ERG Expression Levels in Prostate Tumors Reflect Functional Status of the Androgen Receptor (AR) as a Consequence of Fusion of *ERG* with AR Regulated Gene Promoters

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Abstract: Expression of the *ERG* proto-oncogene, is activated in 50-70% of prostate tumors by androgen receptor (AR) mediated signals due to the fusion of AR regulated promoters (primarily *TMPRSS2* and to a lesser extent *SLC45A3 and NDRG1*) to the *ERG* protein coding sequence. Our previous studies of quantitative expression levels of *ERG or TMPRSS2-ERG* fusion transcripts have noted that relatively low or no *ERG* expression in prostate tumors significantly associated with progressive disease. Here, we have tested the hypothesis that *ERG* expression levels in prostate tumor cells reflect AR transcriptional regulatory function in a given biological context of the tumor progression. Therefore, tumors with lower *ERG* may represent a subset with attenuated AR signaling. Expression of *ERG* and other AR regulated genes were evaluated in a GeneChip dataset obtained from a panel of laser capture micro-dissected well/moderately differentiated (WD) or poorly differentiated (PD) tumor cells derived from primary tumors of patients, who had no prior androgen ablation treatment. Overall, *ERG* expression pattern was similar to that of other AR regulated genes. Strikingly low frequency of *ERG* expression was noted in PD tumor cells (30%) in comparison to WD tumor cells (80%), suggesting for subdued AR function in a significant fraction of tumors with genomic alterations of *ERG*. By integrating *ERG* into a panel of defined AR target genes, we developed a cumulative AR Function Index (ARFI), which if validated may have future potential in stratifying patients for targeted therapy on the basis of overall AR functional status in primary tumors.

Keywords: *ERG*, oncogene, prostate cancer, androgen receptor, *TMPRSS2-ERG*.

INTRODUCTION

Although prostate cancer (CaP) is being detected at early stages by PSA screening, the prognosis of individual patients and consequently treatment decisions remains a challenge. Identification of molecular markers representing central pathways that can distinguish between aggressive and indolent forms of CaP at early stages would offer improved prognostic and therapeutic decisions. With the exception of serum PSA, currently there are no rational (tumor biology based) prognostic or therapeutic molecular markers in the clinical practice of CaP. While 80% of CaP patients respond well to surgery, radiation therapy or watchful waiting, about 20% will develop metastasis that is often fatal to patients [1].

In this regard, it is important to note that the most common genomic alterations in CaP are gene fusions that involve fusion of AR regulated promoter sequences, such as, *TMPRSS2* and to a lesser extent *SLC45A3 and NDRG1* [2-4], to protein coding sequences of genes of the ETS family of transcription factors, predominantly the ETS Related Gene, *ERG* [5-7].

Initially, CaP development is driven by the androgen receptor (AR) pathway [8-11]. Frequent alterations of AR structure and/or function are well recognized during CaP progression especially with metastatic disease. Other genetic pathways that are often altered in these late stage androgen-independent tumors include *p53* mutations, *BCL2* overexpression and mutations or reduced expression of *PTEN* [12]. Importantly, both p53 and PTEN pathways may affect AR functions [13-15]. CaP associated alterations of AR function by mechanisms involving AR mutations, AR gene amplification, altered AR mRNA or AR protein levels, changes in AR interaction with co-activators/co-repressors, furthermore, ligand-independent AR activation by growth factors/ cytokines, may all contribute to CaP progression [16-18]. Although, there is a need for exploring AR dysfunction in pathologic specimens, identification of patients with functional defects of AR poses a great challenge. Moreover, recent demonstration of alternative pathways supplying

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androgen in castration-resistant prostate cancer adds to the complexity of monitoring functional defects of AR [19].

Our previous studies of quantitative expression levels of *ERG or TMPRSS2-ERG* fusion transcripts have noted fluctuations of *ERG* transcripts in prostate tumors with relatively low or no *ERG* expression significantly associating with progressive disease [20,21]. We reasoned that *ERG* expression levels in prostate tumor cells may reflect upstream AR transcriptional function in a given biologic context of the tumor progression. Here we have evaluated *ERG* expression patterns in relation to well defined panel of AR regulated genes [22,23] in a cohort of prostate tumor specimens that has been well characterized in previous studies [20,24] in the context of moderate and aggressive CaP. Using expression characteristics of the AR regulated genes and *ERG*, we have developed a cumulative measure of AR functional status in prostate tumors as the Androgen Receptor Function Index (ARFI). We report that attenuated *ERG,* as well as ARFI may reflect AR dysfunction in subset of prostate tumors that associate with poor prognosis.

