected in diseased cells were not observed in corresponding benign epithelial cells with the exception of case 5. A more sensitive method using allele-specific amplification for mutations failed to generate any signal from corresponding benign controls (data not shown). As for case 5, six mutations, including four substitutions, one deletion and one insertion, were detected not only in cancer cells in a homoplasmic state but also in surrounding benign epithelial cells at an estimated mutant frequency of 70%. Reanalysis of independent benign epithelial cells microdissected from the same case failed to detect any of the mutations. This exception could not be explained by coamplification of nuclear pseudogenes but provides evidence for the induction of multiple mtDNA mutations proceeding identifiable changes in histology and/or pathology.

MtDNA Instability. MtDNA instability (i.e., small deletions/insertions) occurred primarily at mononucleotide sequence repeats and a dinucleotide microsatellite in the control region. Although only 6.13 6P,13C,1 195 T→C (6.13)He
4 11C 235 G→C Ho
11 10P 313 C→T He
11 11C 315 C→T Ho
5 5C,5B 489 T→C (5C)Ho, (5B)He
4 4C 499 G→A Ho
14 14C,14C 16993 T→C Ho
4 14C 16111 C→T Ho
1,4 1C,4C 16182 A→C (1He, (4)Ho)
1,4 1C,4C 16183 A→C (1He, (4)Ho)
1,4 1C,4C 16189 T→C (1He, (4)Ho)
4 4C 16217 T→C Ho
2 2C,1 16218 C→T Ho
1 1C 16232 C→A He
1 1C 16249 T→C Ho
1 1C 16274 G→A He
5 5C,5B 16298 T→C (5C)Ho, (5B)He
1 1C 16304 T→C Ho
1 1C 16311 T→C He
11 11C 16403 C→T Ho
12 12P 16459 C→T Ho
13 13C,1 16474 G→C He
5 5C,5B 16519 T→C (5C)Ho, (5B)He
5.6 5C,5B,6P 243–244 (A2)→(A1) (5C)Ho, (5B,6)He
2,4,5,6,7, 9,11,15 Multipleb 303–309 Code/del He,(11)Ho
2.4,5,6,7, 9,11,15 Multipleb 303–309 Code/del He,(11)Ho
1 1C,1P 514–523 (CA)x→(CA)x Ho
12 12C,12P,12B 16184–193 (CT)x→(CT)x Ho

4 C, cancer; P, PIN; B, benign; C1–1C12, cancer focus 1 or 2.
5 Ho, homoplasmic; Ho, near homoplasmic; He, heteroplasmic.
6 Multiple cancer foci were detected with small insertions or deletions.

5 Internet address: http://www.megasoftware.net.
four nucleotide positions were detected with deletions or insertions, one of the sites was clearly a mutational hotspot for mtDNA instability, because alterations at this position were detected in 8 of 16 cancer patients (Table 1). The hotspot for instability has a unique sequence motif between nucleotides 303 and 316 (CCCCCCTCCCCCCC), and the instability always occurred at the first polymorphic C tract that was referred to previously as D310 (15). Most D310 instabilities detected in this study involved small insertions in cancer and PIN foci, but more complex patterns with both primary and secondary instabilities were also observed (Fig. 1B). The heteroplasmic nature of the D310 instability and frequent detection in more than one diseased focus from each case suggest a widespread instability of mononucleotide runs in prostate cancer-containing tissues. Both oxidative damage and mtDNA polymerase γ were reported to cause DNA instability at mononucleotide runs (16, 17). It is likely that the concerted action of both processes is responsible for the observed instability. It needs to be pointed out that this mutational hotspot was recognized as a major target for mtDNA alterations in human tumors in a previous study (18). However, no alteration was detected in 16 cases of prostate cancer in this study. This discrepancy may be explained by different analytical approaches used in the two studies. Comparison of these small deletions/insertions and substitution mutations reveals subtle but interesting differences. Both substitution mutation and mtDNA instability occurred in high frequency in cancer and PIN, but they did not always occur together. Among the 14 cases that had substitution mutation and/or instability, only half of the cases contained both types of mutations. Statistical analysis shows independence between two types of change. This independence suggests that different mutagenic processes underline the two types of alteration.

