Androgen Receptor Gene Alterations and Chromosomal Gains and Losses in Prostate Carcinomas Appearing During Finasteride Treatment for Benign Prostatic Hyperplasia

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ABSTRACT
Finasteride is commonly used for the treatment of benign prostatic hyperplasia and has been suggested to prevent prostate cancer development. To gain insight into the molecular effects of finasteride on prostate cancer development, we studied six prostate cancers diagnosed during finasteride treatment for benign prostatic hyperplasia. Comparative genomic hybridization detected genetic alterations in four tumors (1–5 changes/tumor). Xq gains and 6q losses were the most common alterations. The recurrent Xq gains motivated us to study the involvement of the androgen receptor (AR) gene. One tumor with Xq gain had a 3-fold amplification of the AR gene, suggesting that tumor development in finasteride-treated patients may require increased AR copy number and expression, as has previously been shown for prostate cancers recurring during hormonal therapy. Furthermore, in another tumor, an Arg726Leu mutation of the AR gene was found. This mutation was also present in the germ-line DNA of the patient. Arg726Leu mutation has previously been reported to affect the trans-activation properties of the AR. In summary, prostate cancers developing during finasteride therapy may have distinct biological properties, such as a low number of chromosomal alterations and frequent involvement of the AR gene. Further studies are needed to explore the role of germ-line AR mutations in these patients.

INTRODUCTION
BPH3 is the most common neoplasia among men. Testosterone and especially DHT have been linked to the etiology of both BPH and prostate cancer. Finasteride, a steroid analogue of testosterone that blocks the conversion of testosterone to DHT by the 5-α-reductase, is widely used for the treatment of BPH. Finasteride also inhibits the growth of prostate cancer in cell lines (1) and prevents the progression of tumors in vivo (2). Studies to evaluate the efficacy of finasteride as a prostate cancer chemoprevention agent are in progress (3). However, it is known that some patients treated with finasteride for BPH do get prostate cancer during the therapy (4). The genetic mechanisms that lead to the development or growth of the prostate cancer during finasteride treatment may be quite different from those that play a role in the development of prostate cancer in men with intact hormonal metabolism.

CGH makes it possible to rapidly screen for DNA amplifications and losses in tumors in a single hybridization (5, 6) and thereby provides a starting point for the identification of cancer-related genes. Frequent genetic alterations reported by CGH in untreated primary tumors include losses of 6q, 8p, 13q, and 16q, whereas gains of 7, 8q, and Xq are common in recurrent, hormone-refractory prostate cancer (7–9). Amplifications detected by CGH at Xq11-q12 in hormone-refractory prostate cancer may reflect the amplification of the AR gene (10), whose increased copy number and overexpression may be advantageous for cell survival and proliferation in androgen-deficient conditions (11).

Here, we studied by way of CGH six prostate carcinomas appearing during finasteride treatment for BPH to identify the genetic aberrations that may facilitate the development and growth of prostate cancer during this treatment. In addition, we explored the question of whether the copy number or structure of the AR gene is altered in these tumors.

MATERIALS AND METHODS

Tumor Specimens. Six prostate carcinomas from patients whose cancer was diagnosed during finasteride treatment for BPH were included in this study. Because prostate carcinomas may arise during finasteride treatment, it is important to accurately exclude the presence of prostate cancer before initiating finasteride therapy for patients with BPH. In all of our patients, prostate cancer was excluded by clinical examination (digital rectal examination and ultrasound), serum prostate-specific antigen measurement, and histological verification. Subsequently, the clinical examination and the PSA test were repeated...
biannually. All patients had initially responded favorably to finasteride treatment, with decreased PSA levels and improved urinary flow, and the duration of therapy had, on average, lasted 22 months (range, 13–29 months) before cancer diagnosis. Formalin-fixed, paraffin-embedded samples were available from all patients. The clinicopathological data are summarized in Table 1. In addition, DNA samples from 70 healthy blood donors were used as controls.

