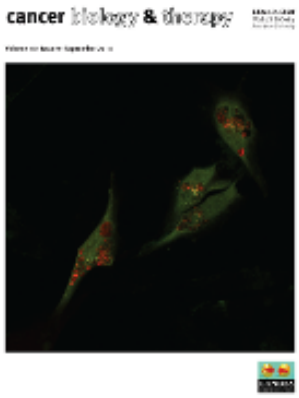



Cancer Biology & Therapy
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/kcbt20

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Published online: 27 Jun 2014.

To cite this article: Kuo-Cheng Huang, Mohammed Alshalalfa, Samar A Hegazy, Michael Dolph, Bryan Donnelly & Tarek A Bismar (2014) The prognostic significance of combined ERG and androgen receptor expression in patients with prostate cancer managed by androgen deprivation therapy, Cancer Biology & Therapy, 15:9, 1120-1128, DOI: 10.4161/cbt.29689

To link to this article: http://dx.doi.org/10.4161/cbt.29689

Cancer Biology & Therapy 15:9, 1120–1128; September 2014; © 2014 Landes Bioscience

# The prognostic significance of combined ERG and androgen receptor expression in patients with prostate cancer managed by androgen deprivation therapy

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**Keywords:** ERG protein expression, immunohistochemistry, Gleason score, tumor volume, hormone therapy, cancer specific mortality

ERG and androgen receptor (AR) are known to function cooperatively in prostate cancer (PCa) progression. However, the prognostic value of combined ERG and AR expression and potential pathways are not well characterized. We assessed ERG and AR protein expression by immunohistochemistry in a cohort of 312 men with PCa diagnosed by transurethral resection of the prostate (TURP). Patients were divided into those with no prior hormonal treatment (designated as PCa/AdvPCa) vs. those with castrate-resistant PCa (CRPC) undergoing channel TURP to relieve obstructive symptoms. The expression status was correlated with various clinical-pathological parameters. The Swedish watchful-waiting cohort was used for validation and characterization of potential gene signatures associated with ERG and AR.

Patients with combined ERG-positive/AR high expression profile demonstrated higher rates of PCa-specific mortality (PCSM) compared with patients with ERG-negative/AR low in patients with no prior treatment ($n = 90$, $P = 0.032$), but this was attenuated in the overall cohort which included the CRPC subgroup ($n = 125$, $P = 0.096$). The prognostic significance to PCSM was validated in the Swedish watchful waiting cohort in univariate (HR: 3.3; 95% CI: 1.9–5.6, $P = 4.25E–5$) and multivariate analysis (HR: 2; 95% CI: 0.97–4.1, $P = 0.057$), which included Gleason score. ERG/AR overexpression status characterized 152 genes signatures including WNT, PI3K/AKT and chemokine signaling pathways known to be deregulated in PCa.

In conclusion, combined ERG/AR overexpression signifies a class of patients at highest-risk of PCSM with specific key genetic alteration likely responsible for disease progression. The prognostic value of combined ERG/AR overexpression and its associated genes should be further investigated as potential prognostic and therapeutic targets in prostate cancer progression.

## Introduction

Prostate cancer (PCa) progression and the development of castration-resistant prostate cancer (CRPC) remains a major issue in patients with this disease. Currently, the relationship between emerging molecular markers and disease prognosis and their potential therapeutic role remain to be elucidated. One of the most common genetic alterations in PCa relates to gene rearrangements between the androgen receptor-regulated gene *TMPRSS2* (21q22.3) and other members of the *ETS* family member of transcription factor, commonly *ERG* (21q22.2). The potential prognostic value and role of *ERG* gene rearrangement in defining molecular subtypes of PCa has been investigated in various cohorts.[1-5] To date, it is suggested that *ERG* is associated with adverse clinical outcome mainly in non-surgical (i.e., expectant and watchful waiting) cohorts compared with surgical cohorts, where it associate with little or no prognostic value.[6-14] Recently, ERG protein expression assessed by immunohistochemistry has been documented as surrogate to *ERG* gene rearrangement.[15,16]

The androgen receptor (AR) is a known key mediator of disease progression.[17] Although androgen ablation is initially very effective treatment causing temporary tumor regression, this is usually followed by disease progression into aggressive and lethal CRPC. Recent investigations have demonstrated that one of the key mediators in driving progression to CRPC is AR reactivation and, in keeping with this, AR protein expression and increased mRNA levels.[18-23]

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Submitted: 04/16/2014; Revised: 06/05/2014; Accepted: 06/22/2014; Published Online: 06/27/2014
http://dx.doi.org/10.4161/cbt.29689

The development of CRPC, involves accumulation of multiple genetic alterations leading to perturbation of several tightly interacting signaling pathways.[17] For example, it is known that *AR*, *ERG*, and *PTEN* genomic aberrations interplay in PCa and are responsible in part for the occurrence of CRPC.[24] For instance, *PTEN* loss has been associated with increased frequency of alterations in the PI3K signaling pathway. Furthermore, selective inhibition of either the PI3K or AR signaling pathways leads to activation of the other due to the relief of feedback inhibition, thereby giving rise to therapeutic resistance in treatments that target just one of these signaling pathways.[25,26] In support, inhibiting both PI3K and AR signaling pathways achieves greater tumor regression, suggesting that combined therapeutic modalities may be required in treating CRPC.[25] Similar reciprocal interaction has been demonstrated experimentally for *ERG* which is under the regulation of AR activity, but *ERG* can in turn regulate AR to promote tumor invasion.[1,27] It is documented that upregulation of either *ERG* or *AR* alone is not sufficient for promotion of tumor progression,[28] suggesting that multiple genetic perturbations and selective cooperation between multiple genes interact in PCa progression.

Based on this tight interaction, we hypothesized that combined assessment of the expression status of *ERG* and *AR* in PCa may be more biologically and clinically relevant than analysis of either one alone. In this study, we investigated the prognostic significance of combined ERG/AR protein expression in a non-surgical cohort consisting of locally advanced PCa patients diagnosed by transurethral resection of prostate (TURP) (designated as PCa/AdvPCa) and locally advanced CRPC patients treated by LH-RH (luteinizing hormone-releasing hormone) agonist, undergoing channel TURP to relieve obstructive symptoms.

