Abstract

The present work focused on the potential involvement of selective adaptations of the androgen receptor pathway in the initiation and progression of prostate cancer. We defined the androgen receptor pathway by selecting 200 genes that were androgen responsive in prostate cancer cell lines and/or xenografts. This androgen receptor pathway gene signature was then used for profiling prostate cancer xenografts and patient-derived samples. Approximately half of the androgen receptor pathway genes were up-regulated in well-differentiated prostate cancer compared with normal prostate. Functionally distinct parts of the androgen receptor pathway were specifically down-regulated in high-grade cancers. Unexpectedly, metastases have down-regulated the vast majority of androgen receptor pathway genes. The significance of this progressive down-regulation of androgen receptor pathway genes was shown for a few androgen receptor–regulated genes. Lower mRNA expression of HERPUD1, STK39, DHCR24, and SOCS2 in primary prostate tumors was correlated with a higher incidence of metastases after radical prostatectomy. HERPUD1 mRNA expression predicted the occurrence of metastases almost perfectly. In vitro experiments showed that overexpression of the stress response gene HERPUD1 rapidly induces apoptosis. Based on the functions of the genes within the distinct subsets, we propose the following model. Enhanced androgen receptor activity is involved in the early stages of prostate cancer. In well-differentiated prostate cancer, the androgen receptor activates growth-promoting as well as growth-inhibiting and cell differentiation genes resulting in a low growth rate. The progression from low-grade to high-grade prostate carcinoma and metastases is mediated by a selective down-regulation of the androgen receptor target genes that inhibit proliferation, induce differentiation, or mediate apoptosis. (Cancer Res 2006; 66(10): 5012–20)

Introduction

The androgen receptor plays a pivotal role in the growth and survival of both normal prostate epithelium and prostate carcinoma. Both normal and malignant prostate tissues regress on androgen deprivation, although this effect on prostate carcinoma is only temporary (1). The function of the androgen receptor, however, markedly differs between the normal prostate and prostate carcinoma. Whereas the androgen receptor is a key regulator for prostatic function and survival in the normal prostate epithelium, the androgen receptor has been converted into an inducer of uncontrolled cell growth in prostate carcinoma (1, 2). The recent identification of frequent chromosomal rearrangement in prostate cancer that results in fusion of TMPRSS2 with ETS transcription factor genes explains this conversion (3). The ETS family members ERG and ETV1 are normally not or lowly expressed in prostate tissues. Fusion of these genes to the strong androgen-regulated TMPRSS2 promoter results in androgen-induced expression of these proto-oncogenes, which likely cause prostate tumorigenesis.

That enhanced androgen receptor activity of itself is unlikely to be sufficient for causing aggressive growth could already be deduced from our knowledge on androgen receptor function in the normal prostate. In vitro experiments indicated that androgens induce differentiation and thereby inhibit growth of prostate epithelial cells (4, 5). In a transgenic mouse model, overexpression of the wild-type (WT) androgen receptor in prostatic epithelial cells resulted in intraepithelial neoplasia but not in carcinomas (6). The growth-stimulating effect of androgens on normal epithelial cells is at least partly mediated by the prostatic stromal cells, which produce and secrete growth factors, the andromedins, in response to androgen exposure (1, 2, 7). During prostatic carcinogenesis, the balance between the differentiation- and proliferation-inducing functions of the androgen receptor might be disturbed. Such a transition would likely be reflected by changes within the androgen receptor pathway genes.

Therefore, we studied the expression levels of androgen receptor pathway genes during progression of prostate carcinoma. Many gene expression profiling studies have been published for prostate carcinoma as well as for other types of cancers. This has led to the identification of gene sets specific for localized prostate carcinoma and metastases or related to the occurrence of relapse after radical prostatectomy (8–14). The functions of the genes within these sets are very diverse. Although these gene sets might have diagnostic and prognostic prospects, their effect on our understanding of the biological mechanisms involved in cancer progression is limited. The use of “functional gene sets,” such as target genes from a transcription factor, might be a more valuable tool to enhance this understanding. This was tested in the present study using an androgen receptor pathway gene signature.

To identify androgen receptor pathway genes, expression microarray analyses were done on the prostate carcinoma cell line LNCaP and on a panel of 13 prostate carcinoma xenografts. In total, 200 androgen receptor pathway genes were identified. To test whether the androgen receptor pathway is changed during prostate carcinoma progression, the expression of these 200 genes...
was assessed in both xenografts and clinical prostate carcinoma specimens. The results indicate that prostate carcinomas down-regulate part of the androgen receptor pathway before acquiring the ability to metastasize. This finding was further supported by quantitative reverse transcription-PCR (RT-PCR) on a selection of genes on an independent set of prostate carcinoma samples.

