Amplification and Overexpression of Androgen Receptor Gene in Hormone-Refractory Prostate Cancer

Marika J. Linja, Kimmo J. Savinainen, Outi R. Saramäki, Teuvo L. J. Tammela, Robert L. Vessella, and Tapio Visakorpi

Laboratory of Cancer Genetics, Institute of Medical Technology, M. J. L., K. J. S., O. R. S., T. V. and Department of Urology, T. L. J., University of Tampere and Tampere University Hospital, FIN-33014 Tampere, Finland, and Department of Urology, University of Washington, Seattle, Washington 98195 (R. L. V.).

Abstract

The expression level of the androgen receptor (AR) gene in androgen-dependent and -independent prostate cancer was determined by using real-time quantitative reverse transcription-PCR assay. Eight benign prostate hyperplasias, 33 untreated and 13 hormone-refractory locally recurrent carcinomas, as well as 10 prostate cancer xenografts, were analyzed. All hormone-refractory tumors expressed AR and showed, on average, 6-fold higher expression than androgen-dependent tumors or benign prostate hyperplasias (P < 0.001). Four of 13 (31%) hormone-refractory tumors contained AR gene amplification detected by fluorescence in situ hybridization. Androgen-independent tumors with gene amplification expressed, on average, a 2-fold higher level of AR than the refractory tumors without the gene amplification. Two xenografts (LuCaP 35 and 69) showed amplification and high-level expression of the AR gene. These xenografts are the first prostate cancer model systems containing the gene amplification. The findings demonstrate that AR is highly expressed in androgen-independent prostate cancer, suggesting that the AR signaling pathway is important in the progression of prostate cancer during endocrine treatment. The two xenografts with the AR gene amplification will enable studies evaluating the functional significance of the amplification and development of new treatment strategies based on high-level expression of AR.

Introduction

Prostate carcinoma is the most common male malignancy in many industrialized countries. Despite the widespread use of serum PSA measurements to detect cancer in asymptomatic men, approximately 30–40% of patients are diagnosed with extracapsular disease (1). For such advanced disease, androgen deprivation remains the only effective treatment strategy (2). Almost all prostate carcinomas are originally androgen-dependent (3). However, during the hormonal therapy, androgen-independent tumor cells eventually emerge, leading to clinical relapse. There are no effective treatments available for such hormone-refractory tumors (2).

Previously, we and others have reported that approximately one-third of prostate carcinomas recurring during androgen deprivation therapy contain an AR gene amplification (4–7). Amplification is not found in any untreated prostate tumors, suggesting that the gene amplification is likely to be involved in the failure of the hormonal treatment.

Gene amplification is believed to lead to overexpression of the target gene of amplification (8). Thus, it has been suggested that amplification of the AR gene could cause overexpression, allowing the cancer cells to continue androgen-dependent growth even in very low levels of androgens left in serum after castration (4, 9). Consistent with this hypothesis, up-regulation of AR levels and its transcriptional activity have been observed in LNCaP cells cultured for about 60 passages in androgen-depleted medium (10). In addition, a preliminary clinical study showed that patients with AR gene amplification responded better to the so-called MAB, which abolishes the effects of adrenal androgens, than patients without the amplification (11).

Our preliminary data have, indeed, suggested that the AR amplification leads to overexpression of the gene in hormone-refractory prostate tumors (5). However, this was found by using poorly quantitative mRNA in situ hybridization technique in a small number of tumors. To study further the role of the AR gene in endocrine treatment failure in prostate cancers, we have here quantified the expression of AR and PSA genes in 54 prostate tumors and 10 prostate cancer xenografts using a real-time RT-PCR system (Light Cycler). The AR gene copy number was determined by FISH. The association of gene expression with gene copy number and tumor type was analyzed.