## Results

### Study population

Mean patients' age for the overall cohort was 76.5 y (range 50.7–93.5 y) with average follow-up time of 23.45 mo (range 1.5–100.2 mo). Data in regards to disease progression in terms of time of hormonal therapy or surgical intervention to relieve symptomatic obstruction (channel TURP) as well as overall and cancer specific mortalities are recorded. A subgroup of the overall cohort (119/312 [38.14%]) of patients had received prior hormonal therapy (designated as CRPC) and the remainder of the patients (193/312 [61.86%]) received no prior hormonal therapy (designated as PCa/AdvPCa). A total of 299/312 (95.8%) and 292/312 (93.5%) patient's samples were available for ERG and AR assessment, respectively. None of the patients in the cohort had received radical prostatectomy. **Table 1** demonstrates patient demographics of the subgroups analyzed. There was significant difference between the two subgroups of patients in relation to PCSM, GS and tumor volume. There was a marginal significance toward higher frequency of ERG positivity in the CRPC subgroup vs. the PCa/AdvPCa subgroup ($P$ = 0.057). No difference was noted in AR expression distribution.

**Table 1.** Patients' demographics in the PCa and CRPC subgroups

| | PCa/AdvPCa | CRPC | *P* value |
|---|---|---|---|
| *PCSM* | | | |
| Yes | 24 (10.2%) | 33 (47.8%) | < 0.001 |
| No | 211 (89.8%) | 36 (52.2%) | |
| *GS* | | | |
| <7 | 99 (41.8%) | 1 (1.4%) | < 0.001 |
| 7 | 46 (19.4%) | 4 (5.8%) | |
| 7 (3+4) | 31 (13.1%) | 0 | |
| 7 (4+3) | 15 (6.3%) | 4 (100%) | |
| >7 | 92 (38.8%) | 64 (92.8%) | |
| *Volume* | | | < 0.001 |
| = <5% | 49 (28.0%) | 0 | |
| >5% | 126 (72.0%) | 46 (100%) | |
| *ERG* | | | 0.057 |
| Negative | 179 (77.2%) | 44 (65.7%) | |
| Positive | 53 (22.8%) | 23 (34.3%) | |
| *AR* | | | 0.857 |
| Low | 108 (48.0%) | 33 (49.3%) | |
| High | 117 (52.0%) | 34 (50.7%) | |

*Not all cases have available information. Low AR refers to negative, weak, and moderate intensity combined. PCSM, prostate cancer specific mortality.

### ERG and AR expression in the incidental/advanced and castration resistant prostate cancer cohorts

Due to differences in the clinical-pathologic characteristics and possible treatment-mediated alteration in ERG and AR expression and/or activity, we sought to investigate the prognostic significance of ERG and AR in the two subgroups separately. Based on individual tissue microarray (TMA) core sample expression, there was no difference in the proportion of cases with high AR expression between ERG-positive and -negative PCa in the overall patients cohort. Specifically, 59/127 (46.5%) vs. 231/429 (53.9%) of cores showed high AR expression in ERG-positive and ERG-negative TMA cores, respectively ($P$ = 0.143). This association was marginal in the PCa/AdvPCa subgroup of patients, where 34/86 (39.5%) vs. 149/293 (50.9%) of TMA cores exhibited high AR expression in ERG-positive and -negative cases, respectively ($P$ = 0.065). In contrast, the CRPC subgroup did not demonstrate such association (25/41 [61.0%] vs. 38/79 [48.1%]) ($P$ = 0.186).

### Combined ERG and AR protein expression status in relation to prostate cancer specific mortality, overall survival and disease progression

A total of 57/304 (18.7%) of patients experienced PCSM. As noted above, a significant difference existed between the PCa/AdvPCa and CRPC subgroups (10.2% vs. 47.8%) ($P$ < 0.001). Neither ERG nor AR expression by itself was significantly associated with higher-rate of PCSM in either the overall or any subgroup of the study cohort. In contrast, within the total cohort, patients with tumors exhibiting ERG-positive/AR high showed non-significant trend toward higher-rate of PCSM compared

