ERG status is unrelated to PSA recurrence
in radically operated prostate cancer in the absence of anti-hormonal therapy

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Abstract

Purpose

About 50% of prostate cancers have TMPRSS2-ERG fusions with concurrent ERG overexpression. The aim of this study was to determine whether clinical differences exist between ERG-positive and ERG-negative cancers in surgically treated patients not exposed to anti-hormonal therapy. A secondary aim was to search for differences between these tumor classes.

Experimental design

A tissue microarray containing samples from more than 2,800 prostate cancers with clinical data was analyzed for ERG alterations by immunohistochemistry and fluorescence in-situ hybridization (FISH). Results were compared with tumor phenotype, biochemical recurrence, and molecular features considered important for prostate cancer. The effect of ERG on androgen receptor (AR)-dependent transcription was analyzed in cell lines.

Results

ERG expression was found in 52.4% of 2805 cancers with a 95% concordance between ERG expression and ERG gene rearrangement detected by FISH. ERG expression was unrelated to clinical outcome and tumor phenotype. Differences in AMACR, annexin A3, Bcl2, CD10, ALCAM, chromogranin A, EGFR, HER2, mTOR, p53 and synaptophysin status were significant but minimal in absolute numbers. The most striking difference was found for AR expression, which was markedly higher in ERG-positive cancers. In vitro studies demonstrated ERG-dependent impairment of AR-mediated transcriptional activity.

Conclusions

The striking similarities between these two types of prostate cancers rules out a major impact of ERG on tumor aggressiveness in early, not hormonally treated cancer. The marked
difference in AR levels between ERG-positive and -negative cancers supports a systematic difference in potential response to hormonal therapy as previously observed in clinical trials.
Statement of translational relevance

Approximately 50% of prostate cancers are molecularly characterized by gene fusions linking the androgen-regulated gene TMPRSS2 with the transcription factor ERG. As a result of this rearrangement, the expression of ERG becomes androgen regulated and thus overexpressed. Despite several previous studies on TMPRSS2-ERG fusion in prostate cancer the clinical significance remains controversial. In this study we show that the ERG status has no influence on the risk of PSA recurrence after radical prostatectomy indicating that ERG does not affect the course of the disease as long as there is no systemic therapy (especially anti-hormonal therapy). In addition our data show a strong association between ERG positivity and high androgen receptor expression levels, which is particularly interesting in the light of recent data suggesting a predictive relevance of ERG status for response to anti-androgen therapy.
### Introduction

Approximately 50% of prostate cancers are molecularly characterized by gene fusions linking the androgen-regulated gene TMPRSS2 with transcription factors of the ETS family (1). The androgen-responsive TMPRSS2 gene encodes a transmembrane serine protease of unknown function. In the most common fusion ERG is fused to 5’-TMPRSS2. Fusion of these genes either occurs through translocation, or more often, through deletion of a 3Mbp intervening sequence between these two genes on chromosome 21(2-4). As a result of this rearrangement, the expression of ERG becomes androgen regulated and, thus, overexpressed in prostatic epithelium. Several studies have investigated clinical and molecular characteristics of fusion versus non-fusion prostate cancers (reviewed in ref. (5)). Studies to date have shown that the rate of fusion positive prostate cancers is higher in Caucasians (50-52%) than in African Americans (31.3%) or Japanese (15.9%) (6, 7). Molecular analyses have recently suggested several molecular aberrations that specifically differ between fusion positive and negative cancers such as PTEN deletions, which were present in a higher percentage of fusion positive cancers as compared to fusion negative cancers (8-11). While such findings suggest significant biological differences, the clinical impact of ETS family gene fusions is still unclear in prostate cancer. While some studies have suggested a worse prognosis of fusion as compared to non-fusion cancers (2, 12-14) other studies either found a favorable prognostic association (15, 16) or did not find any association with clinical outcome (17, 18). These conflicting results may partially be due to differences in cohort size and composition, therapy and clinical endpoints between different studies (reviewed in (5)).

The analysis of TMPRSS2-ERG fusions in prostate cancer has so far been hampered by the need for fluorescence in situ hybridization (FISH) analysis. Antibodies specifically staining ERG have only recently been described (19). Early results reported a high sensitivity and
specificity (>95%) of immunohistochemically detectable ERG expression for the presence of TMPRSS2-ERG gene fusions (19). Since immunohistochemistry is much faster, less cumbersome to perform and sometimes easier to interpret than FISH, the availability of anti-ERG antibodies now drastically facilitates the assessment of ERG rearrangement status and the evaluation of its diagnostic, prognostic and predictive impact.