MATERIALS AND METHODS

Patient Cohort Selection

Prostate tissue specimens and clinical data used in this study were obtained under an IRB-approved protocol at Walter Reed Army Medical Center. Informed consent was obtained from each subject. For specimen selection over 300 radical prostatectomy specimens of a PSA-screened patient cohort with no prior androgen ablation treatment was evaluated. Forty specimens of age, race and tumor differentiation matched patients were identified from the initial cohort. The selected 40 specimens represented two contrasting groups of the continuum of CaP cases: The first group of 20 RP specimens was presented with prostatectomy specimen Gleason sum 6-7 and with no PSA recurrence. In contrast, the second group of 20 RP specimens was presented with prostatectomy specimen Gleason sum 8-9 and with PSA recurrence in 65% of cases. PSA recurrence was defined as two consecutive times of PSA $\geq$ 0.2 ng/ml with follow up from surgery to Median (range) = 43.2 (2.7-71.8) months.

Laser Capture Microdissection and RNA Isolation

Tumor and benign prostate epithelial cells were laser-captured microdissected (LCM, Arcturus Pixel 2) by the pathologist from OCT embedded and H&E stained frozen prostate sections of radical prostatectomy specimens (2000 laser shots for one sample). Total RNA was isolated from the LCM samples with the MicroRNA kit (Stratagene, La Jolla, CA) and quantified using RiboGreen dye (Molecular Probes, Eugene, OR) and VersaFluor fluorimeter (BioRad, Hercules, CA). Total RNA was converted to cDNA (Sensiscript, Qiagen, Valencia, CA). Linear RNA amplification was performed by using RiboAmp RNA amplification kit (Arcturus, Mountain View, CA). Two nanograms of total RNA from LCM derived cells from each patient was used for the first round of amplification. During the second round of amplification after cDNA synthesis and purification the samples were biotinylated during *in vitro* transcription which was used for the GeneChip analysis. Real time QRT-PCR (TaqMan) for quality control was essentially performed as described [20].

GeneChip Data Analyses

Primary data of 80 GeneChip analysis (HG U133A array, Affymetrix, Santa Clara, CA, USA) and procedures representing matched tumor and benign prostate epithelial cells from 40 CaP patients were described previously [24]. RMA (robust multi-chip average) normalized expression values of AR-regulated genes, *PMEPA1* (Gene ID: 56937)*, PSA/KLK3* (Gene ID: 354)*, NKX3.1* (Gene ID: 4824)*, ODC1* (Gene ID: 4953) and *AMD1* (Gene ID: 262) respectively, were log10-transformed, Mean values and Standard Deviation (STD) was calculated and Z score normalized by the following formula: Z= (individual intensity-mean intensity)/STD. The Z score normalized expression intensities (I) representing the relative value of each gene were compared to the mean of expression intensities. Integration of *ERG* (Gene ID: 2078) expression into cumulative intensity (CI), where CI_{max}= +3 and CI_{min}= -3, resulted in the Androgen Regulated Network index (ARFI) that was mathematically defined in the following formulas.