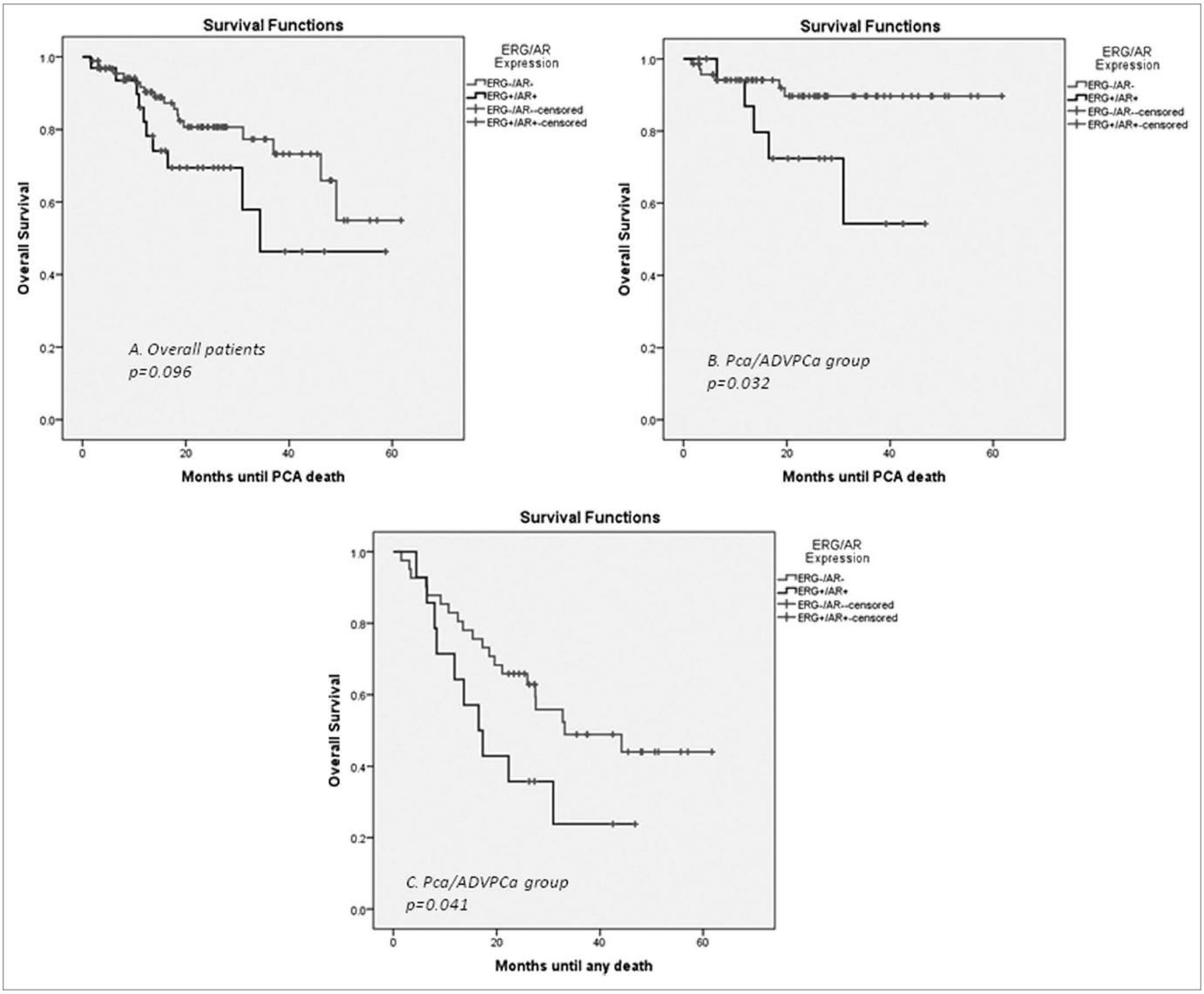


**Figure 1.** Kaplan–Meier survival curves. (**A**) Kaplan–Meier survival curves to cancer specific mortality of PCa patients with ERG-positive/AR high expressing tumors compared with ERG-negative/AR low expressing tumors in the overall study cohort ($n$ = 125, $P$ = 0.096). (**B**) Kaplan–Meier survival curves of cancer specific mortality of PCa patients with ERG-positive/AR high vs. ERG-negative/AR low expression in the subgroup of patients with PCa/AdvPCa (i.e., no prior hormonal treatment) ($n$ = 90, $P$ = 0.032). (**C**) Kaplan–Meier survival curves to overall mortality of PCa patients with ERG-positive/AR high vs. ERG-negative/ AR low expression in the PCa/AdvPCa patients group ($n$ = 90, $P$ = 0.041).

with patients with ERG-negative/AR low status (10/33 [30.3%] vs. 18/92 [19.5%]) ($P$ = 0.096) (**Fig. 1A**). The incidence of PCSM was comparable between patients whose tumors were ERG-positive/low AR, ERG-negative/high AR and ERG-negative/low AR; (12.8%, 15.2%, and 18.4%) vs. 29.4% in ERG-positive/AR high. The association between ERG-positive/AR high expression profile and PCSM was more significant in the PCa/AdvPCa subgroup compared with ERG-negative/AR low (5/19 [26.3%] vs. 6/71 [8.4%]) ($P$ = 0.032) (**Fig. 1B**). Assessing the overall mortality in this subgroup, patients with ERG-positive/high AR tumors still showed significantly worse overall survival compared with patients with ERG-negative/low AR tumors (10/19 [52.6%] vs. 20/71 [28.2%]) ($P$ = 0.041) (**Fig. 1C**). No significant difference in PCSM was noted in the CRPC subgroup between ERG-positive/AR high and ERG-negative/low AR (5/14 [35.7%] vs. 12/21 [57.1%], respectively) ($P$ = 0.515). Moreover, patients with ERG-positive/high AR tumors had no significant difference in disease progression in terms of the time until additional treatments are needed for symptomatic relief (i.e., TURP for CRPC patients and hormonal therapy for PCa/AdvPCa patients) compared with patients with ERG-negative/low AR expression in either the PCa/AdvPCa ($P$ = 0.75) or in CRPC subgroup ($P$ = 0.29).

**Identification of genes associated with ERG-positive/high AR status**

To define the molecular signature of ERG-positive/AR high PCa, we utilized the publicly available Swedish GSE8402 cohort data sets. We first defined 197 genes as ERG signature, 272 genes

as AR signature, and 270 as ERG/AR signature. The ERG and AR signatures had only 9 genes in common (**Fig. 2**). One hundred fifty-two mostly upregulated genes were specific to the combined ERG+/high AR expression status and were designated as ERG-positive/high AR specific signature. The top 10 genes, their expression direction pattern, and known involved pathways are shown in **Table 2**.

The 152 signature genes were analyzed to construct a functional protein network using the Reactome FI plug-in implemented in Cytoscape[29] (**Fig. 3**) which showed these genes to be grouped into non-random highly interconnected functional modules, suggesting biologically relevant pathways that are perturbed in ERG-positive/high AR tumors. The major biological pathways deregulated in the ERG-positive/AR high PCa includes WNT, cell cycle, VEGF, and PI3K/AKT signaling pathways.

**Validation of the prognostic value of the combined ERG-positive/high AR status in relation to prostate-cancer specific mortality**

We utilized the Swedish cohort to validate our ERG-positive/AR high signature, which documented significant association with poor outcome and lethal disease. Specifically, 25/26 PCa patients with ERG-positive/high AR had lethal outcome compared with 40/62 in the ERG-negative/AR low group. ERG-positive/high AR samples were significantly at higher-risk of PCSM compared with ERG-negative/AR low. HR: 3.3; 95% CI: 1.9–5.6, $P = 4.25E{-}5$ (**Fig. 4**). There was no prognostic difference between tumors with ERG-negative/low AR, ERG-positive/low AR, or ERG-negative/high AR status, confirming our own data above that the concomitant expression of ERG and AR defines a higher-risk group of patients (not shown).

In multivariable analysis, ERG-positive/AR high was still marginally significant and showed better correlation with the clinical outcome (HR: 2; 95% CI: 0.97–4.1. $P = 0.057$) compared with either ERG (HR: 1.39; 95% CI: 0.78–2.45, $P = 0.254$) or AR (HR: 0.89; 95% CI: 0.62–1.27, $P = 0.528$) expression alone. However, it did not outperform GS (**Table 3**).

## Discussion

Previous studies had shown that *ERG* gene rearrangements and protein expression are significantly associated with lethal PCa in none surgically treated patients.[12,13] AR expression and amplification have been found to play a major role in PCa progression and the development of CRPC.[30] Moreover, a strong reciprocal interaction was demonstrated experimentally between ERG and AR,[1,27] suggesting that both cooperate functionally in PCa progression.

Although ERG-positive/AR high expression profile conferred adverse clinical outcome compared with ERG-negative/low AR tumors in our PCa/AdvPCa subgroup, this difference was attenuated when including the CRPC subgroup which may suggest that the prognostic value of the two combined markers is more relevant in tumors not initially subjected to hormonal manipulation. However, as previously documented, it is likely that ERG and AR intensity levels are altered by the prior hormonal therapy within the CRPC group.[15] Moreover, our inability to document any differences in disease progression in terms of the time until the requirement for additional therapy to relief symptoms may be reflective of the limited number of patients within each subgroup ($n = 52$, 23, and 31) given the higher number of overall and PCSM mortality rates noted above. Therefore, it is hypothesized that patients with ERG-positive/AR high expression profile signify a subgroup of PCa patients with higher-risk of disease progression and likely require more aggressive management.