In this study we utilized a tissue microarray (TMA) composed of tissue samples from 3261 prostate cancer patients who had undergone radical prostatectomy. The aim of this study was to determine the prognostic significance of ERG status in a homogenously treated series of prostate cancers that were not exposed to anti-hormonal therapy. Taking advantage of a comprehensive database on molecular alterations in these tumors we were able to analyze molecular differences between ERG-positive and -negative prostate cancers.
Materials and Methods

Patients. Radical prostatectomy specimens were available from 3261 patients, treated in the Department of Urology, University Medical Center Hamburg-Eppendorf between 1992 and 2005 (Table 1). Follow-up data were available for 2891 patients, ranging from 1 to 219 months (mean 72 months). None of the patients received neo-adjuvant endocrine therapy. Additional (salvage) therapy was initiated in case of a biochemical relapse (BCR). In all patients, prostate specific antigen (PSA) values were measured quarterly in the first year, followed by biannual measurements in the second and annual measurements after the third year following surgery. Recurrence was defined as a postoperative PSA of 0.2 ng/ml and rising thereafter. The first PSA value above or equal to 0.2 ng/ml was used to define the time of recurrence. Patients without evidence of tumor recurrence were censored at the last follow-up. All prostatectomy specimens were analyzed according to a standard procedure. All prostates were completely paraffin-embedded, including whole-mount sections as previously described (20). All hematoxylin and eosin (H&E) stained histological sections from all prostatectomy specimens were reviewed for the purpose of this study and one 0.6 mm thick tissue core was punched out from a representative cancer area and transferred onto a TMA format as described (21). The 3261 cores were distributed among 7 TMA blocks each containing 129 to 522 tumor samples. From the resultant TMA paraffin blocks, serial histological sections were prepared. The first set of sections was routinely H&E-stained, while the second and third sets were stained for AMACR and 34BE12 to detect presence or absence of cancer in each tissue spot. A further set of sections was then used for ERG immunohistochemistry. For internal controls, each TMA block also contained different various control tissues, including normal prostate tissue.
Immunohistochemistry (IHC). Freshly cut TMA sections were analyzed in one day in one experiment for each antibody. The antibody ERG (clone EPR3864, dilution 1:450, Epitomics) was used for ERG protein detection. Slides were deparaffinized and exposed to heat induced antigen retrieval for 5 minutes in an autoclave at 121°C at pH7.8. Bound primary antibody was visualized using the DAKO EnVision™ Kit (DAKO). Only nuclear ERG staining was scored. For each tumor sample the staining intensity was judged on a scale of 0-4. Additional immunohistochemistry data have previously been generated on a different TMA containing different samples from the identical tumor blocks of the same tumor set by using the following antibodies (pretreatments and dilutions): AMACR (clone 13H4, DAKO, pH9, 1:200), annexin A3 (ANXA3, clone tgc7, TGCBiomics, pH2.5, 1:8100) (22), androgen receptor (AR, clone 2F12, Novocastra, pH9.0, 1:10), Bcl2 (clone 124, DAKO, pH9.0, 1:250), CD10 (MME, clone 56C6, Novocastra, pH9, 1:50) (23), CD166 (ALCAM, clone MOG/07, Novocastra, pH7.8, 1:2700), chromogranin A (CHGA, clone LK2H19, Biocare, 1:500), EGFR (clone 31G7, Zymed, Pronase type XIV, 1:100), FOXP1 (polyclonal, Abcam, pH9, 1:1350), HER2 (HercepTest, DAKO) (24), mTOR (polyclonal, Cell Signaling Technology, pH2.0, 1:75), p53 (clone D01, Oncogene, pH7.8, 1:3,600) (25), PSMA (clone 3E6, DAKO pH6.0, 1:150) (26), SSTR2 (somatostatin receptor 2, polyclonal, Atlas Antibodies, pH6.0, 1:150), synaptophysin (clone SY38, DAKO, pH9.0, 1:20). With the exception of some previously published immunohistochemistry data ((24), (22), (25), (23)) the following scoring system was used: The staining intensity (0, 1+, 2+, 3+) and the fraction of positive tumor cells were recorded for each tissue spot. A final score was built from these two parameters according to the following scores: Negative scores had staining intensity of 0, weak scores had staining intensity of 1+ in ≤70% of tumor cells or staining intensity of 2+ in ≤30% of tumor cells; moderate scores had staining intensity of 1+ in >70% of tumor cells, staining intensity of 2+ in >30% and ≤70% of tumor cells or staining intensity of 3+ in ≤30% of tumor.
cells and strong scores had staining intensity of 2+ in >70% of tumor cells or staining intensity of 3+ in >30% of tumor cells.