**CGH.** Paraffin-embedded tumor DNA was extracted using the PuregeneTM-kit (Gentra Systems, Inc., Minneapolis, MN). CGH was carried out as described earlier (8). Briefly, DNA samples from prostate tumors were labeled with FITC-dUTP (DuPont, Boston, MA), and normal reference male DNAs were labeled with TexasRed-dUTP (DuPont, Boston, MA), and normal reference male DNAs (MN). CGH was carried out as described earlier (8). Briefly, donors were used as controls.

- **Table 1** Clinicopathological characteristics of prostate cancer patients at the time of diagnosis and genetic abnormalities in the prostate carcinomas appearing during finasteride treatment seen by CGH, FISH, and SSCP

<table>
<thead>
<tr>
<th>Patient (sample type)</th>
<th>TNM*</th>
<th>Grade</th>
<th>Duration of finasteride treatment (mo)</th>
<th>Gains</th>
<th>Losses</th>
<th>AR gene mutation</th>
<th>AR gene amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (TURP) T2NXM0</td>
<td>II</td>
<td>5</td>
<td>18</td>
<td>Xqcen-Xq12</td>
<td>6q11-q21, 13q21</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>2 (TURP) T1NXM0</td>
<td>I</td>
<td>3</td>
<td>13</td>
<td>lp32-p36.1, 8q24.1</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>3 (Prostatectomy) T2NXM0</td>
<td>II</td>
<td>6</td>
<td>22</td>
<td>lp13-p35</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>4 (TURP) T3NXM0</td>
<td>II</td>
<td>5</td>
<td>29</td>
<td>2q22-q24, 4q21-qtel, 6q14-q21, 18q12-qtel</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>5 (Prostatectomy) T2NXM0</td>
<td>II</td>
<td>6</td>
<td>21</td>
<td>Xq</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>6 (TURP) T2NXM0</td>
<td>II</td>
<td>5</td>
<td>29</td>
<td>Xq</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

* TMN, tumor-node-metastasis.

**FISH.** Nuclei for FISH analysis of paraffin-embedded tumors were isolated as described elsewhere (13). Briefly, the sections were deparaffinized with xylene, dehydrated in an ethanol series, and placed in 1 ml of Carlsberg solution [0.1% Sigma protease XXIV, 0.1 μl Tris, 0.07 μl NaCl (pH 7.2)] for 1 h at 37°C. The nuclear suspension was pipetted on Vectabond-treated (Vector Laboratories, Burlingame, CA) slides and air-dried.

A P1 probe to the human AR gene (LCG-P1AR) and a probe specific for the pericentricromeric repeat region of chromosome X (DXZ1/BamX7) were used. Probes were labeled with either biotin-14-dATP (Life Technologies, Inc., Gaithersburg, MD) or digoxigenin-11-dUTP (Boehringer Mannheim) using nick translation (BioNick kit, Life Technologies, Inc.) according to the instructions of the manufacturer.

Prior to FISH, the slides were pretreated by heating them in 50% glycerol/0.1× SSC, (1× SSC in 0.15 μl NaCl, 0.015 μl sodium citrate) solution at 90°C for 3 min to decondense the chromatin and to improve hybridization efficiency. The slides were denatured in 70% formamide/2× SSC (pH 7) at 74°C for 5 min, dehydrated in an ethanol series, and treated with 8 mg/ml protease potassium (Sigma Chemical Co., St. Louis, MO) in a 20 mmol/l Tris/2 mmol/l CaCl2 buffer at 37°C for 7.5 min followed by dehydration.

Thirty nanograms of a biotin-labeled P1 probe for the AR gene and 5 ng of a digoxigenin-labeled reference probe for the alphoid repeat of the X chromosome (DXZ1) were mixed with 10 μg of placent DNA and pipetted on denatured and proteinase potassium-treated cells on slides. Hybridization was carried out in a moist chamber for 24 h at 37°C. After hybridization, the slides were washed three times in 50% formamide/2× SSC (pH 7), twice in 2× SSC, once in 0.1× SSC at 45°C for 10 min each, and once in 4× SSC at room temperature for 5 min. Immunofluorescence detection of bound probes was done in three staining steps (20 min each) at room temperature. The biotin-labeled P1 probe was detected with avidin-FITC, and the digoxigenin-labeled DXZ1 probe was detected with anti-digoxigenin-rihodamine. The slides were counterstained with 1 μl 4,6-diamidino-2-phenylindole in a Vectashield-antifade solution (Vector Laboratories).