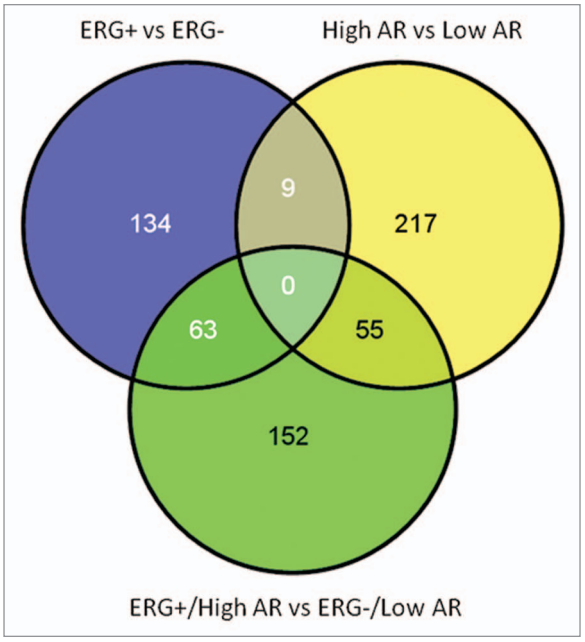


**Figure 2.** Venn diagram showing the intersection among the ERG, AR, and ERG$^+$/AR high signature. Only 9 genes were in common between ERG and AR signatures. When combining the status of ERG and AR, 270 genes were identified using ROC analysis; 152 genes were overlapped with ERG or AR signatures.

Experimental observation from recent reports suggest that neither ERG nor AR overexpression alone is sufficient to enhance PCa progression and that both are required for disease progression.[28] Our data documenting insignificant prognostic difference in any other ERG/AR expression combination is further support of the recent in-vitro data documenting attenuation of the aggressive behavior of PCa cells by targeting the ERG, AR and WNT pathways.[31,32]

Finally, as a first step toward elucidating the mechanistic basis of this aggressive PCa molecular subtype, we defined 152 signature genes specific to ERG-positive/AR high PCa (**Fig. 3**). Based on known function and interactions, these genes are grouped into non-random highly interconnected functional modules (**Fig. 3**), as most of the major biological pathways involved by these genes, including WNT,[32,33] VEGF, PTEN/PI3K/AKT/mTOR,[24,34] and chemokine[35] signaling pathways, have previously been implicated in various stages of PCa progression and many have been found to interact with ERG and AR. Indeed,

**Table 2.** Top 10 differentially expressed genes related to ERG/AR

| Gene | Description | Direction of expression in ERG-positive/AR high PCa | Pathways |
|---|---|---|---|
| CDK2AP1 | Cyclin-dependent kinase 2 associated protein 1 | Up | Cell cycle |
| DRG2 | Developmentally regulated GTP binding protein 2 | Up | Cell growth and differentiation |
| PSMB10 | Proteasome subunit, beta type, 10 | Down | Ubiquitin mediated proteolysis |
| RAB3B | Member RAS oncogene family | Up | Protein transport and tight junctions |
| EPM2AIP1 | EPM2A (Laforin) interacting protein 1 | Up | Unknown |
| SACM1L | SAC1 suppressor of actin mutations 1-Like | Down | Synthesis of PIPs at the ER membrane |
| FUBP1 | Far upstream element (FUSE) binding protein | Up | Myc regulation |
| SEC13 | Sec13 homolog | Up | RNA transport and mTOR signaling |
| GPRASP1 | G protein-coupled receptor associated sorting protein 1 | Up | G-protein mediated lysosomal degradation |
| TUBGCP3 | Tubulin, gamma complex associated protein 3 | Up | $G_2$/M transition and cell cycle |

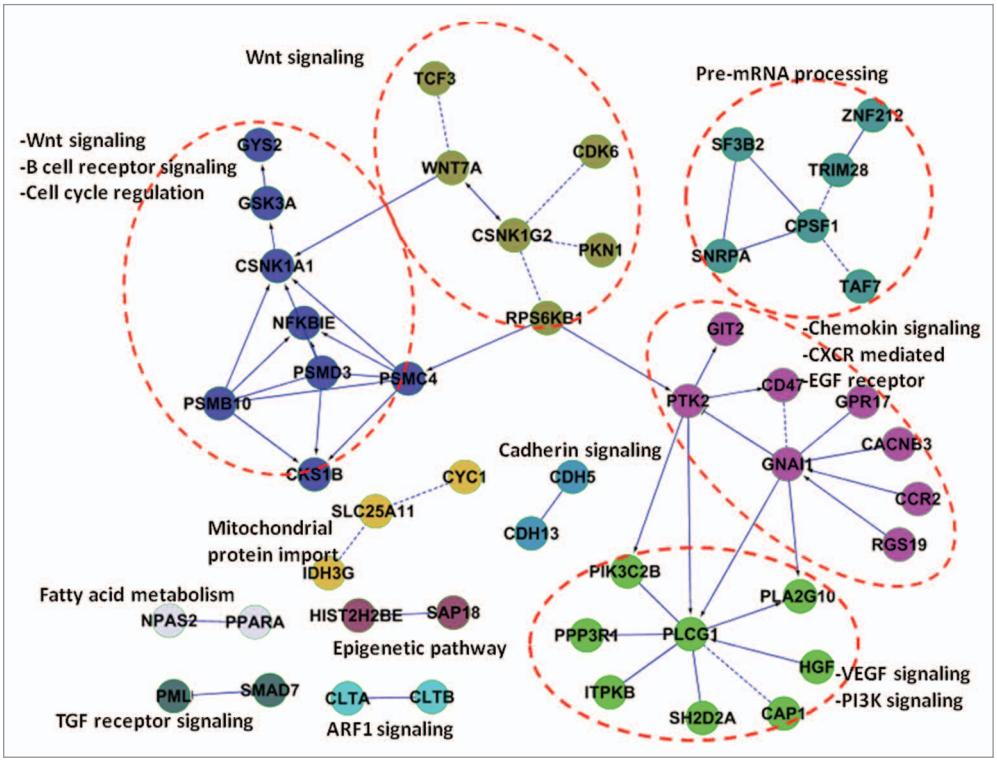


**Figure 3.** Functional protein network of the 152 genes with enriched pathways. The 152 genes are functionally related as they are highly interacting. The genes are enriched in pathways such as Wnt pathway, cell cycle, EGF, and PI3K signaling.

analysis of the individual signature genes suggest that ERG and AR combined expression leads to deregulation of multiple cellular processes including, cell cycle, cell growth and differentiation, transcriptional regulation, specific protein turnover as well as intra- and extra-cellular signaling (**Table 2**). However, thus far only some of the identified genes have been studied in the context of prostate cancer biology. Notably, we have found the gene encoding a CDK2 inhibitor *CDK2P1* to be upregulated as an ERG-positive/AR high gene signature. Overexpression of a *CDK2AP1* had been found in one study to lead to a reduction in cellular growth in vitro using different prostate cancer cell lines.[36] However, this has not been studied in vivo and the effect of *CDK2AP1* expression may be dependent on complex interaction between the cell cycle and the AR pathway since this same study also found CDK2AP1 expression to correlate positively with AR re-expression in certain cell line.