Materials and Methods

Prostate carcinoma xenografts, cell lines, and patient-derived tissues. Thirteen prostate carcinoma xenografts have been established at our laboratory (15–17), and their main characteristics are given in Table 1. Additional information about the xenografts is provided in Supplementary Data. Xenografts were collected either from intact male mice or 7 to 14 days after castration. Tissues were frozen at −80°C. The xenograft PC329 has been lost, and tissues taken from castrated mice are not available anymore. The prostate carcinoma cell line LNCaP has been well described (18). The LNCaP cell line was maintained in RPMI 1640 with 5% FCS and penicillin/streptomycin (Invitrogen, Merelbeke, Belgium). Before R1881 treatment, cells were androgen deprived for 72 hours in medium containing 5% dextran-filtered, charcoal-stripped FCS with a medium replacement after 36 hours. After androgen deprivation, the medium was supplemented for 2, 4, 6, or 8 hours with 1 nmol/L R1881 or ethanol vehicle.

Normal and tumor specimens from patients used for quantitative real-time RT-PCR analysis were obtained from the frozen tissue bank of the Erasmus Medical Center (Rotterdam, the Netherlands). The specimens were collected between 1984 and 2001. Additional information about these specimens is provided in Supplementary Data.

RNA amplification, cDNA labeling, and cDNA microarray hybridizations. Total RNA (3 μg) was used for a T7-based linear mRNA amplification protocol (19). Amplified RNA (2 μg) was used to produce Cy3- or Cy5-labeled cDNA. The cDNA microarrays were manufactured at the Central Microarray Facility at the Netherlands Cancer Institute (Amsterdam, the Netherlands) and contained >18,000 features that have been selected from the Research Genetics Human Sequence Verified Library (Invitrogen). Xenograft cDNA was labeled with Cy3 and cohybridized with Cy5-labeled cDNA prepared from RNA extracted from 13 cell lines, including the 2 prostate carcinoma cell lines Du145 and LNCaP. Four microarrays were used per xenograft on tissue samples collected in separate experiments. Two samples were taken from intact male mice and another two after castration.

The normalization and flagging procedure is provided in Supplementary Data.

The programs Cluster and Treeview were used for hierarchical clustering (20). Comparisons with other microarray databases were done using Sequence Retrieval System 7 (Lion Bioscience AG, Heidelberg, Germany) as published recently (21).

Quantitative real-time RT-PCR analysis. Quantitative real-time RT-PCR analysis was done with a ABI Prism 7700 Sequence Detection System using AmpliTaq Gold according to the manufacturer’s specifications (Applied Biosystems, Foster City, CA). The probes and primers were validated with Taqman Gene Expression Assays (Applied Biosystems). The assay identification numbers and PCR settings are given in Supplementary Data. The amount of target gene expressed was normalized to an endogenous reference and relative to a calibrator. The endogenous reference was glyceraldehyde-3-phosphate dehydrogenase; a mixture of cDNAs of the prostate carcinoma xenografts was used as the calibrator.

Criteria for up-regulation and down-regulation in the microarray analyses and statistics. For the LNCaP time series, spots were considered to be up-regulated or down-regulated by R1881 when both dye swaps gave a ratio >1.62 (2 log 0.7) for at least one time point. For the xenografts, spots were considered to be up-regulated or down-regulated by castration when the average ratios of two experiments between the intact and castrated mice were >2 (2 log 1). Additionally, both individual comparisons between xenografts grown on an intact mouse versus castrated mouse had to yield a ratio >1.62 (2 log 0.7). The significant analysis of microarrays method (SAM; version 1.21; ref. 22) was used to detect significantly differentially expressed spots between normal prostate and prostate carcinoma samples of patients. χ2 analyses were used for comparisons of proportions as indicated in Results. The significance of overlap between the recurrence-associated gene set published by Henschall et al. (14) and our androgen receptor pathway genes was determined by Venn Mapper (23). Box plots, Mann-Whitney U test, Kaplan-Meier test, log-rank test, and Cox regression analyses on quantitative real-time RT-PCR results were done using SPSS11 software (Chicago, IL.). Ps < 0.05 were considered significant.

HERPUD1 overexpression. LNCaP and the liver cancer cell line Hep3B were transfected with HERPUD1 constructs using FuGene (Roche, Mannheim, Germany) according to the manufacturer’s specifications. The HERPUD1 expression constructs are described in Supplementary Data. HERPUD1-transfected cells were detected with either anti-HERPUD1 (24) or FITC-labeled anti-MYC-Tag (Invitrogen). A rabbit anti-caspase-3 active was obtained from R&D Systems (Minneapolis, MN). Nuclei of the cells were counterstained with 0.5 μg/ml Hoechst 33342 (Invitrogen). The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction was done on pDsRed2-mHERP1 transiently transfected cells using the DeadEnd Fluorometric TUNEL kit (Promega, Madison, WI). To determine the proportion of apoptotic cells, at least 100 transfected cells per treatment were counted. The transfection experiments were done in triplicate.