The Olympus BX60 (Olympus Corp., Tokyo, Japan) epifluorescence microscope was used for scoring signal copy numbers from a minimum of 100 nuclei/hybridization. The AR gene amplification was defined as previously explained (10, 11) and...
was based on the presence of individual tumor cells with tight clusters of AR signals, with more than five AR signals per cell or with a >2-fold higher number of signals with the AR probe than with DXZ1.

**Structural Analysis of the Human AR Gene.** All six tumors were screened for the presence of mutations in the AR gene by using standard SSCP analysis. To this purpose, genomic fragments containing exons B to H of the AR gene were amplified as described (14, 15). Standard PCR conditions were: 25 cycles of 1 min at 94°C, 2 min at 55°C or 60°C, and 2 min at 72°C in the presence of 1 μCi of [32P]dATP in a 15-μl reaction mix (Perkin-Elmer Thermal Cycler). One microliter of the reaction product was added to 9 μl of a solution containing 98% formamide, 10 mM EDTA (pH 8), and xylene cyanol and bromphenol blue as dye markers. After denaturation (5 min at 100°C), the solution was chilled on ice, and 1.5 μl was loaded onto a 6% nondenaturating polyacrylamide gel in 0.5× Tris-borate EDTA and 5 or 10% glycerol. Electrophoresis was done overnight at 7 W at room temperature. Subsequently, the gel was dried and exposed to X-ray film for 24–72 h at −80°C using intensifying screens. The sensitivity of the SSCP conditions used was estimated to be ~90%, as judged from the analysis of known point mutations in the AR in patients with androgen insensitivity syndrome. Nontumor DNA served as a control. Fragments showing an aberrant PCR-SSCP pattern on gel were cloned into the vector pCRII (Invitrogen, Palo Alto, CA) and sequenced using the standard dideoxy chain termination method.

**RESULTS**

**CGH.** CGH revealed only few genetic aberrations in the prostate carcinomas appearing during finasteride treatment. On average, there were 0.7 (range, 0–2) gains and 1.0 (range, 0–4) losses/tumor. One sample showed only chromosomal gains, whereas three samples showed both gains and losses. Three samples did not reveal aberrations at all (Table 1). The most frequently gained chromosome arms were Xq (29%) and 1p (29%), and the most often lost chromosome arm was 6q (29%).

**FISH.** To study the possible involvement of AR gene amplification in the appearance of prostate cancer during finasteride treatment, FISH analyses were performed on all carcinomas using a P1 probe (LCG-P1AR) for the AR gene and a probe specific for the pericentromeric repeat region of chromosome X (DXZ1/BamX7). Tumor 1 showed a low-level AR gene amplification (the 3-fold AR gene copy number relative to the DXZ1 copy number; Fig. 1). This tumor also revealed a gain at Xq11-q12 by CGH. Other samples had only the monosomic or diploid AR gene copy number.

**Structural Alterations of the AR Gene.** We also studied whether tumors showed mutations of the AR gene. Exons B to H of the AR gene were screened for aberrations in all six tumors using PCR-SSCP and sequencing. One aberrant SSCP pattern was observed in exon 5 (Fig. 2). Sequence analysis revealed a CGC-to-CTC mutation in codon 726, leading to an Arg-to-Leu substitution in the translated protein. The DNA extracted from the peripheral blood leukocytes of this patient contained the same mutation as the cancer cells. Seventy DNA samples from healthy blood donors were studied, and Arg726Leu mutation was not found among them.