*RAB3B* is another ERG-positive/AR high gene signature that is upregulated. *RAB3B* is a member of the RAB GTPase family and is overexpressed in prostate cancer patients. Moreover, overexpression of RAB3B via a transcriptional regulatory network composing of AR and other transcription factors iNKX3-1 (NK3 homeobox 1) and FoxA1 (Forkhead box A1) has been found to promote prostate cancer cell survival.[36,37] The reason why both putative tumor-suppressors *CDK2AP1* and *RAB3B* are upregulated in the more aggressive ERG-positive/AR high prostate cancer in the present study is not clear. However, it is not uncommon for clinically aggressive and therapeutically resistant cancers to overexpress tumor-suppressors genes that have initially been implicated to function as tumor suppressors in vitro and in mouse xenograft models. This is illustrated in the case of the putative tumor suppressor gene Drg1 (differentiation-related gene 1), the expression of which has been associated with aggressive hepatocellular carcinoma and resistance to irinotecan chemotherapy in colorectal cancer.[38]

Other ERG-positive/AR high gene signatures are implicated in regulation of gene expression. For instance, *FUBP1* (far-upstream element binding protein 1) is an upregulated ERG-positive/AR high gene signature that encodes a protein which had been found to regulate c-myc proto-oncogene transcription and to be frequently expressed in prostate cancer.[39] Although the study failed to document any association with clinic-pathological variables or c-myc protein expression, its role in prostate cancer biology remains to be elucidated since the FUBP family of proteins has been shown to bind a variety of RNAs suggesting functions other than *c-myc* expression regulation.[39]

In conclusion, we characterize and validate a molecular subtype of prostate tumors combining ERG and AR overexpression in association to PCa progression in terms of PCa-specific as well as overall mortality. We further identifies gene signatures associated with ERG-positive/AR high expression profile and show that these can be grouped into non-random functionally interacting modules affecting pathways implicated in ERG and/or AR-mediated PCa progression. Patients with ERG-positive/AR high tumors are at higher-mortality risk and should be managed more aggressively. Our findings are in keeping with the strong cooperatively between ERG and AR in PCa and underpin the need for combined gene analysis in achieving better PCa prognostication.

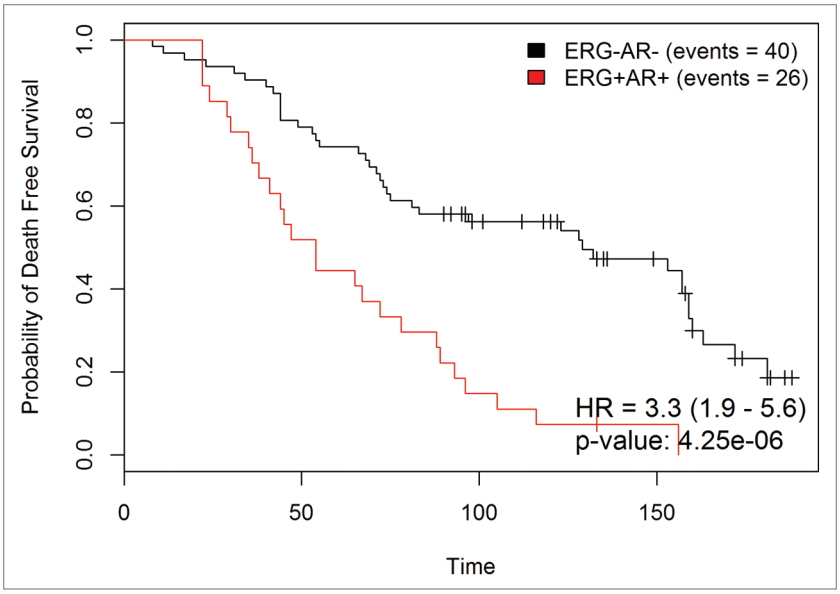


**Figure 4.** Kaplan–Meier survival curve (Swedish cohort: GSE8402) showing that PCa samples with ERG-positive/high AR expression profile are at higher risk of cancer specific deaths (HR:3.3 [1.9–5.6],*P* = 4.25E-6) compared with PCa samples with ERG-negative/low AR expression profile.

**Table 3.** Multivariate analysis of GS, ERG, AR, and ERG/AR overexpression to cancer specific mortality in the Swedish cohort

| | HR (CI) | *P* value |
|---|---|---|
| AR | 0.89 (0.62–1.27) | 0.5289 |
| ERG | 1.39 (0.78–2.45) | 0.2549 |
| ERG/AR | 2.0 (0.97–4.1) | 0.0575 |
| Gleason (≤7 vs. >7) | 3.3 (2.44–4.47) | 6.00e–15 |

## Materials and Methods

### Study population and tissue microarray construction

The study cohort consisted of 312 men diagnosed with PCa by TURP and managed expectantly by observation, radiotherapy or hormonal manipulation between 2005 and 2009. To assess the relation and interaction with hormonal therapy, we subdivided the cohort into two subgroups. The first ($n$ = 193), consisted of tumors with no prior hormonal therapy, referred to asPCa/AdvPCa. The second group represented men with CRPC ($n$ = 119), who were initially treated by LH-RH agonist as monotherapy, but subsequently progressed to require channel TURP to relieve obstructive symptoms. Clinical follow-up was collected from the Alberta Tumour Registry in regard to overall survival, cancer-specific mortality, and dates of hormonal treatment implementation. Prostate cancer-specific mortality (PCSM) was defined as patients with evidence of metastatic disease who progressed while on hormonal therapy and died of PCa based on medical records. The complete cohort was assembled onto two tissue microarrays (TMAs) with an average of two cancer cores (2–6) per patient including adjacent benign prostate tissue as control for a total of 714 cores using a manual tissue arrayer (Beecher Instruments). Each block was assembled without prior knowledge of any clinical or pathological staging information. All Clinical and pathological data were obtained with approval of the institutional review board at University of Calgary, Faculty of Medicine.