Results

Using expression microarray analyses, we followed two strategies to identify androgen receptor pathway genes. First, LNCaP cells were treated with R1881 to detect genes that were up-regulated within 8 hours by androgen. Secondly, we assessed which genes were down-regulated by androgen deprivation in androgen receptor–expressing prostate carcinoma xenografts.

R1881-induced genes in LNCaP. LNCaP cells were treated with R1881 for short times (2, 4, 6, and 8 hours) to enrich for genes directly regulated by the androgen receptor. RNA from

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**Table 1. Panel of prostate cancer xenografts**

<table>
<thead>
<tr>
<th>Tumor model</th>
<th>Androgen dependence</th>
<th>AR status*</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC82</td>
<td>Androgen dependent</td>
<td>AR+</td>
<td>Prostate</td>
</tr>
<tr>
<td>PC295</td>
<td>Androgen dependent</td>
<td>AR+</td>
<td>Lymph node</td>
</tr>
<tr>
<td>PC310</td>
<td>Androgen dependent</td>
<td>AR+</td>
<td>Prostate</td>
</tr>
<tr>
<td>PC329</td>
<td>Androgen dependent</td>
<td>AR+</td>
<td>Prostate</td>
</tr>
<tr>
<td>PC346</td>
<td>Androgen responsive</td>
<td>AR+</td>
<td>TURP</td>
</tr>
<tr>
<td>PC346B</td>
<td>Androgen independent</td>
<td>AR+</td>
<td>TURP</td>
</tr>
<tr>
<td>PC346I</td>
<td>Androgen independent</td>
<td>AR+</td>
<td>TURP</td>
</tr>
<tr>
<td>PC346B1</td>
<td>Androgen independent</td>
<td>AR+</td>
<td>TURP</td>
</tr>
<tr>
<td>PC374</td>
<td>Androgen independent</td>
<td>AR+</td>
<td>Skin</td>
</tr>
<tr>
<td>PC133</td>
<td>Androgen independent</td>
<td>AR−</td>
<td>Bone</td>
</tr>
<tr>
<td>PC135</td>
<td>Androgen independent</td>
<td>AR−</td>
<td>Prostate</td>
</tr>
<tr>
<td>PC324</td>
<td>Androgen independent</td>
<td>AR−</td>
<td>TURP</td>
</tr>
<tr>
<td>PC339</td>
<td>Androgen independent</td>
<td>AR−</td>
<td>TURP</td>
</tr>
</tbody>
</table>

Abbreviations: AR, androgen receptor; TURP, transurethral resection of the prostate.
*Genes are designated as AR+ when the androgen receptor is detectable by immunoblotting and the xenograft expresses prostate-specific antigen.
†PC324 shows some androgen receptor expression but does not express prostate-specific antigen. Although no mutations in the androgen receptor have been detected, PC324 is considered to have a nonfunctional androgen receptor pathway.
R1881-treated and control cells were compared directly by hybridization to the same microarray. This was done in duplicate with reversed Cy dye labeling. Spots considered to be up-regulated or down-regulated by R1881 when both dye swaps gave a ratio >1.62 (2 log 0.7) for at least one time point. One hundred thirty-six up-regulated genes (represented by 163 spots) and 60 down-regulated genes (represented by 60 spots) were detected (Supplementary Fig. S1; Supplementary Table S1).

Genes affected by castration in prostate carcinoma xenografts. We assessed the expression profiles of 12 xenografts collected either from intact male mice or 7 to 14 days after castration. Eight xenografts have functional androgen receptor expression. Three of these xenografts depend on androgens for growth, whereas four xenografts grow androgen independently (Table 1; see Materials and Methods). One xenograft (PC346) is hormone responsive. It shows reduced albeit continuous growth after castration, which is stimulated by administration of androgens. Four other xenografts lack expression of a functional androgen receptor and grow independently of androgens.

We did two castration experiments per xenograft yielding two samples taken from intact male mice and another two after castration. Based on the selection criteria given in Materials and Methods, 293 spots were up-regulated or down-regulated by castration in at least one of the 12 xenografts. A hierarchical clustering of these 293 spots showed the overlap in castration-affected genes among the xenografts (Fig. 1A). A high number of genes were up-regulated or down-regulated in the androgen-dependent and hormone-responsive xenografts. Few genes were affected by castration in the androgen receptor–expressing, androgen-independent xenografts. As expected, very few genes were affected by castration in the xenografts lacking androgen receptor expression. Another observation is that a subset of the genes are up-regulated or down-regulated in the majority of androgen receptor–expressing xenografts, whereas other genes are castration responsive in only one or two xenografts. Evidently, xenograft-specific androgen receptor pathway genes exist in addition to common androgen receptor pathway genes.