Genetic Heterogeneity between Cancer and PIN. It has long been speculated that PIN is the most likely preinvasive stage of adenocarcinoma because of their frequent coexistence and some shared pathological features (4). To test the clonal relationship between these two lesions, we dissected prominent PIN lesions coexisting within the same cancer focus used for genetic analysis. As demonstrated in Fig. 1C, different homoplasmic mutations were detected in a pair of cancer and PIN from case 11, but none of them in a benign control. Among 8 cases of paired cancer and PIN, 10 substitution mutations were detected in cancer cells from 2 cases, 6 substitutions in PINs from 4 cases, but none of the substitutions was shared by both lesions. The only case where both cancer and PIN shared a common mutation was a homoplasmic deletion of a dinucleotide repeat in case 1. This mutation appeared to be widely distributed in neoplastic cells, because it was also confirmed in an unrelated PIN lesion but not detected in a second benign control. Interestingly, 8 heteroplasmic substitution mutations were also detected in the cancer focus of this case but not in corresponding PINs, suggesting that the substitution mutations evolved more recently and most likely during the progression of this specific cancer. The extensive genetic alterations observed in cells from different pathological stages provide an informative marker for the evaluation of both a possible clonal relationship and the extent of divergence between neoplastic lesions. The heteroplasmatic D310 instability is not very informative in this analysis because of its high frequency of occurrence and likely independence in origin. Although clonality was observed between a paired cancer and PIN in a single case, the genetic heterogeneity observed in this study suggests a rapid and independent accumulation of genetic changes for the two types of lesions.

MtDNA Sequence Polymorphism and Clustering. The control region of mtDNA is highly polymorphic and contains two hypervariable regions, HV1 and 2. A total of 57 polymorphic sites were identified in the region from 16 prostate cancer patients, which included 54 sites with nucleotide substitutions and 3 sites with length variation. With the exception of 3 newly identified substitutions, T206C, C437T, and A487G, the remaining sequence variations were listed in the Mitomap database.7 On the basis of their sequence variation, each of the 16 patients has a unique mtDNA haplotype. The evolutionary relationship among the 16 mtDNA haplotypes was reconstructed using a Neighbor-Joining algorithm (Fig. 2). A published African reference sequence was used as an outgroup (19). It is interesting to note that cases 1, 4, and 6 were closely related with one another and clustered into a single subgroup in the Neighbor-Joining tree. This grouping was also confirmed by minimum parsimony analyses in MEGA 2. Coincidentally, multiple somatic mutations were detected in each of the 3 cases, a total of 20 somatic substitution mutations including 4 mutations detected in >1 of the 3 cases. The nonrandom distribution of somatic mutations raises the possibility that certain constellations of sequence variation might be prone to somatic

---

7 Internet address: http://www.mitomap.org.

---

Fig. 1. Fluorograms of somatic mutations detected in the mtDNA control region using an automated Li-cor DNA sequencer. A, multiple homoplasmic substitution mutations. As indicated by arrows, four substitution-mutations were identified in cancer cells in a 40-bp region. The 12-G tract caused by three mutational events in the cancer cell led to slippage of downstream DNA sequencing. A total of 10 near homoplasmic substitutions and one event of instability were detected in the D-loop region of this patient’s cancer cells (case 4), and none of the mutations were detected in surrounding benign epithelial cells. B, examples of mtDNA instability at the D310 tract. Sequence instability with two- and three-base insertions at the first C tract were only detected in cancer cells in case 4. Whereas a complex pattern of instability was observed in case 15 where not only the dominant C-tract length differed between benign and two cancer foci, the secondary instability was also detectable in all three target-cells. C, genetic heterogeneity between paired cancer and PIN lesions. As indicated by arrows, two homoplasmic substitutions were detected in cancer cells, whereas a homoplasmic deletion was obvious at the D310 tract in the PIN. None of the mutations was observed in benign cells.
It is also important to recognize some differences between these two types of variations. A substantial number of somatic mutations occurred at sites where evolution rates were zero or below the average rate, especially in the HV 2 region, a functionally more important region. For example, two independent substitution mutations of C313T and C315.1T were detected in a mononucleotide repeat of six cytosines located one base downstream of the D310 tract in a conserved sequence block (CSB II). In contrast with the highly polymorphic and instable D310 tract, the second mononucleotide repeat was highly conserved, and no instability was reported in human cancers. Assuming that the individual hypervariable polymorphic sites in the D-loop are neutral in function, these unique somatic mutations might confer functional consequences for cancer cells. On the other hand, newly arisen variants identified in pedigree analysis differed from their parental type almost exclusively by single nucleotide changes in the D-loop (21), whereas new mutants detected in cancer cells frequently contained multiple mutations. This unique difference suggests a greatly accelerated process in mtDNA mutagenesis in prostate cancers. The cause of this acceleration and its molecular mechanism are fundamental questions that need to be addressed in mitochondrial carcinogenesis.