### ERG and AR protein expression assessment by immunohistochemistry

ERG immunohistochemistry was performed as previously described.[13] AR immunohistochemistry was performed on Leica Bond Max platform (Leica Microsystems). Four µm thick formalin-fixed paraffin-embedded sections were subjected to heat-induced antigen retrieval for 30 min using Leica Epitope Retrieval Solution 2. Slides were then incubated with androgen receptor mouse monoclonal antibody from Santa Cruz clone AR441 (sc-7305) for 15 min at a 1:200 dilution. Bond Polymer Refine Detection kit (Leica Microsystems) was used for HRP detection following hematoxylin counter stain. The correlation between AR gene amplification status and AR protein expression in prostate cancer as assessed by immunohistochemistry was previously demonstrated in Sicar et al.[40]

### Pathological analysis

Histological diagnosis of TMA cores were confirmed by two study pathologists (SAH and TAB) on the initial slides. GS was assessed according to the 2005 ISUP criteria.[41] For each patient, the predominant two patterns of PCa were sampled. ERG

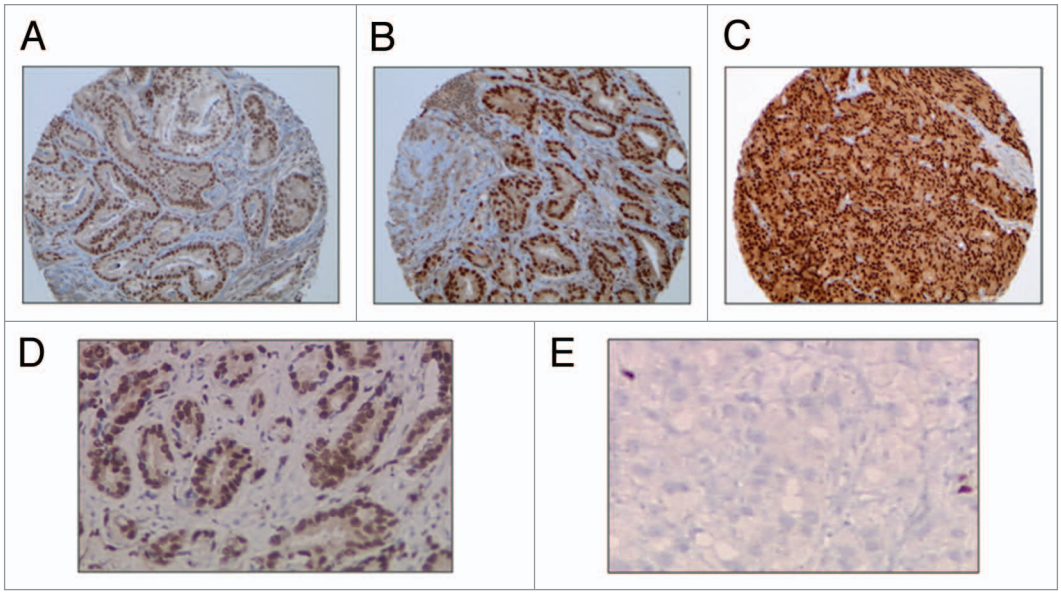


**Figure 5.** Representative images of ERG and AR immunohistochemistry in prostate cancer. (**A**) AR low expression in a Gleason score 6 PCa, (**B**) AR high expression in a Gleason score 6 PCa. (**C**) AR high expression in a Gleason score 8 PCa. (**D**) ERG-positive expression in a Gleason score 6 PCa. (**E**) ERG-negative expression in a PCa (note endothelial cells positivity acting as internal control).

immunohistochemistry was assessed as positive vs. negative based on previous correlation with *ERG* gene rearrangement detected by break apart fluorescent in situ hybridization assay.[15] ERG expression was strongly and consistently expressed in endothelial cells, acting as internal control. AR immunohistochemistry was categorized as high vs. other lower intensities (**Fig. 5**). Specifically, the AR immunostaining intensity was graded semi-quantitatively on a grading scale of 0–3 by two pathologists who assigned a grade of 3 to the highest intensity of a given sample, grade 1 to the lowest staining intensity above faint non-specific stromal and endothelial cell staining, and grade 2 to staining intensities between grade 1 and 3. Grade 0 was assigned to those samples with staining intensity no higher than the non-specific staining in the stroma. The high AR PCa expressers were designated as those with immunostaining intensity grade 3, and the low AR PCa expressers were those with immunostaining intensity grades 0–2.

**Development of ERG/AR signature**

The Swedish GSE8402 cohort ($n$ = 281) data sets was used to define a molecular signature for ERG and AR[42] (Information regarding this cohort can be accessed through the NCBI Gene Expression Omnibus site http://www.ncbi.nlm.nih.gov using the GEO accession identification number GSE8402). Forty-four samples were ERG rearranged (ERG-positive) as determined by fluorescent in situ hybridization (FISH) and 190 expressed high AR mRNA as determined by PAM clustering implemented in cluster R package. ROC area under curve was used to filter signature genes differentially expressed between ERG-positive vs. ERG-negative, high vs. low AR and ERG-positive/AR high vs. ERG-negative/AR low PCa. AUC 0.7 was used as a threshold to filter significant genes. As a result; 152 genes were identified in the ERG-positive/AR high subgroup. The Reactome Functional Interaction plug-in in Cytoscape[29] was used to build the network between these genes to better understand the systems biology behind the combination of ERG and AR overexpression in PCa.

**Statistical analysis**

Patient characteristics were presented as frequencies and percentages for categorical variables, and as means and ranges for continuous variables. Chi-square test was used to test for associations between ERG and AR protein expression. The Kaplan–Meier approach along with the log-rank test was used for survival analyses to test associations between ERG and AR expression and each of PCSM overall mortality, and time till development of castrate resistant disease. Multivariate analysis was conducted using Cox regression models including ERG, AR and GS to associate variables to lethal disease. In all statistical tests a $P$ value < 0.05 was considered significant.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Acknowledgments

This work was supported in part by the Prostate Cancer Foundation Young Investigator Award (T.A.B.). This work was also supported by Prostate cancer Canada and is proudly funded by the Movember Foundation (B2013-01).