Materials and Methods

Tumor and Xenograft Samples. Freshly frozen specimens from 8 BPHs, 33 androgen-dependent untreated primary prostate carcinomas (32 prostatectomy specimens and 1 TURP specimen), and 13 locally recurrent hormone refractory prostate carcinomas (TURP specimens) were obtained from the Tampere University Hospital (Tampere, Finland). Of the untreated primary carcinomas, 12 were grade I, 15 were grade II, and 6 were grade III, according to the WHO classification (12). The Tumor-Node-Metastasis distribution of the cases was pT1, 2; pT2, 15; pT3, 13; pT4, 2; pTX, 1; pN0, 30; pN+, 1; pNX, 2; M0, 32; and M+, 1. The hormone-refractory tumor specimens were obtained from patients experiencing urethral obstruction during the hormonal monotherapy. The endocrine therapy modalities were either orchietomy (four cases), luteinizing hormone-releasing hormone analogue (four cases), estrogen (one case), MAB (three cases), and unknown (one case). The time from the beginning of the therapy to TURP varied from 16 to 60 months. The specimens were histologically examined for the presence of tumor tissue using H&E-stained slides. Only samples containing more than 60% of cancerous or hyperplastic tissue were selected for the analyses. The fraction of carcinomas cells in the androgen-dependent and hormone-refractory tumors was, on average, equal. In addition, freshly frozen samples from 10 prostate cancer xenografts (LuCaP 23.1, 23.8, 23.12, 35, 41, 49, 58, 69, 70, and 73) were analyzed. All xenografts, except LuCaP 49 and LuCaP 58, have been established from hormone-refractory human prostate carcinomas and propagated in intact male mice.

RT-PCR. One to three 20-μm frozen sections were cut using a cryotome. Total RNAs were isolated from the sections using Qiagen RNeasy Mini Kit (Qiagen, Inc., Valencia, CA), and used for the first-strand cDNA synthesis with Superscript II reverse transcriptase and oligo d(T)12–18 primer according to the manufacturer’s protocol (Life Technologies, Inc., Gaithersburg, MD).
For preparing the standard curve, total RNA from prostate cancer cell line LNCaP (American Type Culture Collection, Manassas, VA), cultured under recommended conditions, was isolated using Trizol reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. After the first strand cDNA synthesis, serial dilutions were made corresponding to about 500, 100, 20, 4, and 0.8 ng of cDNA.

Primers for the AR, PSA, and TBP genes were designed with the assistance of the Primer3 program. To avoid amplification of any genomic DNA, the forward and reverse primers for each gene were chosen from different exons. The sizes of PCR products were designed to be under 400 bp to optimize the RT-PCR measurements. Primer and probe sequences for the genes are given in Table 1.

The PCR reactions were performed in the LightCycler apparatus (13) using the LC DNA Hybridization Probes Kit (Roche Diagnostics, Mannheim, Germany). Thermocycling for each reaction was done in a final volume of 20 μl containing 2 μl of cDNA sample (or standard), 4 mM MgCl₂, 0.5 μM of each primer, 0.2 μM both fluorescein- and LC Red 640-labeled probes (0.4 μM LC Red 640 probe in TBP reactions) as well as 1X ready-to-use reaction mix including Taq DNA polymerase, reaction buffer, and deoxynucleotide triphosphate mix. After 10 s of initial denaturation at 95°C, the cycling conditions of 45 cycles consisted of denaturation at 95°C for 1 s, annealing at 55°C (for AR and PSA) or 58°C (for TBP) for 10 s, and elongation at 72°C for 10 (AR and TBP) or 13 (PSA) s. The LightCycler apparatus measured the fluorescence of each sample in every cycle at the end of the annealing step. After the PCR reaction, the software program plotted logarithmic values of fluorescence against cycle number. After proportional background adjustment, the fit-point method was used to determine the cycle in which the log-linear signal was distinguished from the background, and that cycle number was used as the crossing-point value. The software produced the standard curve by measuring the crossing point of each standard and plotting them against the logarithmic values of concentrations. The concentrations of unknown samples were then calculated by setting their crossing points to the standard curve. The expression values of concentrations. The concentrations of unknown samples were then calculated by setting their crossing points to the standard curve. The expression values of AR and PSA were normalized by the housekeeping gene TBP value and multiplying by 100.