Because androgen-dependent xenografts regress after androgen deprivation, castration was expected to affect both cell cycle–related and androgen-regulated genes. We tested this assumption by selecting all spots down-regulated by castration in at least one androgen-dependent xenograft (n = 132; Fig. 1A) and clustered these spots based on expression levels in the xenografts (Fig. 1B). The clustering discriminated two groups of genes. The genes of group I (n = 65; 71 spots) showed highest expression in the androgen-dependent xenografts and also, for a part, in androgen receptor–expressing, androgen-independent xenografts. In contrast, the genes of group II (n = 59; 61 spots) showed low expression levels in androgen-dependent xenografts compared with all others. The two groups clearly differed in proportion of spots that were up-regulated by R1881 in LNCaP: 19 from 71 in group I and 4 from 61 in group II (P = 0.002, χ² analysis). To acquire more insight in the functions of these two groups of genes, we made use of a data set of genes that are differentially expressed during the cell cycle in HeLa cells and for which the expression is related to the cell proliferation rate (25). The proportion of cell cycle–related genes was much higher within group II [(26 of 59 genes (44%)] than within group I [4 of 65 genes (6%); P = 10⁻⁶, χ² analysis; Fig. 1B]. The genes of group II therefore very likely represent cell proliferation–related genes that are down-regulated as a second-term effect of castration due to the cease of cell division. Their higher expression levels in...
hormone refractory than androgen-dependent xenografts simply reflect the higher proliferation rate of hormone refractory xenografts. Group I likely represents genes that are more directly associated with the androgen pathway. Forty-nine genes of group I were not present in the set of 136 R1881 up-regulated genes in LNCaP and included in the androgen receptor pathway signature set. Fifteen additional genes were selected based on their down-regulation by castration in the androgen receptor–expressing, androgen-independent xenografts of the PC346 group whose growth is unaffected by castration. Combining this gave a set of 200 androgen receptor pathway genes. We investigated the expression of these genes in xenografts and clinical prostate carcinoma samples to assess the changes of the androgen receptor pathway during prostate carcinoma progression. The complete microarray results have been submitted to GEO (accession GSE4048).

**Expression levels of the androgen receptor pathway genes in prostate carcinoma xenografts.** The 200 androgen receptor pathway genes were hierarchically clustered on expression in the xenografts (Fig. 2). As expected, most androgen receptor pathway genes were much higher expressed in the androgen-dependent xenografts than in the androgen-independent xenografts lacking androgen receptor expression. The androgen receptor–expressing, androgen-independent xenografts showed high expression for only a fraction of the androgen receptor pathway genes, indicating interference of other pathways/mechanisms that prevent transcriptional activation of the majority of these genes by the androgen receptor.

**Expression levels of the androgen receptor pathway signature genes in primary prostate carcinoma and metastases in patients.** Expression levels of the androgen receptor pathway genes were also evaluated in clinical tumor samples. From the 200 androgen receptor pathway genes, 171 were represented in a microarray study on 41 normal prostate specimens, 62 primary prostate tumors, and 9 lymph node metastases published by Lapointe et al. (13). The prostate carcinoma samples in this study contained at least 90% tumor tissue. The metastases have not been subjected to androgen ablation therapy. The 171 androgen receptor pathway genes were sufficient to separate normal prostate, low-grade prostate carcinoma, and high-grade prostate carcinoma and metastases in discrete groups by unsupervised hierarchical clustering (Fig. 3A). Data reported by Stuart et al. (26) were used to indicate genes for which the expression levels are associated with the content of either stroma, benign prostatic hyperplasia (BPH), or tumor tissue. Subsets of androgen receptor pathway genes were differentially expressed among the sample groups (Fig. 3A). One subset was higher expressed in normal prostate (Fig. 3A, green), and nearly all genes within this subset were stroma associated. Approximately half of the 171 androgen receptor pathway genes were highly expressed in well-differentiated prostate carcinoma (Fig. 3A, blue and red), and most of these genes were tumor associated. This implies that the up-regulation of these androgen-up-regulated genes in prostate carcinoma is due not only to a higher content of epithelial cells but also to an increased expression in tumor cells compared with normal epithelial cells. A subset of these genes is down-regulated in high-grade prostate carcinoma (Fig. 3A, red). Only a few genes remain relatively highly expressed in metastases (Fig. 3A, orange). The metastases showed also low expression for the well-known androgen receptor target gene prostate-specific antigen (KLK3). In marked contrast, the androgen receptor itself is higher expressed in the metastases than in the majority of primary prostate carcinomas (Fig. 3A).