**Fluorescence in Situ Hybridization (FISH).** A two-color ERG break-apart FISH probe consisting of two BAC clones one each at 5' ERG (spectrum-green labeled RP11-95I21 and RP11-360N24) and the other at 3' ERG (spectrum-orange labeled RP11-720N21 and RP11-315E22) with approximately a 55-kb genomic gap between the two sets was made. One freshly cut 4µm TMA sections from block one of our TMA set containing 522 tumors was used for FISH analysis. The slide was deparaffinized and pretreated enzymatically using a commercial kit (Paraffin pretreatment reagent kit, Vysis) according to the manufacturer's instructions. For hybridization, the slide was dehydrated in 70%, 85% and 100% ethanol, air dried, and denaturated for 10 min at 72°C in 70% formamide-2 X SSC solution. After overnight hybridization at 3°C in a humidified chamber, the slide was washed and counterstained with 0.2 µM DAPI in antifade solution. The stained slide was manually interpreted with an epifluorescence microscope. Tumors were defined as “normal” when two pairs of overlapping red and green signals were seen per cell nucleus. An ERG translocation was assumed if at least one split signal consisting of separate red and green signals was observed per cell nucleus. An interstitial deletion of 5' ERG sequences was assumed if at least one green signal per cell nucleus was lost. Tumors were defined as FISH positive if ERG translocation and/or interstitial deletion were present in at least 60% of the tumor cell nuclei in the corresponding tissue spot.

LPL and c-MYC data were taken from a previous publication (27).

**Cell culture, transfection, and luciferase reporter assays.** The LNCaP (DSMZ), DU-145 (DSMZ), RWPE-1 (ATCC) and VCaP (ECACC) prostate/prostate cancer cell lines were obtained as indicated, subcultured according to the supplier’s instructions and stocks were
frozen at passage 2. Cell line identity verification procedures used can be found at the homepage of the respective supplier. Transfections were performed using polyethylene imine (Polysciences). For the reporter gene assay, 5x10⁴ cells were seeded on 24-well plates for transfection. Expression vectors for AR (PSG5-AR, kindly provided by Dr. Roland Schuele, University of Freiburg, Germany) and ERG (pMSCV-ERG, kindly provided by Dr. Pierre Pandolfi, Boston MA, USA) were co-transfected with a probasin luciferase reporter (probasin-Luc, kindly provided by Dr. Roland Schuele). Transfection efficiency was normalized by co-expression of renilla using pCMV-Renilla. Twenty-four h after transfection, the medium was changed and cells were further maintained in the medium containing charcoal-stripped 10% (vol/vol) FBS with or without 100 nM dihydrotestosterone (DHT) for 24 h. For depletion experiments, VCaP cells were cotransfected with probasin-Luc and pCMV-Renilla together with endoribonuclease-prepared siRNA (esiRNA) against green fluorescence protein (GFP), AR and ERG. Cells were lysed and tested using a dual luciferase assay kit (Promega) and a Berthold luminometer.

**Total RNA extraction and real-time quantitative PCR.** Total RNA from cells was extracted using Trizol and RNeasy system (Macherey-Nagel, Germany). RNA was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Darmstadt, Germany). Real time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as described previously (28). For all other genes Assay-on-Demand primer/probe sets supplied by Applied Biosystems were used (Assay IDs are available upon request). Relative expression was calculated by normalization to a selected housekeeper mRNA (GAPDH) by the DDCt method (29).

**Statistics.** Statistical calculations were performed with JMP statistical software (Version 8.0, SAS institute). Contingency tables were calculated with the \( \chi^2 \)-test and Fisher’s exact test to
analyze differences between groups. Survival curves were calculated by the Kaplan-Meier method and compared with the Logrank test.
Results

Technical issues. As in all TMA studies, a fraction of the cases were non-informative due to complete lack of tissue samples, absence of unequivocal cancer tissue, or non-interpretable FISH signals. The percentage of non-interpretable samples was 14.0% for ERG immunostaining in this study.