## References


1. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, Varambally S, Cao X, Tchinda J, Kuefer R, et al. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 2005; 310:644-8; PMID:16254181; http://dx.doi.org/10.1126/science.1117679
2. Soller MJ, Isaksson M, Elfving P, Soller W, Lundgren R, Panagopoulos I. Confirmation of the high frequency of the TMPRSS2/ERG fusion gene in prostate cancer. Genes Chromosomes Cancer 2006; 45:717-9; PMID:16575875; http://dx.doi.org/10.1002/gcc.20329
3. Tomlins SA, Mehra R, Rhodes DR, Smith LR, Roulston D, Helgeson BE, Cao X, Wei JT, Rubin MA, Shah RB, et al. TMPRSS2:ETV4 gene fusions define a third molecular subtype of prostate cancer. Cancer Res 2006; 66:3396-400; PMID:16585160; http://dx.doi.org/10.1158/0008-5472.CAN-06-0168
4. Helgeson BE, Tomlins SA, Shah N, Laxman B, Cao Q, Prensner JR, Cao X, Singla N, Montie JE, Varambally S, et al. Characterization of TMPRSS2:ETV5 and SLC45A3:ETV5 gene fusions in prostate cancer. Cancer Res 2008; 68:73-80; PMID:18172298; http://dx.doi.org/10.1158/0008-5472.CAN-07-5352
5. Hermans KG, van der Korput HA, van Marion R, van de Wijngaart DJ, Ziel-van der Made A, Dits NF, Boormans JL, van der Kwast TH, van Dekken H, Bangma CH, et al. Truncated ETV1, fused to novel tissue-specific genes, and full-length ETV1 in prostate cancer. Cancer Res 2008; 68:7541-9; PMID:18794142; http://dx.doi.org/10.1158/0008-5472.CAN-07-5930
6. Rubin MA, Maher CA, Chinnaiyan AM. Common gene rearrangements in prostate cancer. J Clin Oncol 2011; 29:3659-68; PMID:21859993; http://dx.doi.org/10.1200/JCO.2011.35.1916
7. Tomlins SA, Bjartell A, Chinnaiyan AM, Jenster G, Nam RK, Rubin MA, Schalken JA. ETS gene fusions in prostate cancer: from discovery to daily clinical practice. Eur Urol 2009; 56:275-86; PMID:19409690; http://dx.doi.org/10.1016/j.eururo.2009.04.036
8. Reid AH, Attard G, Ambroisine L, Fisher G, Kovacs G, Brewer D, Clark J, Flohr P, Edwards S, Berney DM, et al.; Transatlantic Prostate Group. Molecular characterisation of ERG, ETV1 and PTEN gene loci identifies patients at low and high risk of death from prostate cancer. Br J Cancer 2010; 102:678-84; PMID:20104229; http://dx.doi.org/10.1038/sj.bjc.6605554
9. Nam RK, Sugar L, Yang W, Srivastava S, Klotz LH, Yang LY, Stanimirovic A, Encioiu E, Neill M, Loblaw DA, et al. Expression of the TMPRSS2:ERG fusion gene predicts cancer recurrence after surgery for localised prostate cancer. Br J Cancer 2007; 97:1690-5; PMID:17971772; http://dx.doi.org/10.1038/sj.bjc.6604054
10. Saramäki OR, Harjula AE, Martikainen PM, Vessella RL, Tammela TL, Visakorpi T. TMPRSS2:ERG fusion identifies a subgroup of prostate cancers with a favorable prognosis. Clin Cancer Res 2008; 14:3395-400; PMID:18519769; http://dx.doi.org/10.1158/1078-0432.CCR-07-2051
11. Darnel AD, Lafargue CJ, Vollmer RT, Corcos J, Bismar TA. TMPRSS2-ERG fusion is frequently observed in Gleason pattern 3 prostate cancer in a Canadian cohort. Cancer Biol Ther 2009; 8:125-30; PMID:19029822; http://dx.doi.org/10.4161/cbt.8.2.7134
12. Demichelis F, Fall K, Perner S, Andrén O, Schmidt F, Setlur SR, Hoshida Y, Mosquera JM, Pawitan Y, Lee C, et al. TMPRSS2:ERG gene fusion associated with lethal prostate cancer in a watchful waiting cohort. Oncogene 2007; 26:4596-9; PMID:17237811; http://dx.doi.org/10.1038/sj.onc.1210237
13. Bismar TA, Dolph M, Teng LH, Liu S, Donnelly B. ERG protein expression reflects hormonal treatment response and is associated with Gleason score and prostate cancer specific mortality. Eur J Cancer 2012; 48:538-46; PMID:22300588; http://dx.doi.org/10.1016/j.ejca.2012.01.001
14. Hoogland AM, Jenster G, van Weerden WM, Trapman J, van der Kwast T, Roobol MJ, Schröder FH, Wildhagen MF, van Leenders GJ. ERG immunohistochemistry is not predictive for PSA recurrence, local recurrence or overall survival after radical prostatectomy for prostate cancer. Mod Pathol 2012; 25:471-9; PMID:22080055; http://dx.doi.org/10.1038/modpathol.2011.176
15. Teng LH, Wang C, Bégin LR, Dolph M, Yilmaz A, Trpkov K, Donnelly B, Bismar TA. ERG protein expression and gene rearrangements are present at lower rates in metastatic and locally advanced castration-resistant prostate cancer compared to localized disease. Urology 2013; 82:394-9; PMID:23746715; http://dx.doi.org/10.1016/j.urology.2013.03.029
16. Braun M, Goltz D, Shaikhibrahim Z, Vogel W, Böhm D, Scheble V, Sotlar K, Fend F, Tan SH, Dobi A, et al. ERG protein expression and genomic rearrangement status in primary and metastatic prostate cancer--a comparative study of two monoclonal antibodies. Prostate Cancer Prostatic Dis 2012; 15:165-9; PMID:22231490; http://dx.doi.org/10.1038/pcan.2011.67
17. Schrecengost R, Knudsen KE. Molecular pathogenesis and progression of prostate cancer. Semin Oncol 2013; 40:244-58; PMID:23806491; http://dx.doi.org/10.1053/j.seminoncol.2013.04.001
18. Brooke GN, Bevan CL. The role of androgen receptor mutations in prostate cancer progression. Curr Genomics 2009; 10:18-25; PMID:19721807; http://dx.doi.org/10.2174/138920209787581307
19. Burnstein KL. Regulation of androgen receptor levels: implications for prostate cancer progression and therapy. J Cell Biochem 2005; 95:657-69; PMID:15861399; http://dx.doi.org/10.1002/jcb.20460
20. Chmelar R, Buchanan G, Need EF, Tilley W, Greenberg NM. Androgen receptor coregulators and their involvement in the development and progression of prostate cancer. Int J Cancer 2007; 120:719-33; PMID:17163421; http://dx.doi.org/10.1002/ijc.22365
21. Jenster G. The role of the androgen receptor in the development and progression of prostate cancer. Semin Oncol 1999; 26:407-21; PMID:10482183
22. Setlur SR, Rubin MA. Current thoughts on the role of the androgen receptor and prostate cancer progression. Adv Anat Pathol 2005; 12:265-70; PMID:16210922; http://dx.doi.org/10.1097/01.pap.0000184179.50672.f7
23. Suzuki H, Ueda T, Ichikawa T, Ito H. Androgen receptor involvement in the progression of prostate cancer. Endocr Relat Cancer 2003; 10:209-16; PMID:12790784; http://dx.doi.org/10.1677/erc.0.0100209
24. Bismar TA, Yoshimoto M, Duan Q, Liu S, Sircar K, Squire JA. Interactions and relationships of PTEN, ERG, SPINK1 and AR in castration-resistant prostate cancer. Histopathology 2012; 60:645-52; PMID:22260502; http://dx.doi.org/10.1111/j.1365-2559.2011.04116.x
25. Carver BS, Chapinski C, Wongvipat J, Hieronymus H, Chen Y, Chandarlapaty S, Arora VK, Le C, Koutcher J, Scher H, et al. Reciprocal feedback regulation of PI3K and androgen receptor signaling in PTEN-deficient prostate cancer. Cancer Cell 2011; 19:575-86; PMID:21575859; http://dx.doi.org/10.1016/j.ccr.2011.04.008
26. Mulholland DJ, Tran LM, Li Y, Cai H, Morim A, Wang S, Plaisier S, Garraway IP, Huang J, Graeber TG, et al. Cell autonomous role of PTEN in regulating castration-resistant prostate cancer growth. Cancer Cell 2011; 19:792-804; PMID:21620777; http://dx.doi.org/10.1016/j.ccr.2011.05.006
27. Yu J, Yu J, Mani RS, Cao Q, Brenner CJ, Cao X, Wang X, Wu L, Li J, Hu M, et al. An integrated network of androgen receptor, polycomb, and TMPRSS2-ERG gene fusions in prostate cancer progression. Cancer Cell 2010; 17:443-54; PMID:20478527; http://dx.doi.org/10.1016/j.ccr.2010.03.018
28. Zong Y, Xin L, Goldstein AS, Lawson DA, Teitell MA, Witte ON. ETS family transcription factors collaborate with alternative signaling pathways to induce carcinoma from adult murine prostate cells. Proc Natl Acad Sci U S A 2009; 106:12465-70; PMID:19592505; http://dx.doi.org/10.1073/pnas.0905931106
29. Wu G, Feng X, Stein L. A human functional protein interaction network and its application to cancer data analysis. Genome Biol 2010; 11:R53; PMID:20482850; http://dx.doi.org/10.1186/gb-2010-11-5-r53
30. Shiota M, Yokomizo A, Naito S. Increased androgen receptor transcription: a cause of castration-resistant prostate cancer and a possible therapeutic target. J Mol Endocrinol 2011; 47:R25-41; PMID:21504942; http://dx.doi.org/10.1530/JME-11-0018
31. Shao L, Zhou Z, Cai Y, Castro P, Dakhov O, Shi P, Bai Y, Ji H, Shen W, Wang J. Celastrol suppresses tumor cell growth through targeting an AR-ERG-NF-κB pathway in TMPRSS2/ERG fusion gene expressing prostate cancer. PLoS One 2013; 8:e58391; PMID:23554889; http://dx.doi.org/10.1371/journal.pone.0058391
32. Li Y, Kong D, Wang Z, Ahmad A, Bao B, Padhye S, Sarkar FH. Inactivation of AR/TMPRSS2-ERG/Wnt signaling networks attenuates the aggressive behavior of prostate cancer cells. Cancer Prev Res (Phila) 2011; 4:1495-506; PMID:21680704; http://dx.doi.org/10.1158/1940-6207.CAPR-11-0077
33. Wu L, Zhao JC, Kim J, Jin HJ, Wang CY, Yu J. ERG is a critical regulator of Wnt/LEF1 signaling in prostate cancer. Cancer Res 2013; 73:6068-79; PMID:23913826; http://dx.doi.org/10.1158/0008-5472.CAN-13-0882
34. Leinonen KA, Saramäki OR, Furusato B, Kimura T, Takahashi H, Egawa S, Suzuki H, Keiger K, Ho Hahm S, Isaacs WB, et al. Loss of PTEN is associated with aggressive behavior in ERG-positive prostate cancer. Cancer Epidemiol Biomarkers Prev 2013; 22:2333-44; PMID:24083995; http://dx.doi.org/10.1158/1055-9965.EPI-13-0333-T
35. Cai J, Kandagatla P, Singareddy R, Kropinski A, Sheng S, Cher ML, Chinni SR. Androgens Induce Functional CXCR4 through ERG Factor Expression in TMPRSS2-ERG Fusion-Positive Prostate Cancer Cells. Transl Oncol 2010; 3:195-203; PMID:20563261; http://dx.doi.org/10.1593/tlo.09328
36. Saito R, Smoot ME, Ono K, Ruscheinski J, Wang PL, Lotia S, Pico AR, Bader GD, Ideker T. A travel guide to Cytoscape plugins. Nat Methods 2012; 9:1069-76; PMID:23132118; http://dx.doi.org/10.1038/nmeth.2212
37. Teng LH, Wang C, Dolph M, Donnelly B, Bismar TA. ERG Protein Expression Is of Limited Prognostic Value in Men with Localized Prostate Cancer. ISRN Urol 2013; 2013:786545; PMID:24027643; http://dx.doi.org/10.1155/2013/786545
38. Huang KC, Dolph M, Donnelly B, Bismar TA. ERG expression is associated with increased risk of biochemical relapse following radical prostatectomy in early onset prostate cancer. Clin Transl Oncol 2014; PMID:24796295; http://dx.doi.org/10.1007/s12094-014-1182-x