The expression of the set of 200 androgen receptor pathway genes in prostate carcinoma samples was also assessed using microarray data published by Dhanasekaran et al. (8). Again, approximately half of the androgen receptor pathway genes were up-regulated in primary prostate carcinoma, whereas only few genes were relatively high expressed in metastases (Supplementary Fig. S2). All androgen receptor pathway genes that according to SAM were differentially expressed between primary prostate carcinoma and metastases in Lapointe et al. and/or Dhanasekaran et al. are listed in Supplementary Table S2. Fifty-nine genes were more highly expressed in primary prostate carcinoma than in metastases, whereas only two genes were more highly expressed in metastases (lymph node) than in primary prostate carcinoma. Taken together, this confirms our results with the xenografts that main parts of the androgen receptor pathway are down-regulated in metastases, whereas only few genes were relatively high expressed in metastases (Supplementary Fig. S2). All androgen receptor pathway genes that according to SAM were differentially expressed between primary prostate carcinoma and metastases in Lapointe et al. and/or Dhanasekaran et al. are listed in Supplementary Table S2. Fifty-nine genes were more highly expressed in primary prostate carcinoma than in metastases, whereas only two genes were more highly expressed in metastases (lymph node) than in primary prostate carcinoma.

We verified this analysis with alternative sets of androgen receptor pathway genes that were selected on up-regulation by

![Figure 2. Expression of androgen receptor pathway genes in prostate cancer xenografts. Two hundred genes were selected on up-regulation in LNCaP and/or down-regulation in androgen receptor–expressing xenografts by castration. Genes (n = 165) with unflagged expression value for at least 50% of the xenografts were hierarchically clustered on expression in xenografts grown on intact mice. Thereafter, expression values of xenografts 1 to 2 weeks after castration were added.](image)
androgens at later time points in either LNCaP or other androgen-dependent prostate carcinoma cell lines (27). The expressions of these gene sets in normal prostate and prostate carcinoma samples were essentially the same as that of our set of 200 genes (see Supplementary Fig. S3). We then confirmed that the expression patterns of the androgen receptor pathway genes did not simply reflect the general variation in gene expression. Compared with all spots of the Lapointe database, the androgen receptor pathway gene sets selected on prostate carcinoma cell lines had much higher proportions of spots ($P < 10^{-6}$) that were either (a) up-regulated in primary prostate carcinoma versus normal prostate, (b) down-regulated in lymph node metastases versus primary prostate carcinoma or normal prostate, or (c) down-regulated in high-grade prostate carcinoma versus low-grade prostate carcinoma (see Supplementary Fig. S4).

**Down-regulation of androgen receptor pathway genes in primary prostate carcinoma predicts development of distant metastases.** The down-regulation of the majority of androgen receptor pathway genes in metastases is intriguing. We addressed the important issue whether this down-regulation occurs after development of the metastasis or that the primary prostate carcinoma down-regulates the androgen receptor pathway genes before acquiring the ability to metastasize. Support for the latter hypothesis was found in the results published previously by Henshall et al. (14) who compared the expression profiles of primary prostate carcinomas that did or did not relapse after radical prostatectomy. For 152 genes represented by 181 spots, relatively low expression in primary prostate carcinoma was found to be associated with fast relapse (14). Ten of these genes were present within our set of 200 androgen-up-regulated genes, which was significantly ($P < 10^{-5}$) more than expected by random chance. Nine of these 10 genes were included in the data set of Lapointe et al. and all up-regulated in the majority of primary prostate carcinoma compared with normal prostate (Fig. 3B). Interestingly, most of these genes had low expression levels in metastases (Fig. 3B). Taken together, this indicates down-regulation of part

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**Figure 3.** Expression of androgen receptor pathway genes in prostate cancer specimens from patients. A, expression data were taken from Lapointe et al. (13), which included 171 genes from our androgen receptor pathway signature set. A hierarchical clustering was done on both genes and samples. Bottom, androgen receptor and KLK3; right, genes significantly up-regulated (red) or down-regulated (green) according to SAM. Association with proportion of stroma, BPH, or tumor as published by Stuart et al. (26). PC I, II, and III, three clustering groups from primary prostate carcinoma (PC) samples on 5,153 variably expressed genes as described by Lapointe et al. (13). PC II and III, relatively high-grade and advanced-stage tumors. All prostate carcinoma samples with early recurrence of metastases after radical prostatectomy ($n = 7$) clustered within this high-grade PC II and III group. B, selection of nine genes included in (A) for which down-regulation in primary prostate cancer has been reported to be associated with fast relapse after radical prostatectomy (14). LN metast, lymph node metastases; normal, normal prostate.
of the androgen pathway to be associated with an enhanced metastatic potential.

