Androgen Receptor Gene Amplification: A Possible Molecular Mechanism for Androgen Deprivation Therapy Failure in Prostate Cancer

Pasi Koivisto, Juha Kononen, Christian Palmberg, Teuvo Tammela, Eija Hyytinen, Jorma Isola, Jan Trapman, Kitty Cleutjens, Arjan Noordzij, Tapio Visakorpi, and Olli-P. Kallioniemi

Laboratory of Cancer Genetics, Institute of Medical Technology, University of Tampere [P. K., J. K., E. H., J. I., T. V., O-P. K.] and Division of Urology [C. P., T. T.], Tampere University Hospital [P. K., J. K., E. H., J. I., T. V., O-P. K.], 33521 Tampere. Finland. Departments of Pathology [J. T., K. C.] and Urology [A. N.]. Erasmus University. Rotterdam, the Netherlands; and Laboratory of Cancer Genetics, National Center for Human Genome Research, NIH, Bethesda, Maryland 20892-4470 [T. V., O-P. K.]

ABSTRACT

Progression of prostate cancer during endocrine therapy is a major clinical problem, the molecular mechanisms of which remain poorly understood. Amplification of the androgen receptor (AR) gene was recently described in recurrent prostate carcinomas from patients who had failed androgen deprivation therapy. To evaluate the hypothesis that amplification of the AR gene is a cause for the failure of androgen deprivation therapy in prostate cancer, we studied whether AR amplification leads to gene overexpression, whether the amplified AR gene is structurally intact, and whether tumors with AR amplification have distinct biological and clinical characteristics. Tumor specimens were collected from 54 prostate cancer patients at the time of a local recurrence following therapy failure. In 26 cases, paired primary tumor specimens from the same patients prior to therapy were also available. Fifteen (28%) of the recurrent therapy-resistant tumors, but none of the untreated primary tumors, contained AR gene amplification as determined by fluorescence in situ hybridization. According to single-stranded conformation polymorphism analysis, the AR gene was wild type in all but one of the 13 AR amplified cases studied. In one tumor, a presumed mutation in the hormone-binding domain at codon 674 leading to a Gly—→Ala substitution was found, but functional studies indicated that this mutation did not change the transactivation properties of the receptor. AR amplification was associated with a substantially increased level of mRNA expression of the gene by in situ hybridization. Clinico pathological correlations indicated that AR amplification was most likely to occur in tumors that had initially responded well to endocrine therapy and whose response duration was more than 12 months. Tumors that recurred earlier or those that showed no initial response did not contain AR amplification. The median survival time after recurrence was two times longer for patients with AR amplification in comparison to those without amplification (P = 0.03, Wilcoxon-Breslow test). In conclusion, failure of conventional androgen deprivation therapy in prostate cancer may be caused by a clonal expansion of tumor cells that are able to continue androgen-dependent growth despite the low concentrations of serum androgens. Amplification and the increased expression of a wild-type AR gene may play a key role in this process.

INTRODUCTION

Prostate cancer is the most common malignancy in men in the United States and Western Europe. The proportion of operable early stage (stages A and B) prostate cancers has increased rapidly in recent years as a result of the widespread use of serum prostate-specific antigen measurements to screen for cancer in asymptomatic men (1). However, many prostate cancers are still diagnosed at stage C or D, when cure by radical surgery is not possible. Patients with advanced disease are usually treated by endocrine therapy, either by classical androgen deprivation (orchietomy or luteinizing hormone-releasing hormone agonists) or by maximal androgen blockade (castration combined with antiandrogens). About 70—80% of the patients respond favorably and disease palliation is achieved for several months or years. Eventually, however, the disease progresses despite the therapy (2).

Molecular mechanisms of recurrence and endocrine therapy failure in prostate cancer have remained unclear. Many investigators have studied whether mutations in the AR3 gene would explain therapy resistance. Mutations leading to activation of AR by other steroids, and paradoxically also by antiandrogens, have been found, but their prevalence in primary and locally recurrent prostate cancer is low and clinical significance unknown (3—8). However, Taplin et al. (9) recently reported AR mutations in 5 of 10 distant metastases from patients who had failed endocrine therapy and suggested that AR mutations would play a major role in the metastatic progression of prostate cancer.