The expression level of AR and PSA were normalized by the housekeeping gene TBP value and multiplying by 100.

Statistical Analyses. The association of the gene copy number and tumor type with the expression level was calculated with the nonparametric Kruskal-Wallis test.

Results

**AR Gene Amplification.** In FISH analyses, 4 of 13 (31%) hormone-refractory prostate cancers showed amplification of the AR gene. As expected, AR gene amplification was not detected in any of the 33 primary tumors or 8 BPHs studied. High-level amplification of the AR gene was found in two xenografts (LuCaP 35 and 69; Fig. 1). In four other xenografts (LuCaP 23.1, 23.8, 23.12, and 70); two copies of the chromosome X centromere, as well as AR, were found. In the remaining four xenografts, one copy of AR and the centromere was detected.

**Expression of AR Gene.** Fig. 2 shows a standard curve of the real-time RT-PCR measurement of AR. The dynamic range was wide, from 0.8 to 500 ng of equivalent total cDNA. The linear relationship between the fractional cycle and the fluorescent threshold was strong ($r^2 = -1$). In addition to TBP, the expression of β-actin was measured and used alternatively for normalization in most of the samples (data not shown). The results were similar with both control genes. Because of the potential problems with the β-actin retropeudogenes, TBP was chosen for normalization in the whole material. Fig. 3A illustrates the relative expression of AR in BPH as well as in primary and hormone-refractory prostate cancers. All refractory tumors expressed the AR. The median expression was 6-fold higher in hormone-refractory tumors than in primary tumors or BPHs ($P < 0.001$). The median value of AR expression in the hormone-refractory tumors with the gene amplification was >2 times higher than in the refractory tumors without the amplification. However, the difference was not statistically significant ($P = 0.057$). The tumor with the highest expression also showed the gene amplification. In the xenografts, there was a clear tendency that the expression of AR increased with the gene copy number (Fig. 4A). The LuCaP 49 xenograft did not show expression at all. In addition, clearly the highest expression of AR was observed in the xenograft LuCaP 69, which contained the highest level of AR amplification.

**Expression of PSA.** The expression of PSA was about the same in all tumor types ($P = 0.055$), as shown in Fig. 3B. Neither was there any statistical significance of association between the expression of

---

Table 1. The primer and probe sequences used in the real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5'-3')</th>
<th>Hybridization probe sequences (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR</td>
<td>CTACCAACGTCCTGAGACTC</td>
<td>GCCGACGAGTGACATCTGGAA</td>
</tr>
<tr>
<td></td>
<td>CAGGCAAGAAGGTCTGGCAAG</td>
<td>TCCACACCCAGTGTTGGAGAA-fluorescein</td>
</tr>
<tr>
<td></td>
<td>GACGATGAGAAAGCTTGAGCT</td>
<td>Red640-CTATTCTCCCCACCCAGACCTAGT</td>
</tr>
<tr>
<td>PSA</td>
<td>GAACAAGAGAAGCTTGAGAT</td>
<td>GCCGGAAGAGGTCTGGCAAG</td>
</tr>
<tr>
<td></td>
<td>AACAGTGGGAGGCTTGAGAT</td>
<td>GGTGCGTACCGGGATGGAAGTC-fluorescein</td>
</tr>
<tr>
<td></td>
<td>AACAGTGGGAGGCTTGAGAT</td>
<td>Red640-ACACATGCTGGCACCACCCCTG</td>
</tr>
<tr>
<td>TBP</td>
<td>GAATATAATCCCAAGCGGTTTG</td>
<td>TCTCCAGAAGCTGAAATACAGTGGCC-fluorescein</td>
</tr>
<tr>
<td></td>
<td>ACTCCTACATCACAGCTCCC</td>
<td>Red640-TGGTTCGCGTCCTATATCTCAGT</td>
</tr>
</tbody>
</table>

---

PSA and AR copy number in the hormone-refractory tumors. LuCaP 49 did not express PSA at all, whereas LuCaP 23.1, 23.8, and 23.12 expressed about 10 times more PSA than the other xenografts (Fig. 4B).