To verify these findings, we did quantitative real-time RT-PCR for five androgen receptor pathway genes on normal prostate and prostate carcinoma specimens obtained at our institute. STK39, SOCS2, HERPUD1, and 24-dehydrocholesterol reductase (DHCR24) or Seladin-1 were selected based on down-regulation in high-grade prostate carcinomas and lymph node metastases within the Lapointe data set. STK39, SOCS2, and DHCR24 were among the 10 genes for which down-regulation was correlated with fast tumor relapse according to Henshall et al. (Fig. 3B). The fifth androgen receptor pathway gene, α-methylacyl-CoA racemase (AMACR), is a well-known marker for prostate carcinoma for which a reduced expression in primary prostate carcinoma has been correlated with recurrence before (28). We included AMACR together with another well-known prostate carcinoma marker Hepsin as positive controls. Compared with normal prostate and nonmetastatic primary prostate carcinoma, HERPUD1, STK39, and SOCS2 were significantly down-regulated in metastatic prostate carcinoma (Fig. 4A).

The expression of HERPUD1, but not STK39, remained low in lymph node metastases. AMACR and Hepsin were the only two genes with significantly higher expression in prostate carcinoma than normal prostate. The Kaplan-Meier results of the quartiles for the occurrence of metastases after radical prostatectomy are shown in Fig. 4B. For HERPUD1 and STK39, none of the primary prostate carcinomas within the three or two quartiles with highest expression were metastatic (Fig. 4B). When the optimal threshold level per gene was selected on receiver operator characteristic curves, low expression levels in primary prostate carcinoma were significantly correlated with an increased rate of occurrence of metastases after radical prostatectomy are shown in Fig. 4B. For HERPUD1 and STK39, none of the primary prostate carcinomas within the three or two quartiles with highest expression were metastatic (Fig. 4B). When the optimal threshold level per gene was selected on receiver operator characteristic curves, low expression levels in primary prostate carcinoma were significantly correlated with an increased rate of development of a distant metastasis for all five androgen receptor pathway genes but not for Hepsin (P < 0.00005 for HERPUD1, P = 0.0001 for STK39, P = 0.02 for DHCR24 and SOCS2, P = 0.03 for AMACR, and P = 0.11 for Hepsin, log-rank test). Low or high HERPUD1 expression almost perfectly predicted recurrence or no recurrence. The HERPUD1 expression value correctly classified the 2 of 24 prostate carcinomas with Gleason score 6 and 2 of 14 prostate carcinomas with Gleason score 7 that developed metastases. In addition, it also correctly classified the 2 of 8 prostate carcinomas with Gleason score 8 that did not develop metastases. Peculiarly, the only missclassified tumor had a Gleason score 10 from which a metastasis became apparent 4 months after radical prostatectomy already. The hazard ratio (and 95% confidence interval) was 1.13 (0.22-5.88; P = 0.88) for the Gleason score and 0.26 (0.11-0.66; P = 0.03) for HERPUD1 mRNA expression (Cox multivariate regression analysis), indicating HERPUD1 to be a better predictor for recurrence than the Gleason score.

**Overexpression of HERPUD1 induces apoptosis.** HERPUD1 (alias HERP, Mif1, SUP, and KIAA0025) is an endoplasmic reticulum (ER)–resident protein that is up-regulated during a variety of stress conditions (24, 29). We selected HERPUD1 to test whether overexpression affects cell viability. Overexpression of HERPUD1 efficiently induced cell death (apoptotic nuclei) in both LNCaP and Hep3B cells (Fig. 5A). The presence of apoptotic nuclei co-occurred with a localization of HERPUD1 in the entire cytoplasm instead of the ER (Fig. 5A). Two deletion mutants of HERPUD1 were tested as comparison. HERPUD1 lacking a transmembrane domain induced apoptosis even more rapidly than WT HERPUD1 (Fig. 5B). In contrast, HERPUD1 lacking the ubiquitin-like domain had a decreased ability to induce apoptosis (Fig. 5B). These findings point to a mechanism, in which release of HERPUD1 from the ER to the cytoplasm induces apoptosis, which is mediated by the ubiquitin-like domain. We confirmed that HERPUD1 induced cell death via activation of apoptosis. HERPUD1 overexpression resulted in the activation of caspase-3 (Fig. 5C and D) and DNA degradation as detected by the TUNEL assay (data not shown).

**Discussion**

The current study shows that focusing on a set of target genes from one transcription factor can be a very valuable tool in unraveling the biological changes occurring during cancer progression. The focus on the expression of androgen receptor pathway genes yielded new insights in the role of the androgen receptor in the progression of prostate carcinoma. Our results indicate that changes in the androgen receptor pathway rather than a generally enhanced androgen receptor activity play a critical role in the development of more aggressive prostate carcinoma. Specific sets of androgen receptor pathway genes are down-regulated during the progression from well-differentiated prostate carcinoma to high-grade prostate carcinoma, and this might be a prerequisite to acquire the ability to metastasize. Only few androgen receptor pathway genes remained highly expressed in metastases compared with primary prostate carcinoma.