Another molecular aberration involving the AR gene may also underlie endocrine therapy failure. In our recent study (10), amplification of the AR gene was found in about 30% of locally recurrent hormone-refractory prostate cancers. In vitro studies of cancer cell lines have revealed amplifications of specific genes associated with the development of drug resistance to cancer chemotherapeutic agents and metabolic inhibitors (11, 12). This prompted us to suggest that AR gene amplification in recurrent prostate cancer would represent an analogous in vivo mechanism responsible for failure of androgen deprivation therapy. AR gene amplification, by leading to increased expression of the AR gene, could allow the cancer cells to continue androgen-dependent growth, although the serum androgen level is drastically reduced after castration (10, 13).

Here, we sought to evaluate this hypothesis by identifying biological and clinical characteristics of prostate carcinomas that contain AR gene amplification as well as by studying whether amplification leads to gene overexpression. Furthermore, because of the proposed role of AR mutations in endocrine therapy failure, we evaluated whether the amplified AR gene is structurally intact.

MATERIALS AND METHODS

Patients and Tumor Specimens. The material consisted of patients with advanced prostate cancer who were treated at the Tampere University Hospital in Finland (n = 51) or at the Dijkzigt Hospital in Rotterdam, the Netherlands (n = 3) during 1972—1995. Conventional androgen deprivation therapy consisting of either orchietomy (37 cases), luteinizing hormone-releasing hormone analogue (6), estrogen (6), or orchietomy and estrogen (5) was the primary treatment for these patients. All patients experienced a local tumor recurrence, as evidenced by symptoms of urethral obstruction and an increase of serum prostate specific antigen (before 1991) or serum prostate-specific antigen measurements to screen for cancer in men (1).
antigen (PSA) levels (after 1991). TNM stage and histological grade distribution of the tumors at the time of diagnosis are summarized in Table 1. The response of the patients to primary endocrine therapy was classified as complete response, partial response, or stable disease according to the clinical criteria of Murphy et al. (14) or the change in serum PSA levels. Paired primary tumor specimens taken before administration of any therapy were available from 26 patients. Formalin-fixed, paraffin-embedded tumor specimens were used in all molecular analyses. These specimens had been collected either by Tru-Cut needle biopsy (17 primary, 12 recurrent tumors) or by transurethral resection (9 primary, 42 recurrent tumors).

**Analysis of Gene Amplification by Interphase FISH.** The most representative paraffin-embedded blocks containing more than 70–80% malignant cells were selected for interphase FISH analysis. Many blocks were trimmed by removing surrounding hyperplastic tissue. FISH analysis of AR gene amplification was done from dissociated nuclei obtained by a proteinase K treatment from 100-μm sections of the tumors (10). The nuclei were deposited on Vectabond-treated (Vector Laboratories, Burlingame, CA) slides and pre-treated by heating in a 50% glycerol/0.1× SSC (0.15 m NaCl and 0.015 m sodium citrate) solution for 3 min at 90°C (15). Thirty ng of a biotin-labeled P1 probe for the AR gene and 5 ng of digoxigenin-labeled reference probe for the alphoid repeat of the X chromosome (DXZ1) were mixed with 10 μg of placental DNA and pipetted on denatured and proteinase K-treated cells on slides. Hybridization was carried out in a moist chamber for 24 h at 37°C. After hybridization, the slides were washed and the biotin-labeled AR probe was detected with avidin-FITC and the digoxigenin-labeled DXZ1 probe with anti-digoxigenin-rhodamine. The slides were counterstained with 4',6-diamidino-2-phenylindole in an antifade solution.

Either a Nikon SA (Nikon Corporation, Tokyo, Japan) or an Olympus BX60 (Olympus Corporation, Tokyo, Japan) epifluorescence microscope was used for scoring signal copy numbers of both probes from a minimum of 70 nuclei per hybridization. Both microscopes were equipped with multiband pass filters as well as both ×60 and ×100 objectives. AR amplification was considered to be present when the average number of AR signals was more than five per cell or when the copy number of the AR gene was at least 2-fold higher than that of the DXZ1. In practice, amplification was easily recognizable based on the finding of individual tumor cells with very high numbers of AR signals, usually arranged in tight clusters (10). Aneuploidy or polysomy for chromosome X were defined as the presence of an equally elevated copy number for both AR and the DXZ1 reference probe and could thereby be clearly distinguished from gene amplification.