Discussion

The emergence of hormone-refractory prostate cancer is a serious clinical problem. Thus, it is not surprising that mechanisms underlying the transition of prostate tumors from the androgen-dependent to the -independent phase have been studied extensively (16). During recent years, evidence has accumulated suggesting that the AR signaling pathway plays a central role in the progression of prostate cancer during hormonal therapy. Immunohistochemical studies have indicated that AR is expressed in both hormone-naive and -refractory tumors (17–21). However, there has been controversial data on the level of expression in these different forms of carcinoma. Some publications, based on either immunohistochemistry, mRNA in situ hybridization, or RT-PCR, have suggested that the expression of AR is high in hormone-refractory tumors (4, 5, 22). Others have suggested heterogeneous or decreased expression of AR in a subset of the hormone-refractory tumors (17, 20, 23). The main shortcoming of all these studies has been that the methodologies used for the measurement of expression have been semiquantitative at best. Here, our strategy was to use the new real-time RT-PCR approach for measuring AR expression. In real-time PCR, the quantification of the template is based on detection of the cycle in which the reaction enters the exponential phase, instead of on measuring the amount of end product. Thus, none of the reagents are rate-limiting in the reaction at the time of measurement of the fluorescence. Several studies have already shown that the real-time RT-PCR is a highly quantitative and reliable method (14, 24–26).

We showed that locally recurrent, hormone-refractory prostate tumors expressed significantly more AR than BPHs or hormone-naive prostate carcinomas. In addition, we did not find any hormone-refractory tumor with low-level expression. All of the tumors also produced the AR protein, as detected by immunohistochemistry (data not shown). The results are somewhat in disagreement with recent data suggesting that a fraction (10–15%) of hormone-refractory metastatic lesions express only low levels of AR because of hypermethylation of the promoter CpG island (23). The discrepancy could well be attributable to the fact that only locally recurrent tumors, rather than metastases, were analyzed here, or that the number of tumors analyzed was just too low to detect such a small subgroup.

Four of 13 (31%) hormone-refractory tumors contained AR gene amplification, the frequency of the amplification being in accordance with previously published data (4–7). The expression of AR seemed...
to be higher in the tumors with the amplification than in those without the amplification, but the difference was not statistically significant. The tumor clearly showing the highest expression of AR also contained the gene amplification. Still, the amplification explained only a part of the high expression level in the androgen-independent tumors. It seems that other mechanisms than amplification, leading to over-expression of the AR, must also be involved. Altogether, the high level expression of AR in the hormone-refractory tumors underlies the importance of the AR signaling pathway in endocrine therapy failure.

To analyze the functionality of the AR signaling pathway in the hormone-refractory tumors, we measured the expression of PSA, which is androgen-regulated (27). PSA was clearly expressed in the hormone-refractory tumors, suggesting that AR is functional. The level of mRNA expression was about the same in BPH and hormone-naïve and -refractory tumors. It has earlier been shown that PSA protein expression is actually higher in BPH than in primary tumors (28). There was no association between AR amplification and PSA expression possible because of the complex regulation of PSA expression. It has actually been suggested that there are also other signaling pathways than AR that regulate the expression of PSA (29).