Immunohistochemistry. A positive ERG immunostaining could be observed in 52.4% (1469/2805) of cases. As previously described, small vessels staining could be used as internal controls for all samples (30). The staining was always nuclear and was observed in invasive cancer, in high grade PIN as well as in a subset of lymphocytes. Representative images are given in Figure 1A-C. The ERG staining intensity was considered 1+ in 18.2%, 2+ in 33.4%, 3+ in 42.9% and 4+ in 5.5% of the positive cases. Since there was no significant difference between ERG staining intensity and ERG gene rearrangement as detected by FISH as well as other parameters, ERG immunostaining intensity 1-4+ were combined as “positive” in subsequent statistical analyses (data not shown). Although ERG immunostaining was statistically associated with tumor stage (p=0.0003), and Gleason grade (p<0.0001), there was no clear trend to explain these p-values (Table 2). For example, ERG was particularly high in pT3a cancers as well as in the Gleason 3+4 group. Accordingly, ERG immunostaining was unrelated to the risk of BCR (p=0.1710, Figure 2A).

ERG expression by immunohistochemistry versus TMPRSS2-ERG fusion by FISH. A subset of 453 cancers was successfully analyzed by FISH on consecutive sections to those used for ERG IHC. There was an overall concordance of 95.8% between FISH and IHC. Two hundred thirty of 247 IHC ERG positive cancers showed ERG gene rearrangements by FISH.
(93.1%) while ERG gene rearrangements were only seen in 2 of 206 cancers (1.0%) with a negative ERG IHC result.

**Relationship with different molecular markers.** The majority of previously examined immunohistochemical stainings and FISH findings differed significantly between fusion-positive and fusion-negative prostate cancers. These include the expression of AR, AMACR, annexin A3, BCL2, CD10, CD166, chromogranin A, EGFR, HER2 and mTOR, p53 and synaptophysin. No significant differences between fusion and non-fusion type cancers were found for c-MYC amplification, SSTR2 expression, and LPL deletion. The results are summarized in Figure 3, which shows that the absolute differences between fusion and non-fusion cancers were mostly low, despite highly significant p-values. The strongest difference was found for AR expression, which was considered strong in 77.2% of ERG positive but in only 57.7% of ERG negative cancers (Figure 4). AR immunostaining was unrelated to the risk of BCR (p=0.5606, Figure 2B). Hence, the combination of AR and ERG immunostaining was also unrelated to the risk of BCR (p=0.0975, Figure 2C).

**Influence of ERG expression on AR-mediated transactivation in prostate cancer cell lines.** Given the strong association between ERG and AR expression in our IHC data, the relationship between these parameters was analyzed in cell lines. Among the different cell lines tested, VCaP was the only cell line, which resembled the prostate cancers in terms of AR and ERG expression. Specifically, the effect of ERG on AR-dependent transcription was further analyzed in ERG-negative LNCaP cells and ERG-positive VCaP cells (Figure 5, Panel A). After transfection, cells were incubated with or without dihydrotestosterone (DHT), and the AR-mediated transcriptional activity was monitored using the AR-dependent probasin promoter chemoluminescence assay. In LNCaP cells, a marked induction of AR-mediated transcriptional activity was observed after exposure to 100 nM DHT. The probasin
luminescence was strongly reduced in cells coexpressing AR and ERG as compared to AR or ERG alone (Figure 5 Panel B). Overexpression of AR and ERG showed no effect on the activity of the GAPDH promoter (data not shown). In order to extend these data we performed depletion experiments using an esiRNA-based approach in VCaP cells. VCaP is the only available prostate carcinoma cell line that harbors a TMPRSS2-ERG translocation and overexpresses AR (Figure 5, Panel C). Transfection of VCaP cells with esiRNA directed against AR resulted in abrogation of DHT-induced probasin luminescence. Inversely, depletion of ERG enhanced the probasin luminescence about 3-fold as compared to the controls. Overall, our data show the functional link between AR and ERG expression in VCaP cells and further indicate that ERG modulates the transcription of AR-dependent genes.
Discussion