**Analysis of Gene Expression by mRNA in Situ Hybridization.** Seven-μm sections were cut from the paraffin blocks representing both primary and recurrent tumors from six patients with AR amplification. Sections were placed on SuperFrost (Menzel-Gläser, Germany) slides, deparaffinized, and subjected to mRNA in situ hybridization as described (16). An oligonucleotide probe against the ligand-binding domain (nucleotides 810–854) of human AR (GenBank no. M34233; Ref. 17) was used. The probe was labeled with 33PdATP in a 15-μl reaction mix (Perkin-Elmer Thermal Cycler). One μl 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× TBE 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 to 72 h at −80°C using intensifying screens. The sensitivity of the SSCP conditions used was estimated to be approximately 90%, as judged from the analysis of known point mutations in the AR in patients with androgen insensitivity syndrome. Nontumor DNA served as control. Fragments showing an aberrant PCR-SSCP pattern on gel were cloned into the vector pCR II (Invitrogen, Palo Alto, CA) and sequenced using the standard dideoxy chain termination method.

**Transfections.** To assay the transactivating function of mutated AR, the mutation was introduced into AR cDNA in the AR expression vector pSVAR (19) utilizing standard PCR technology with mutated primers. PCR fragments used for construction of the expression vector containing the mutated AR cDNA were completely sequenced. Functionality of the AR was analyzed in Hep3B cells, which were cotransfected with wild-type or mutant pSVAR, and the reporter vector GRE-pLuc with the calcium phosphate precipitation method. Following transfection, cells were incubated for 24 h in the absence or in the presence of the synthetic androgen R1881 (10−9 M) or the anti-androgen OH-flutamide (10−9 M). Cell lysates were prepared, and luciferase activity was measured as described (20). All experiments were done in 4-fold, and corrections were made for protein content of the samples.

**Statistical Analyses.** BMDP Statistical Software Package (21) was used in all statistical analyses. Clinicopathological correlations were analyzed using the Pearson’s χ2 test (BMDP4F program). The statistical significance of survival differences between patient groups was determined with Mantel-Cox and Breslow tests (BMDP1L).

**RESULTS**

**Prevalence of AR Amplification.** According to interphase FISH analysis, 15 of the 54 (28%) recurrent prostate cancers showed specific amplification of the AR gene using FISH. The mean AR copy number in these tumors ranged from 2.7 to 28/cell, with individual cells showing much higher copy numbers, ranging from 5 to more than 60 AR copies/cell in the various tumors. Aneuploidy or polysomy for chromosome X, seen in 10 tumors (19%), was distinguished from AR gene amplification based on the finding of an equally high copy number for both the AR and the DXZ1 probe. The remaining 29 recurrent tumors (53%) had only a single copy of AR and DXZ1. AR amplification was detected both in tumors from Finnish and Dutch patients.

AR gene amplification was not detected in any of the 26 primary tumors. Between 1000 and 1500 nuclei/specimen were screened from 10 primary tumors available from those patients whose tumors had AR gene amplification at the time of recurrence. No
evidence of cells with unambiguous gene amplification was found even in this extensive analysis. Occasional doublet signals that were observed are likely to reflect the presence of the S-phase and G2-M cells.

Clinicopathological Characteristics and Predictive Value of AR Amplification. AR gene amplification was not significantly associated with the age of the patient, tumor size, presence of distant metastases, nor with the tumor grade evaluated at the time of diagnosis (Table 1). In contrast, the appearance of AR amplification was associated with both the degree and duration of response to primary endocrine therapy (Fig. 1). Recurrent tumors from patients who had not initially responded to androgen deprivation did not show AR gene amplification (Fig. 1A). In contrast, two of the three patients with complete response and one third of those with a partial response to primary endocrine therapy had AR-amplified recurrent tumors. AR gene amplifications were not found in any of the 11 recurrent specimens from patients who progressed less than 1 year after initiation of androgen deprivation therapy (Fig. 1B). In contrast, AR amplification was found in 30–38% of the cases whose response duration was more than 1 year.