**Adaptations of the androgen receptor pathway between normal prostate and primary prostate carcinoma.** We selected 200 androgen receptor pathway genes up-regulated by androgens in the prostate cancer cell line LNCaP and/or down-regulated on castration in prostate cancer xenografts. We then assessed the expression of these genes in clinical prostate carcinoma samples. A proportion of the androgen receptor pathway genes were stroma associated. Because prostate carcinoma contains less stromal cells than normal prostate, these stroma-associated genes were down-regulated in prostate carcinoma. The majority of the genes up-regulated in primary prostate carcinoma were tumor associated. This implies that these androgen receptor pathway genes are up-regulated in the malignant epithelial cells rather than simply reflecting the increase of percentage of epithelial cells (26). Approximately half of the androgen receptor pathway genes are up-regulated in prostate carcinoma, indicating an enhanced androgen receptor activity that however does not up-regulate all target genes. Apparently, prostate carcinoma cells activate androgen receptor pathway genes that are less or not androgen responsive in normal prostate epithelial cells.

**Adaptations of the androgen receptor pathway between low- and high-grade primary prostate carcinoma.** The expression levels of the androgen receptor pathway signature within the data set of Lapointe et al. clearly differed between low- and high-grade prostate carcinoma (13). A subset of 33 androgen receptor pathway genes was higher expressed in low-grade prostate carcinoma than in most of the high-grade tumors. A function has been ascribed to 28 from the 33 genes (see Supplementary Table S3). Thirteen genes have been described to affect cell growth from which 6 act as proliferation inhibitors, 1 as proliferation inducer, and 3 as mediators of apoptosis. Six genes are a marker or inducer of differentiation, whereas two genes are involved in cell adhesion. Down-regulation of these 33 genes therefore is expected to be beneficial for cell proliferation and prevention of apoptosis, and this likely explains the difference in proliferation rate between low- and high-grade prostate carcinoma.

**Adaptations of the androgen receptor pathway between high-grade primary prostate carcinoma and metastases.** Fifteen genes up-regulated in low-grade prostate carcinoma...
remained highly expressed in high-grade prostate carcinoma and were down-regulated in metastases (see Supplementary Table S3). Twelve of these 15 genes have a known function, and most are involved in metabolism, exocytosis, transport, protein folding, or signal transduction. \( \text{DHCR24} \) has recently been shown to be a key mediator of \( \text{Ras} \)-induced senescence (30). \( \text{Tumor protein D52} \) (TPD52) has been reported previously to be up-regulated in prostate carcinoma (31, 32). Apparently, high TPD52 expression is not beneficial for growth of metastases.

Eleven androgen receptor pathway genes remained highly expressed in lymph node metastases as indicated by their higher expression levels in these samples compared with normal prostate cancer. 1st quartile, comprises the 25% of the patients with the lowest expression. RP, radical prostatectomy.

Figure 4. Expression of five androgen receptor pathway genes as measured by quantitative real-time RT-PCR on an independent set of prostate cancer samples. A, box plots, expression of five androgen receptor pathway genes in prostate cancer samples. Well-known prostate cancer marker gene \( \text{Hepsin} \) was included as comparison. Top, significant differences \((P < 0.05, \text{Mann-Whitney U test})\); boxes, interquartile range, which contains 50% of values; O or *, whiskers extending from the highest to lowest values, excluding outliers; line across the box, median; PC → no MET or PC → MET, primary prostate cancers that did or did not develop distant metastases; LN-MET, prostate cancer metastases in the lymph node. Expression in lymph node metastases was not assessed for \( \text{SOCS2}, \text{DHCR24}, \) and \( \text{Hepsin} \). X axis, number of patients per sample group. B, Kaplan-Meier curves for the metastasis-free time of patients with different expression levels in the primary prostate cancer. 1st quartile, comprises the 25% of the patients with the lowest expression.
Overexpression of HERPUD1 induces apoptosis. A, green, Hep3B cells transiently transfected for 1 day with HERPUD1; arrows, condensed apoptotic nuclei in the cells with HERPUD1 distributed over the entire cytoplasm instead of localized at the ER. B, deletion of the transmembrane domain (del TM) accelerates induction of apoptosis, whereas deletion of the ubiquitin-like domain (del Ub-like domain) slows down induction of apoptosis by HERPUD1 overexpressed in Hep3B cells. Representative of three experiments. a, b, and c, significant differences daily between a and b, b and c, and a and c (P < 0.01, Bonferroni). C, overexpression of HERPUD1 (green) results in activation of caspase-3 (red). Hep3B cells were transiently transfected for 2 days with HERPUD1. Left, counterstaining of the nuclei with Hoechst 33342. D, activation of caspase-3 in HERPUD1-transfected Hep3B cells during time. a and b, significant differences between days (P < 0.01, Bonferroni).