$$ARGI = \Sigma (I_{PMEPA1} + I_{PSA} + I_{NKX3.1} + I_{ODC1} + I_{AMD1})$$

$$ARFI = CI = ARGI + I_{ERG}$$

Detection of *TMPRSS2-ERG* Fusion Junctions by Quantitative RT-PCR

The data presented here for detecting *TMPRSS2-ERG* fusion transcripts was described before [21]. Briefly, the following primers were used and marked with the *ERG* exon nomenclature of Owczarek *et al.* [25] in the study: 5'-TAGGCGCGAGCTAAGCAGGAG-3' (*TMPRSS2* exon 1) 5'-GGCGTTGTAGCTGGGGGTGAG-3' (*ERG* exon 10). Quantitative gene expression analysis was performed by TaqMan-based QRT-PCR on ABI 7700 (PE Applied Biosystems, Foster City, CA). The TaqMan primers and probe for *TMPRSS2-ERG* fusion A: forward primer 5'-CCTGGAG CGCGGCAGGAA-3' (from *TMPRSS2* exon 1) reverse primer 5'- CGCGGTCATCTCTGTCTTAGC- 3' (*ERG* exon 8) and TaqMan probe 6FAM-GCCTACGGAACGCCAC AC-MGBNF-Q. *TMPRSS2-ERG* fusion B: forward primer 5'- GGAGCGCGGCAGGTTATT -3' (from *TMPRSS2* exon 1) reverse primer 5'- TTGGTCAACACGGCTTTCCT -3' (*ERG* exon 3) and TaqMan probe 6FAM- AGGATCTTTGG AGACCC -TAMRA; *TMPRSS2-ERG* fusion C: forward primer 5'-GAGCGCCGCCTGGAG-3' (*TMPRSS2* exon 1) reverse primer 5'-TCGTTCGTGGTCATGTTTGG-3' (*ERG* exon 9) and TaqMan probe FAM-GCAGGAACTCTC CTGATG-TAMRA. Thermal cycling conditions: 95°C for 10 min, 50 cycles at 95°C for 15sec, and 60°C for 1min. RNA samples without reverse transcription were included as the negative control in each assay.

RESULTS

Evaluation of AR Function by Monitoring the Expression of Androgen Regulated Genes

Transcription of *ERG* fusion transcripts is under the control of androgen regulated gene promoters in CaP [2-4]. Thus, we reasoned that the expression of androgen regulated

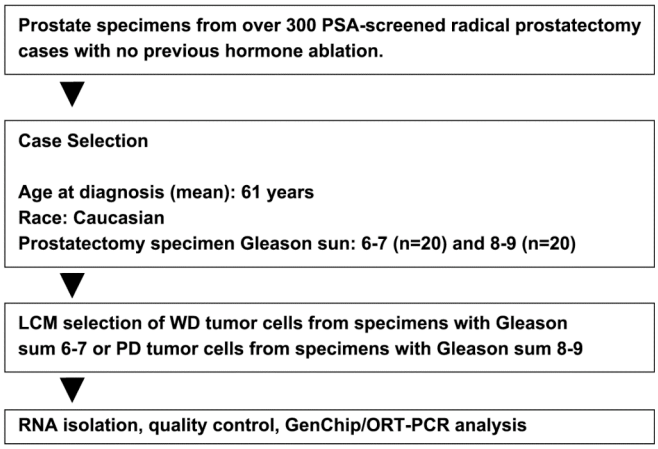
genes (ARGs) and *ERG* expression may reflect the overall function of the AR transcription factor within the same CaP cells. Androgen-inducible genes were selected from previous reports where androgen dose and the time kinetics of gene expression responses were established in prostate cancer cells (LNCaP) (Table **1**) [22,23]. These studies revealed ARGs with prompt and robust expression characteristics in response to androgen induction. Indeed, ARGs, such as, *PSA, PMEPA1, NKX3.1, ODC1* and *AMD1*, represent functional read out of androgen receptor by their reported response to androgenic stimuli as well as by their wide dynamic range of expression [16,22,23].

Table 1. Accession Numbers of Selected AR Regulated Genes with Corresponding References

KLK3 (PSA)	NM_001648	Segawa *et al.*, Oncogene 2002
NKX3.1	NM_006167	Segawa *et al.*, Oncogene 2002
ODC1	NM_002539	Segawa *et al.*, Oncogene 2002
AMD1	NM_001634	Xu *et al.*, Int J Cancer 2001
PMEPA1	NM_199169	Xu *et al.*, Int J Cancer 2001

We investigated a relationship between the expression of *ERG* and well characterized AR regulated genes in a defined panel of prostatectomy specimens from PSA-screened patients, who had no prior androgen ablation treatment. *ERG* expression was evaluated in relation to the expression of ARGs in human CaP by using Affymetrix oligonucleotide microarray (HG U133A GeneChip) data generated from laser capture microdissected tumors (n=40) from OCT embedded frozen prostate specimens [20]. These specimens were obtained from age and race (Caucasians) matched cases selected from a cohort of over 300 patients. Patients and specimens were stratified by clinico-pathologic features and in the subsequent steps cells were isolated by LCM from the specimens (Table **2**). The patient cohort for this study panel represents the two contrasting groups of the continuum of prostate cancer cases.