The analysis of 2805 prostate cancers demonstrated ERG expression in 52.4%. This is in the range of previous studies, which found ERG expression in 48% (30), and 28% (31) by immunohistochemistry, in 50% by quantitative RT-PCR (32). Also TMPRSS2-ERG gene fusion by FISH has been described in 47-55% of prostate cancers (1, 7, 33, 34). In our study ERG expression analyzed by immunohistochemistry was strongly associated with the presence of ERG gene rearrangement detected by FISH at a concordance of 95.8%. This figure corresponds to recent data by Park et al. (30) describing a sensitivity of 95.7% and a specificity of 96.5% of immunohistochemistry for the identification of TMPRSS2-ERG fusions. Some of the few cases with ERG expression in the absence of a FISH detected fusion may be due to other molecular changes. Rare ERG fusions have also been found with other androgen dependent genes such as SLC45A3 (35) and NDRG1(36). We assume that the two cases with ERG rearrangement in the absence of detectable ERG expression represent IHC failures caused by, for example, suboptimal tissue fixation, a preanalytical problem that cannot always be avoided (37). It is also possible that ERG expression is halted in some fusion positive prostate cancers through specific molecular mechanisms such as inactivation of the androgen receptor pathway. It is noteworthy, however, that ERG expression was never seen in non-neoplastic prostate epithelium, either in this study or that of Park et al. (30). Given this specificity of ERG expression for neoplastic prostate epithelium, i.e. prostate cancer and high grade PIN, ERG immunostaining may indeed represent a highly diagnostic marker as previously suggested (30), albeit in 50% of the cases. Based on the current data, it appears possible, that ERG positive atypical small acinar proliferation (ASAP) may be judged as cancers in the future.
Our data demonstrate that the ERG status is unrelated to the clinical disease course in radically operated prostate cancers. There was no discernable difference with respect to PSA relapse between 1192 ERG-positive and 1086 ERG-negative cancers. Although ERG immunostaining was statistically associated with tumor stage (p=0.0003) and Gleason grade (p<0.0001) there was no clear trend despite these p-values and the absolute numbers differed only little. Previous studies investigating the possible clinical/prognostic relevance of ERG expression have described conflicting data. Studies analyzing between 59 and 445 cases with FISH or RT-PCR have reported fusion-positive cancers to be associated with either poor prognosis or more aggressive cancer (2, 12, 38, 39). In contrast, Saramaki et al. (15) have analyzed 253 cases with FISH and found fusion-positive cancers to be significantly associated with longer progression-free survival. In line with our study, several other investigations failed to detect a prognostic difference between fusion-negative and fusion-positive cancers in cohorts of 521 (18) and 214 (17) cancers. Our study on 2,891 cancers with clinical follow-up data used PSA recurrence as its clinical endpoint. We thus believe that our data strongly exclude a significant difference in the biologic behavior of ERG positive and ERG negative cancers in the absence of anti-hormonal or other systemic therapy.

It was the secondary aim of this study to determine, whether differences exist in molecular features that were previously discussed as potentially relevant for prostate cancer between fusion-positive and fusion-negative cancers. For this purpose, ERG data were compared with a variety of molecular features that were available from previous studies (22-26). Our analysis showed statistically significant differences for most analyzed features that were previously analyzed on our TMA, including androgen receptor (AR), AMACR, annexin A3, Bcl2, CD10, CD166, Chromogranin A, EGFR, HER2 and mTOR, p53 and synaptophysin. For most of these parameters the absolute differences were rather small, however. These data show, that pathway alterations involving these genes are neither essential nor exclusive for either one of
these two main prostate cancer subgroups. Molecular analyses have recently suggested several molecular aberrations that specifically differ between fusion positive and fusion negative cancers, such as PTEN deletions, which were found in a higher percentage of fusion positive cancers as compared to fusion negative cancers (8-11). Carver et al (9) found in 14 of 15 ERG FISH positive samples reduced or absent PTEN expression compared to 13 of 25 ERG FISH negative samples. Accordingly, King et al (8) found in 14 of 57 ERG FISH positive samples a PTEN loss compared to 3 of 64 ERG FISH negative samples. Expression screening studies had also described a number of molecular differences between fusion-positive and fusion-negative prostate cancer (40-42). However, most of the latter studies suggest only comparatively small changes in the expression levels of most individual differentially expressed genes. We have calculated the linear fold changes from the expression data provided by Jhavar et al. (41) and Taylor et al. (42) and found less than 2-fold expression differences in the vast majority of genes (Figure 6). Only 2 genes (CRISP3 and HLA-DMB) had a more than 3 fold expression difference between ERG positive and ERG negative tumors and one gene (TDRD1) had more than 4 fold expression difference between ERG positive and ERG negative tumors (42). Overall the available data seem to suggest that only a small number of genes show distinct difference between ERG positive and ERG negative tumors.