(see Supplementary Table S2). SIM2 and AMACR have been reported to positively affect prostate carcinoma growth (33, 34). Within our xenograft panel, both genes have a low expression level in all xenografts lacking functional androgen receptor, suggesting the androgen receptor as an important inducer for their expression in prostate carcinoma. Only two genes, NOL8 and GIP2, are higher expressed in lymph node metastases than in primary prostate carcinoma. However, both genes are highly expressed in lymphocytes, and their high expression in lymph node metastases might therefore be due to contaminating lymphocytes.

**Down-regulation of androgen receptor pathway genes is correlated to the ability to metastasize.** Ten of the androgen receptor pathway genes with high expression in primary prostate carcinoma and low expression in metastases were included in a set of 152 genes (181 spots) for which down-regulation in primary prostate carcinoma was associated with fast relapse after radical prostatectomy (14). We confirmed this correlation for five androgen receptor pathway genes using quantitative real-time RT-PCR on an independent set of prostate carcinoma samples. For *AMACR*, this correlation has been reported recently using immunohistochemistry on tissue microarrays as well (28). In our experiments, HERPUD1 and STK39 were better predictors for development of metastases than *AMACR*. When using the most optimal threshold level of 3, low or high expression of HERPUD1 mRNA in primary prostate carcinomas correlated perfectly with the occurrence or nonoccurrence of distant metastases with the exception of one prostate carcinoma with Gleason score 10. None of the prostate carcinomas with STK39 mRNA relative expression higher than 7.8 developed a metastasis. Both HERPUD1 and STK39 are stress response proteins and have been reported before to be up-regulated by androgens in LNCaP cells (24, 35–37). HERPUD1 is an ER-resident protein that is up-regulated during the unfolded protein response and after cellular stresses, such as amino acid deprivation and oxidative stress. The unfolded protein response results in a temporary inhibition of protein synthesis. For chronic stress, this leads to activation of apoptotic pathways (38, 39). Interestingly, the HERPUD1 gene is localized at chromosome 16q, a region with high frequency of copy number losses in prostate carcinoma and other types of cancer (40). HERPUD1 is considered to be a protein that protects against apoptosis because knockout of this gene causes cells to become less tolerant to stress (39). Our transfection experiments in both LNCaP and Hep3B cells indicate that also high expression of HERPUD1 is not favorable to cells. HERPUD1 expression induces apoptosis, and this apoptotic potential is affected by domain mutations of HERPUD1, showing a functional significance. Therefore, we expect that HERPUD1 acts as an apoptosis inhibitor on a shorter term and as an apoptosis inducer on a longer term.

In summary, our results point out that prostate carcinoma cells disable the HERPUD1- and STK39-related stress responses to acquiring the potency to metastasize. The HERPUD1 status promises to have high clinical utility as a biomarker to predict tumor recurrence. Its expression level might become a valuable criterion for decisions about “watchful-waiting” protocols or aggressiveness of therapy. Because down-regulation can only be detected when occurring in the majority of the cells, our findings on HERPUD1 also supports the earlier proposed view that the characteristics of the bulk of the tumor rather than that of a small subset of cells determine the metastatic capability (41).

**Evolution of the androgen receptor pathway during prostate carcinoma progression.** Our results lead to the following model. In nonmalignant prostatic epithelial cells, the androgen receptor regulates genes that are mainly involved in the differentiation into a secretory epithelial cell and in this cell’s function of production of prostate fluid with all its components,
such as vesicles, proteins, and metabolites. Cell growth and apoptosis of normal epithelium are likely regulated indirectly by stromal-epithelial interactions. Tumorigenesis and androgen-dependent growth of prostate cancer are acquired traits often caused by chromosomal rearrangements leading to a fusion gene of the androgen-regulated TMRPSS2 promoter to the oncogenic ETS family members (3). Androgen regulation in early-stage cancer cells will drive not only growth but also differentiation and normal prostate functions. These cells will become more aggressive by selective down-regulation of androgen receptor pathway genes that inhibit proliferation, induce differentiation, or mediate stress responses and apoptosis. This down-regulation results in a higher proliferation rate and enhances the potency to metastasize.

The present results provide novel insight and are expected to have important consequences for the direction of future research. Taking advantage of the differentiation-promoting and cell growth-inhibiting effects of the androgen receptor might be a more promising route to therapy development than attempts to fully block androgen receptor activity. For this, it will be essential to reveal mechanisms that mediate the selective down-regulation of the differentiation-promoting and proliferation-inhibiting genes. Eventually, this may lead to development of drugs that prevent or reverse the down-regulation of differentiation-promoting and cell growth–inhibiting genes.

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