Patients with AR gene amplification had a more favorable overall survival than those with no amplification (Fig. 2A). Since the AR gene is only amplified at the time of recurrence, we also evaluated the median postrecurrence survival of the patients which was almost two times longer in the AR-amplified cases as compared with the nonamplified ones (Fig. 2B). The survival differences were most prominent during the first 48 months of follow-up.

AR Amplification Leads to Increased mRNA Expression. According to mRNA in situ hybridization, the AR gene was highly expressed when the gene was amplified (Fig. 3). Based on a semi-quantitative analysis of six cases, a wide range of expression levels was seen in both primary and recurrent tumors. However, gene expression was systematically higher in the recurrent AR-amplified tumors than in the corresponding primary unamplified tumors (Table 2).

Amplification and Mutations of the AR Gene. With one exception, the coding region of the AR gene was not structurally altered in any of the 13 tumors with AR amplification studied using PCR-SSCP and sequencing. In the only structural aberration detected, codon 674 (GGT) in exon 4 was changed into GCT, leading to Gly → Ala substitution. Although normal control DNA from the same patient was not available, an Ala at position 674 has never been found in any of many hundreds of DNA samples analyzed, arguing against a polymorphism. The mutation was introduced into AR cDNA and tested for functionality. Comparison of wild-type and mutated AR-transactivating activity in Hep3B cells did not reveal any difference (data not shown).

DISCUSSION

The present results emphasize the close association between AR gene amplification and failure of androgen deprivation therapy in prostate cancer as originally reported by us in a smaller series (10). Several new findings that emerged from the present study substantiate the hypothesis that AR amplification is directly contributing to the development therapy failure by allowing cells to resume hormone-dependent growth in the low concentrations of androgens. First, amplification occurs exclusively in the recurrent tumors from patients whose disease has progressed during the therapy. Not even individual amplified cells were found in the untreated primary tumors, many of which came from patients with late-stage metastatic prostate carcinoma. Thus, AR amplification is not involved in the genesis of
A. Overall survival (from diagnosis to death). The median survival for AR-amplified cases is 135 months in comparison to 70 months in the nonamplified cases. B. Survival after recurrence. The median post-recurrence survival for AR-amplified cases is 55 months in comparison to 24 months in the nonamplified cases.

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Fig. 2. Survival of the prostate cancer patients according to the AR gene amplification. A, overall survival (from diagnosis to death). The median survival for AR-amplified cases is 135 months in comparison to 70 months in the nonamplified cases. B, survival after recurrence. The median post-recurrence survival for AR-amplified cases is 55 months in comparison to 24 months in the nonamplified cases.

Although it does not exclude the fact that there may be other important genes in the same amplicon. Results from the mRNA in situ hybridization are compatible with those from previous immunohistochemical studies (7, 22) in that the AR gene is virtually always highly expressed in both primary and recurrent prostate cancer. However, immunohistochemical studies are less quantitative than the mRNA in situ hybridization used here, and detection of the increased level of expression in this subgroup of recurrent tumors has previously not been reported.

Fourth, the amplified AR is usually not mutated and has thereby retained specificity for androgens. Although 1 of the 15 AR-amplified cases showed a mutation in the steroid-binding domain, this had no influence on the transactivation properties of the receptor, suggesting that this mutation did not lead to impaired steroid specificity. The finding of predominantly wild-type AR gene in the amplified tumors supports the concept that recurrent AR-amplified tumors may still be androgen dependent. Our results on AR mutations are compatible with previous studies of both primary and locally recurrent prostate cancers that have shown a very low frequency of AR mutations (3–5, 7–8). A recent study indicated that micrometastases of prostate cancer may much more often contain AR gene mutations (9).

Fifth, prostate cancer is known to be an extraordinarily hormone-dependent tumor type. Since the AR protein functions as a key mediator of androgen-dependent growth, amplification and consequent increased expression of such a receptor gene is a logical mechanism by which the growth of tumor cells could be maintained when this critical ligand for the receptor is present at a very low concentration. There are more than 20 analogous in vitro examples of specific gene amplifications that have been found when cultured tumor cells have been deprived of an essential agent for growth or treated by chemotherapeutic drugs (12). For example, DHFR gene amplification is selected for during methotrexate treatment in response to deprivation of folic acid in vitro (11) and the recent report by Goker et al. (23) suggests that a similar mechanism may be operational in therapy-resistant leukemic cells in vivo. AR amplification was never seen in the tens of thousands of cells screened from many different untreated tumors. Furthermore, close to 100% of the cells in the recurrent tumors usually had AR amplification. These findings, taken together with the fact that a long period of therapy (12 months) was required before AR amplification emerged, all resemble the situation in the previous in vitro studies where a strong selection force has been applied on the cultured cells to induce a stepwise selection for gene amplification over many cell generations (24, 25).