The androgen receptor plays a central role in prostate cancer development and progression. It is conceivable, that this may be especially true for fusion positive cancers, since the AR-dependent gene TMPRSS2 directly controls expression of the oncogenic ERG fusion protein. Previous studies have shown that the 5'fusion partners of ERG, such as TMPRSS2, are among the most androgen-responsive genes (43, 44). It is therefore not surprising, that the strongest difference between fusion positive and fusion negative cancers was found for AR expression in this study. This observation prompted us to further study the functional consequences of ERG-AR interaction. Downregulation of the AR-dependent probasin
promoter under the influence of ectopically induced ERG in LNCaP cells demonstrated that ERG could impair the expression of AR-mediated genes. This finding is consistent with the data reported by Yu et al. (45), showing a significantly reduced expression of AR as well as repression of AR dependent promoters such as those of TMPRSS2 and KLK3 in ERG overexpressing prostate cell lines. It is possible, that significantly elevated levels of AR in ERG expressing cancer cells in vivo, as shown by our immunohistochemical experiments, may reflect a compensatory receptor upregulation as a result of decreased levels of one or several AR regulated genes. A potential cross-talk between ERG rearrangement and AR signaling was also suggested by Rickmann et al. (46). Depending on the level of androgen signaling they found that ERG exerts dual activities on the expression of Trefoil factor 3 (TFE3), a differentially regulated gene with respect to ERG rearrangement.

Our data describing a strong association between ERG positivity and high AR expression levels is particularly interesting in the light of recent data suggesting a predictive relevance of ERG status for response to anti-androgen therapy. Attard et al. (47) demonstrated that men with androgen-resistant cancers had a higher maximal prostate-specific response when treated with the anti-androgen abiraterone acetate if the tumors were ERG positive compared to ERG negative tumors. This is also in the line with a recent study by Karnes et al. (48), which showed that patients with ERG positive tumors showed a more significant treatment effect in response to adjuvant androgen deprivation than patients with ERG negative tumors. This could be due to the fact, that ERG positive tumors are particularly dependent on a functional AR since ERG can only be overexpressed in the presence of AR. The lack of prognostic significance of ERG expression in our study using BCR as an endpoint does not rule out a predictive value of ERG for anti-androgen therapy. In prostatectomy patients, anti-androgen therapy will only be applied after BCR, which represents the clinical endpoint of our study.
Tumor heterogeneity is a major issue in tumor biology. Moreover, in case of prostate cancer over 90% of affected prostate glands contain multiple independent cancers (49, 50). As a consequence, heterogeneous molecular findings will often remain undetected in TMA studies, especially in case of multifocal or large cancer. Despite of this, earlier TMA studies have shown, that one core per tumor is sufficient for finding associations between molecular markers and clinico-pathological parameters in prostate cancer, at least if the TMAs contain large numbers of patient samples (22, 24-26, 51). Attempts to better represent prostate cancer in TMAs by taking up to 10 different cores from one selected donor block may not sufficiently improve representativity since the median number of tumor containing blocks ranges between 10 and 11 in our laboratory (unpublished data).

In summary, our data show that TMPRSS2-ERG fusion is unrelated to prostate cancer phenotype and that differences in many important molecular features are little between fusion positive and fusion negative cancers. The lacking influence of the ERG status on the risk of PSA recurrence after radical prostatectomy indicates that ERG does no affect the course of the disease as long as there is no systemic therapy. Up-regulation of AR in fusion-positive cancers could however argue for a specific response type to hormone therapy as suggested in some early studies.
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Figures

1. Representative images of ERG immunohistochemistry and ERG gene rearrangement detected by FISH. A) negative staining in prostate cancer, positive staining in adjacent blood vessels, B) positive staining in prostate cancer, negative staining in non-neoplastic prostate epithelium, C) positive staining in prostate cancer and adjacent high grade PIN, D) FISH image showing an ERG rearrangement.

2. Influence of (A) ERG immunostaining, (B) Androgen Receptor immunostaining and (C) combined ERG and Androgen Receptor immunostaining on PSA recurrence.