**DISCUSSION**

The following findings suggest that prostate cancers arising in patients who have received finasteride may be different from those developing in hormonally intact men. First, tumors from finasteride-treated patients have relatively few genetic changes, and the pattern of changes may be somewhat different from that
described in untreated primary prostate cancer (7, 16). Second, alterations of the AR gene may often take place, and they may involve either copy number changes or structural alterations of the gene. Only one-half of the tumors arising during finasteride treatment showed genetic changes by CGH, with losses being more common than gains. The higher percentage of losses than gains is in agreement with previously published CGH studies of untreated primary prostate cancers (7–9, 16, 17). However, the overall number of alterations in these previous studies has been higher than in the present study, which may, in part, be caused by the fact that prostate carcinomas here are of earlier clinical stage and better histological differentiation than untreated primary prostate carcinomas in most of the previous studies (7, 16). For example, Visakorpi et al. (7) found genetic aberrations in 74% of 31 untreated primary prostate cancers and reported, on average, 0.5 gains and 2.4 losses/tumor.

Despite the small number of cases, which makes definitive frequency comparisons difficult, there was a tendency for genetic changes seen by CGH in finasteride-treated tumors to be qualitatively different from those seen in untreated primary prostate cancers. The most commonly gained chromosomal regions in the present material were Xq and 1p, whereas 6q was the site of the most common losses. Whereas 8p losses and 8q gains are frequently seen as the most common genetic alterations in prostate cancer by CGH (7, 9, 16, 17), only one tumor in our study showed gain at 8q24. A smaller than expected frequency of deletions was also found at 13q and 16q (7, 9, 16, 17).

Because the AR protein is a key mediator of growth signaling in the prostate, much research activity has focused on the involvement of the AR gene in prostate cancer development (18). These alterations may affect both the copy number and the structure of the AR gene. The AR gene may undergo amplification during prostate cancer progression during hormonal therapy (10). Two of the tumors from finasteride-treated patients revealed a gain at Xq11–q12, the site of the AR gene, and one of these showed a 3-fold amplification of the AR gene by FISH. This is interesting in the light of the previous results, which suggest that AR gene amplification only arises in tumors from men who have received androgen deprivation therapy for prostate cancer and that the increased copy number of the AR gene also leads to increased mRNA expression (11). The finding of an increased copy number of AR genes in one of these finasteride-treated patients suggests that a decrease of androgen availability induced by 5α-reductase blockade is also capable of leading to a clonal selection of AR gene amplification.

Mutations of the AR gene have been studied by several groups in primary prostate cancers and in hormonally treated prostate cancers, but only a few mutations have been found (18), with the exception of distant metastases from hormonally treated patients (19). We found one tumor from a finasteride-treated patient with a mutation in the steroid binding domain at codon 726 leading to an Arg-to-Leu substitution. This mutation was also present in the germ-line DNA of this patient. We also studied DNA samples from 70 healthy blood donors, and Arg726Leu mutation was not found. This suggests that the Arg726Leu mutation is not a polymorphism without clinical significance. The Arg726Leu germ-line mutation has previously been reported by Elo et al. (20) who found it in 1 of 29 Finnish prostate carcinoma cases. To the best of our knowledge, the patient with Arg726Leu germ-line mutation in the study by Elo et al. (20) is not related to our patient, which raises the question about the role of Arg726Leu germ-line mutation in the etiology of prostate cancer among Finnish and other populations. Moreover, Elo et al. (20) showed that Arg726Leu mutation in the AR gene also leads to activation of the AR by low (10 nM) estradiol concentration. It is known that finasteride treatment leads to elevated serum concentrations of estradiol (21), and therefore, it is tempting to speculate that the Arg726Leu mutation may provide a growth advantage for prostate cancers appearing during finasteride treatment.

Taken together, we have shown here by CGH that prostate cancers appearing during finasteride treatment have only few genetic alterations and that, in some cases, alterations in the AR gene may be involved in the adaptation of the DHT-deficient milieu. Further studies are also needed to demonstrate the importance of Arg726Leu germ-line mutation of the AR gene in the etiology of prostate cancer and especially finasteride-associated prostate cancer among Finnish and other populations.

REFERENCES


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