Patients whose tumors contained AR amplification at the time of recurrence had a somewhat more favorable prognosis than those whose tumors were not amplified. It is unexpected that DNA amplification, usually associated with genetically highly unstable (26–28) and aggressive tumors (29–30), is showing an association with favorable prognosis. One can speculate that the AR-amplified tumor cells, although being genetically unstable, are still subject to a rigorously regulated hormone-dependent growth and therefore show a lower level of malignant potential. Based on this material, however, it is difficult to separate the effects of a favorable response to androgen deprivation and survival from one another and, thus, the prognostic findings should be confirmed in a prospective setting.

The discovery of AR gene amplification and the emerging understanding of the molecular mechanisms of therapy failure may in the future translate into new therapy options for patients with prostate cancer. The results illustrate the fact that recurrent hormone-refractory tumors may not always be androgen independent as often thought (31). These cells may in fact be highly dependent on the residual low levels of circulating androgens that originate from the adrenals and are...
Table 2 Comparison of AR copy numbers by interphase FISH analysis and AR expression levels by mRNA in situ hybridization in six paired specimens from patients whose tumors were amplified for AR at the time of recurrence

<table>
<thead>
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<th>Patient</th>
<th>Primary tumor</th>
<th>Recurrent tumor</th>
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<tr>
<td></td>
<td>Copy no. by FISH</td>
<td>Expression by mRNA ISH</td>
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<tr>
<td>1</td>
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<td>6</td>
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*Average number of AR copies per cell (corrected for the number of copies of reference probe).
*Expression by mRNA in situ hybridization (ISH) were semiquantitatively scored to have either low (+), moderate (++), or high (+++) hybridization intensity.
*N.S., no sample available for study.

Fig. 3. Androgen receptor gene copy number analysis using FISH and mRNA in situ hybridization of a tissue sections from an untreated primary tumor and a locally recurrent tumor after failure of androgen deprivation therapy. The specimens came from the same patient. A, interphase FISH analysis of nuclei from the primary tumor with probes for AR (green) and DXZ1 (red). Only one copy of both AR and DXZ1 is seen. B, interphase FISH analysis of nuclei from the recurrent tumor demonstrating high level AR amplification. C, mRNA in situ hybridization analysis of an entire tissue section from the untreated primary tumor shows moderate expression level of the AR gene; D, mRNA in situ hybridization of the locally recurrent, therapy-resistant tumor shows highly elevated AR expression. The intensity of mRNA in situ hybridization is expressed in pseudocolors.

converted to bioactive dihydrotestosterone in the prostatic tissue by 5α-reductase (32). The critical question for additional studies is whether more complete removal of these androgens could improve survival in the subgroup of patients with AR-amplified tumors. It should be emphasized that all of the patients evaluated here received conventional androgen deprivation and were mostly treated in the 1980s. Studies are now required to assess whether MAB allows for the eradication of tumors that have recurred after androgen deprivation as well as whether AR amplification also arises if the patients have received MAB as the first-line hormonal therapy. We have thus far documented one patient whose tumor recurred with AR amplification and was subsequently treated with MAB. An excellent initial treatment response was obtained, but the relapse was short-lived (33). Such short-term responses have previously been documented in about 20–35% of unselected patients who have received MAB therapy after castration has failed (34, 35). AR amplification could turn out to be a useful marker to identify patients who are most likely to benefit from MAB as a second-line therapy. Finally, the results from the retrospective studies reported here warrant the incorporation of AR copy number analysis in prospective studies of hormonal therapy in prostate cancer. We have now gained extensive experience on such analyses and strongly recommend the use of the highly sensitive inter-
phase FISH technique for this purpose. Southern and slot blot studies are complicated by the inadequate specificity of most AR cDNA probes and the poor histological representativeness of many specimens obtained by biopsy or transurethral resection.

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