3. Molecular findings in ERG positive and ERG negative tumors. The figure describes previously collected molecular findings obtained by FISH and IHC using various methods according to specific properties to the analyzed gene/protein. Overall the data show that for the majority of analyzed parameters the difference between ERG positive and ERG negative tumors is rather low in absolute numbers but statistically significant. The color code varies between the analyzed parameter: AMACR, CD166, Chromogranin A (CHA), EGFR, mTOR, p53, SSTR2, synaptophysin (SYN): weak (dark grey), moderate (light grey), strong (medium grey). Bcl2: low (dark grey), high (light grey). CD10: apical (dark grey), cytoplasmatic (light grey), mixed staining (medium grey). C-Myc: gene polysomy (dark grey), gene gain (light grey), gene amplification (medium grey). HER2 immunostaining: 1+, 2+ and 3+ combined. Lipoprotein lipase (LPL): gene loss. annexin A3 (ANX3): weak (dark grey), moderate (light grey), strong staining (medium grey).

4. Comparison of Androgen Receptor (AR) expression levels in ERG positive and ERG negative prostate cancer. AR expression was considered strong in 77.2% of ERG positive cancers but in only 57.7% of ERG negative cancers.

5. Modulation of ligand induced AR-dependent transcription. Panel A: Expression levels of AR and ERG were determined in different prostate cancer cell lines by Taqman PCR. Relative expression was calculated by normalization to the housekeeper mRNA GAPDH.
Panel B: Effect of ERG, AR and AR+ERG overexpression on the activity of probasin promoter in LNCap cells. The bars demonstrate the promoter activity before (light grey) and after (dark grey) DHT stimulation. Mock, PIG (pMSCV-IRES-GFP; vector control), and PMLRARa (pMSCV) were used as controls. Two independent experiments are depicted (except for PIG).

Panel C: Effect of ERG, AR and AR+ERG depletion using endoribonuclease-prepared siRNAs (esiRNAs) on the activity of probasin promoter before (blue) and after (red) DHT stimulation in VCap cells. Mock and esiGFP were used as controls.

6. **Comparison of the linear fold changes of genes upregulated in fusion positive (ERG+) and fusion negative (ERG-) prostate cancers from published datasets.** The data from Taylor et al. (42) were re-analyzed to identify ERG+ and ERG- tumors according to the ERG expression levels. The data of 149 ERG-deregulated genes from Jhavar et al. (41) were taken from supplementary Table 1 and log. fold changes were converted to linear fold changes. ERG (lin. fold change = 9.4 in the Taylor data set and 1.4 in the Jhavar dataset) was excluded from analysis.
REFERENCES


Figure 1: Representative images of ERG immunohistochemistry and ERG gene rearrangement detected by FISH A) negative staining in prostate cancer, positive staining in adjacent blood vessels B) positive staining in prostate cancer, negative staining in non-neoplastic prostate epithelium C) positive staining in prostate cancer and adjacent high grade PIN D) FISH image showing an ERG rearrangement
Figure 2

Panel A: PSA recurrence free survival for ERG expression.
- 0 (n=887)
- 1 (n=177)
- 2 (n=328)
- 3 (n=415)
- 4 (n=50)

Panel B: PSA recurrence free survival for AR expression.
- Negative (n=97)
- Weak (n=179)
- Moderate (n=320)
- Strong (n=1266)

Panel C: PSA recurrence free survival for ERG/AR expression.
- ERG negative/AR low (n=334)
- ERG negative/AR high (n=464)
- ERG positive/AR low (n=206)
- ERG positive/AR high (n=687)

Statistical significances:
- ERG: p=0.1710
- AR: p=0.5606
- ERG/AR: p=0.0975
Figure 3
Figure 4

![Bar chart showing AR IHC staining intensities for negative and positive samples.]