**Conclusions.** LCM is a powerful technique for the procurement of pure target cells for precise genetic analysis. With this approach, we have revealed a total of 34 different somatic mutations in the control region of mtDNA distributed in 14 of 16 cases of prostatectomy specimens. The massive induction of lesion-associated mutations suggests active mitochondrial mutagenesis in both prostate cancer and its preinvasive lesions. Inspection of these somatic mutations provides new insights into prostate cancer genetics. Both substitution mutation and mtDNA instability occurred in high frequencies in prostate cancer cells, but they appeared to be governed by different mechanisms. Genetic heterogeneity observed between paired cancer and PIN lesions suggests frequently independent pathologic processes. Comparison with genetic polymorphism in the same sequence, on the other hand, reveals novel patterns of somatic mutations. The nonrandom distribution of somatic mutations in a subgroup of closely related haplotypes raises the possibility that certain constellations of genetic

### Table 2 Relative evolution rate and sequence polymorphism at sites where somatic substitutions had occurred in the two hypervariable segments

<table>
<thead>
<tr>
<th>Position</th>
<th>No. of mutation</th>
<th>Rate</th>
<th>Mutation</th>
<th>Polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>73</td>
<td>3</td>
<td>5.35</td>
<td>A→G</td>
<td>A→G</td>
</tr>
<tr>
<td>94</td>
<td>1</td>
<td>0</td>
<td>G→A</td>
<td>–</td>
</tr>
<tr>
<td>106</td>
<td>1</td>
<td>0</td>
<td>G→A</td>
<td>–</td>
</tr>
<tr>
<td>150</td>
<td>1</td>
<td>6.12</td>
<td>C→T</td>
<td>C→T</td>
</tr>
<tr>
<td>174</td>
<td>1</td>
<td>0</td>
<td>C→T</td>
<td>–</td>
</tr>
<tr>
<td>195</td>
<td>2</td>
<td>6.22</td>
<td>T→C</td>
<td>T→C</td>
</tr>
<tr>
<td>207</td>
<td>1</td>
<td>3.29</td>
<td>G→A</td>
<td>G→A</td>
</tr>
<tr>
<td>235</td>
<td>1</td>
<td>2.02</td>
<td>G→C</td>
<td>A→G</td>
</tr>
<tr>
<td>313</td>
<td>1</td>
<td>0</td>
<td>C→T</td>
<td>–</td>
</tr>
<tr>
<td>315.1</td>
<td>1</td>
<td>0</td>
<td>C→T</td>
<td>–</td>
</tr>
<tr>
<td>10693</td>
<td>1</td>
<td>2.98</td>
<td>T→C</td>
<td>T→C</td>
</tr>
<tr>
<td>16111</td>
<td>1</td>
<td>2.57</td>
<td>C→T</td>
<td>C→T</td>
</tr>
<tr>
<td>16182</td>
<td>2</td>
<td>0.84</td>
<td>A→C</td>
<td>A→C,A→G</td>
</tr>
<tr>
<td>16183</td>
<td>2</td>
<td>3</td>
<td>A→C</td>
<td>A→C</td>
</tr>
<tr>
<td>16189</td>
<td>2</td>
<td>4.78</td>
<td>T→C</td>
<td>T→C</td>
</tr>
<tr>
<td>16217</td>
<td>1</td>
<td>1.38</td>
<td>T→C</td>
<td>T→C</td>
</tr>
<tr>
<td>16218</td>
<td>1</td>
<td>0.54</td>
<td>C→T</td>
<td>C→T</td>
</tr>
<tr>
<td>16232</td>
<td>1</td>
<td>0.21</td>
<td>C→A</td>
<td>C→A,C→T</td>
</tr>
<tr>
<td>16249</td>
<td>1</td>
<td>1.64</td>
<td>T→C</td>
<td>T→C</td>
</tr>
<tr>
<td>16274</td>
<td>1</td>
<td>2.78</td>
<td>G→A</td>
<td>G→A</td>
</tr>
<tr>
<td>16298</td>
<td>1</td>
<td>1.95</td>
<td>T→C</td>
<td>T→C</td>
</tr>
<tr>
<td>16304</td>
<td>1</td>
<td>1.69</td>
<td>T→C</td>
<td>T→C,T→G</td>
</tr>
<tr>
<td>16311</td>
<td>1</td>
<td>4.78</td>
<td>T→C</td>
<td>T→C</td>
</tr>
</tbody>
</table>

| a | From Meyer et al. (20). |
| b | From the Mitomap database. |
| c | Total no. of mutations in HV 1 and 2. |
| d | Average evolution rate. |
variation in the human population may impact on the process of mtDNA mutagenesis. Meanwhile, the remarkable correlation both in position and type between somatic mutations and hypervariable polymorphic sites suggests a common, but greatly accelerated process in mtDNA mutagenesis in human cancers.

Acknowledgments

We thank Alan Warbritton and Janet Taylor for tissue sectioning and staining, Bridgett Green and Terri Teague-Ross for technical assistance, Brett Thorn for statistical support, Tucker Patterson and Carol Sweeney for useful comments on the manuscript. We also thank Robert Heflich and Dan Casciano for their comments on the manuscript.

References

Extensive Somatic Mitochondrial Mutations in Primary Prostate Cancer Using Laser Capture Microdissection


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/22/6470

Cited articles
This article cites 19 articles, 8 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/22/6470.full#ref-list-1

Citing articles
This article has been cited by 14 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/62/22/6470.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.