39. Weber A, Kristiansen I, Johannsen M, Oelrich B, Scholmann K, Gunia S, May M, Meyer HA, Behnke S, Moch H, et al. The FUSE binding proteins FBP1 and FBP3 are potential c-myc regulators in renal, but not in prostate and bladder cancer. BMC Cancer 2008; 8:369; PMID:19087307; http://dx.doi.org/10.1186/1471-2407-8-369
40. Sircar K, Yoshimoto M, Monzon FA, Koumakpayi IH, Katz RL, Khanna A, Alvarez K, Chen G, Darnel AD, Aprikian AG, et al. PTEN genomic deletion is associated with p-Akt and AR signalling in poorer outcome, hormone refractory prostate cancer. J Pathol 2009; 218:505-13; PMID:19402094; http://dx.doi.org/10.1002/path.2559
41. Epstein JI, Allsbrook WC Jr., Amin MB, Egevad LL; ISUP Grading Committee. The 2005 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma. Am J Surg Pathol 2005; 29:1228-42; PMID:16096414; http://dx.doi.org/10.1097/01.pas.0000173646.99337.b1
42. Sboner A, Demichelis F, Calza S, Pawitan Y, Setlur SR, Hoshida Y, Perner S, Adami HO, Fall K, Mucci LA, et al. Molecular sampling of prostate cancer: a dilemma for predicting disease progression. BMC Med Genomics 2010; 3:8; PMID:20233430; http://dx.doi.org/10.1186/1755-8794-3-8