One of the most critical problems in studying the progression of prostate cancer has been the lack of good model systems. Of the five commercially available cell lines (PC-3, DU145, LNCaP, MDA-Pca2a, and NIH-H660), only two express AR (LNCaP and MDA-Pca2b). Both of them contain AR mutations (30, 31). In addition, there has been no cell line or xenograft that demonstrates AR gene amplification. Therefore, we screened 10 recently established prostate cancer xenografts derived from eight patients with hormone-refractory prostate cancer. Two of the xenografts contained the amplification. As far as we know, these are the first prostate cancer model systems that were found to have amplification of the AR gene. We have shown previously that the amplified AR gene in hormone refractory tumors is usually of the wild type (5, 32). The fact that neither one of the xenografts contains mutations in the AR gene further strengthens their utility for studying the functional consequences of the gene amplification. We analyzed also the expression of AR in the xenografts. All but LuCaP 49 expressed AR. LuCaP 49 represents a rare form of prostate tumors, i.e., small cell carcinomas, and these are
known not to express AR (33). Interestingly, it seemed that one additional copy of AR was able to increase the expression of the gene, suggesting that even a small increase in gene dosage could have biological significance. We have shown earlier that ~20% of locally recurrent prostate carcinomas contain two copies of AR (5), which thus may have biological significance. The exact copy number of AR in the cases of amplification (LuCaP 35 and LuCaP 69) could not be evaluated because of the clustering of the signals in the FISH analyses. However, based on the sizes of the clusters, it was evident that LuCaP 69 contained a higher copy number of AR. It also expressed the gene more than the others. All xenografts, except LuCaP 49, expressed AR protein product as detected by immunohistochemistry (data not shown). However, because of the nonquantitative nature of AR immunohistochemistry, only RT-PCR data were used for the comparison of expression levels.

In most of the xenografts, the level of expression of PSA was about the same. LuCaP 49 did not express the gene as was expected from the fact that it is a small cell carcinoma. LuCaP 23.1, LuCaP 23.8, and LuCaP 23.12 expressed more PSA than the others. These three xenografts, derived from a single parental xenograft LuCaP 23, are known to express high levels of PSA (34). The growth of these xenografts in mice is also very androgen-dependent. Although eight of the xenografts were derived from tumors that were androgen-independent in the patient, they grow as androgen-dependent or -responsive tumors in mice. Castration of the mice decreases the expression of PSA and the volume of the tumors, especially in LuCaP 35 and derivatives of LuCaP 23, but also in LuCaP 23- and 41-bearing mice. In addition, LuCaP 58 and 73 show slower growth in castrated than in intact male mice. LuCaP 23.1, 23.8, 23.12, and 35 eventually become androgen-independent when propagated in castrated mice for longer period of time. The growth of LuCaP 49 is fast and truly androgen-independent in intact mice (34).6

Mechanisms other than gene amplification that are related to AR and have been suggested to be important in the emergence of androgen-independent prostate cancer, include mutations in the AR and alterations in the coregulators of the AR as well as ligand independence or alternative activation of AR (30, 31, 35–39). Although, in general, AR mutations seem to be quite rare in hormone-refractory tumors, about 25% of tumors treated with antiandrogens (flutamide) do show specific mutations (35). Thus, it seems that the treatment selects for the mutations, which alter the transactivational properties of the AR protein. Such mutated forms of AR can be activated by other hormones or antiandrogens (22, 30, 35). In addition, it has been suggested that other signaling pathways, especially HER-2/neu, could activate AR in the presence of low levels of androgens (36, 38). In vitro data has also suggested that AR coregulators may alter the transactivational properties of AR (39, 40). However, practically nothing is known about the expression of these coregulators in prostate cancer in vivo.

In conclusion, our finding of high-level expression of AR in hormone-refractory tumors support the notion that the AR signaling pathway is important in the emergence of androgen-independent prostate cancer. One key mechanism for the overexpression seems to be gene amplification. As AR has emerged to be a key player in the progression of prostate cancer, the critical issue is whether it could be targeted by novel therapies. Evidently, castration is not enough. Although it seems that MAB is somewhat more beneficial for patients with the amplification than without, neither is it efficient enough (11). New treatment modalities, based on the high expression of AR in the cancer cells, have been suggested (41, 42). The development of such therapies will be boosted by our finding of two model systems containing the gene amplification.

Acknowledgments

We thank Mariita Vakkuri, Heli Lehtonen, and Maarit Ohranen for technical assistance.

References

Amplification and Overexpression of Androgen Receptor Gene in Hormone-Refractory Prostate Cancer


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/61/9/3550

Cited articles
This article cites 39 articles, 17 of which you can access for free at:
http://cancerres.aacrjournals.org/content/61/9/3550.full#ref-list-1

Citing articles
This article has been cited by 90 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/61/9/3550.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.