- **AR IHC strong**
- **AR IHC moderate**
- **AR IHC weak**
- **AR IHC negative**

**ERG IHC**

- Negative (n=1131)
- Positive (n=1265)

**p<0.0001**
Figure 6: Comparison of the linear fold changes of genes upregulated in fusion positive (ERG+) and fusion negative (ERG-) prostate cancers from published datasets. The data from Taylor et al. (42) were re-analyzed to identify ERG+ and ERG- tumors according to the ERG expression levels. The data of 149 ERG-deregulated genes from Jhavar et al. (41) were taken from supplementary Table 1 and log fold changes were converted to linear fold changes. ERG (lin. fold change = 9.4 in the Taylor data set and 1.4 in the Jhavar dataset) was excluded from analysis.
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<th>Characteristic</th>
<th>No. on TMA n=3,261</th>
<th>No. (%) with complete follow-up n=2,891</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follow-up (months)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>72.1</td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td>68.9</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td>0.03-219</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>83 78 (94.0)</td>
<td></td>
</tr>
<tr>
<td>50-60</td>
<td>998 912 (91.4)</td>
<td></td>
</tr>
<tr>
<td>60-70</td>
<td>1.807 1699 (94.0)</td>
<td></td>
</tr>
<tr>
<td>&gt;70</td>
<td>175 169 (96.6)</td>
<td></td>
</tr>
<tr>
<td>Preoperative PSA (ng/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;4</td>
<td>513 478 (93.2)</td>
<td></td>
</tr>
<tr>
<td>4-10</td>
<td>1.673 1544 (92.3)</td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td>641 608 (94.9)</td>
<td></td>
</tr>
<tr>
<td>&gt;20</td>
<td>225 212 (94.2)</td>
<td></td>
</tr>
<tr>
<td>pT category (AJCC 2002)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT2</td>
<td>2.08 1907 (91.7)</td>
<td></td>
</tr>
<tr>
<td>pT3a</td>
<td>609 579 (95.1)</td>
<td></td>
</tr>
<tr>
<td>pT3b</td>
<td>372 361 (97.0)</td>
<td></td>
</tr>
<tr>
<td>pT4</td>
<td>42 42 (100.0)</td>
<td></td>
</tr>
<tr>
<td>Gleason Score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤3+3</td>
<td>1.426 1307 (91.7)</td>
<td></td>
</tr>
<tr>
<td>3+4</td>
<td>1.311 1238 (94.4)</td>
<td></td>
</tr>
</tbody>
</table>
Table 1: Clinicopathological features of the entire study cohort of 3,261 patients.

Patients with PSA recurrence: n=728 (25.2%); median PSA recurrence: 29.0 months.
Numbers do not always add up to 3,261 in the different categories because of cases with missing data (AJCC=American Joint Committee on Cancer)
Table 2: ERG expression and tumor phenotype

<table>
<thead>
<tr>
<th>pT category</th>
<th>all</th>
<th>ERG negative (%)</th>
<th>ERG positive (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pT2</td>
<td>3261</td>
<td>2805 47.6</td>
<td>52.4</td>
<td>0.0003</td>
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<tr>
<td>pT3a</td>
<td>2080</td>
<td>1740 50.6</td>
<td>49.4</td>
<td></td>
</tr>
<tr>
<td>pT3b</td>
<td>609</td>
<td>552 40.6</td>
<td>59.4</td>
<td></td>
</tr>
<tr>
<td>pT4</td>
<td>372</td>
<td>335 44.5</td>
<td>55.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>37   46.0</td>
<td>54.0</td>
<td></td>
</tr>
<tr>
<td>Gleason Score</td>
<td>≤3+3</td>
<td>1426 52.2</td>
<td>47.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>3+4</td>
<td>1311 41.8</td>
<td>58.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4+3</td>
<td>313 49.6</td>
<td>50.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥4+4</td>
<td>55   68.8</td>
<td>31.2</td>
<td></td>
</tr>
<tr>
<td>pN category</td>
<td>pN0</td>
<td>1544 45.5</td>
<td>54.5</td>
<td>0.8714</td>
</tr>
<tr>
<td></td>
<td>pN+</td>
<td>96   46.4</td>
<td>53.6</td>
<td></td>
</tr>
<tr>
<td>Preoperative PSA (ng/ml)</td>
<td>&lt;4</td>
<td>513 46.3</td>
<td>53.7</td>
<td>0.0488</td>
</tr>
<tr>
<td></td>
<td>4-10</td>
<td>1673 45.8</td>
<td>54.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10-20</td>
<td>641 52.2</td>
<td>47.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;20</td>
<td>225  51.0</td>
<td>49.0</td>
<td></td>
</tr>
</tbody>
</table>
ERG status is unrelated to PSA recurrence in radically operated prostate cancer in the absence of anti-hormonal therapy

Sarah Minner, Malaika Enodien, Huseyin Sirma, et al.

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