Table 2. Work Flow of Patient Selection, Specimen Processing and Gene Expression Analyses in the Study



GeneChip derived gene expression values were RMA normalized, expression values of androgen regulated genes including *ERG* were log10-transformed and *Z* score normalized and represented in a heat-map diagram (Fig. **1**). Consistent with our hypothesis, in a subset of prostate tumors *ERG* was lost or decreased, as well as, the selected panel of androgen-inducible genes exhibited completely lost or attenuated expression of androgen-inducible genes indicating attenuated AR function. The significant overlap that was observed between *ERG* and ARGs expression features suggests for a similar AR dependent transcriptional regulation of these genes in the respective biological context of tumor cells.

Defining the Androgen Receptor Function Index

The observed concordance between the expression of ARGs and *ERG* prompted us to develop an integrated formula for the definition of AR function. Towards this goal RMA normalized expression values of AR-regulated genes,

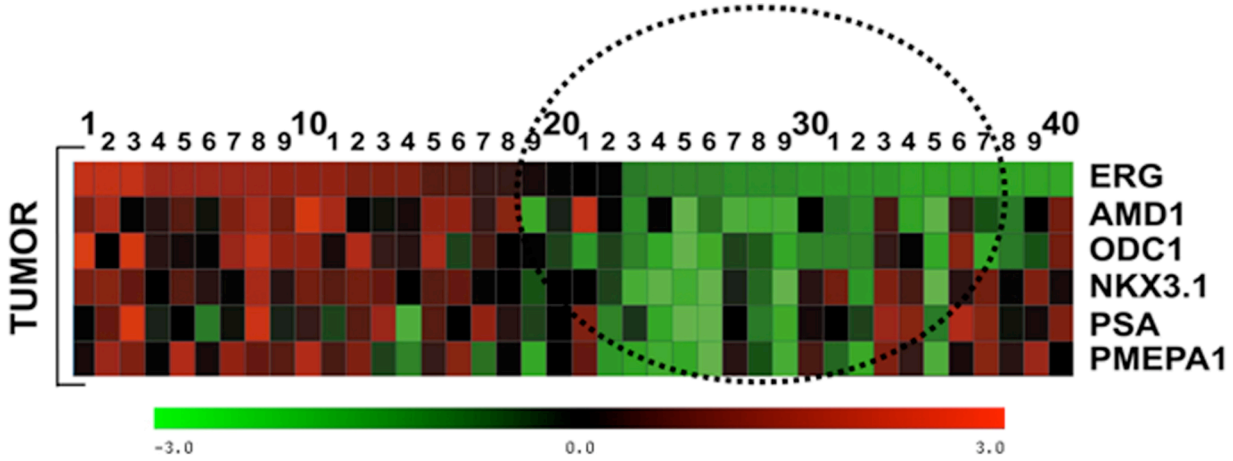


Fig. (1). Decreased expression of androgen-inducible genes indicates the dysfunction of androgen receptor in a subset of prostate tumors. Tumor cells were selected by LCM from 40 prostate tumors. Gene expression was analyzed by microarrays. Expression values of androgen regulated genes including *ERG* were log10-transformed and Z score normalized and represented in a heat-map diagram. Circle highlights lost or decreased expression of ARGs in prostate tumors. Heat-map on a red (high, max = +3) to green (low, min = -3) color scale is shown in the insert.

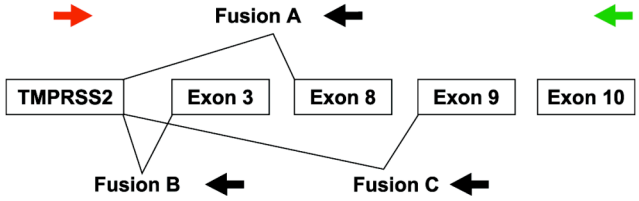
PMEPA1, PSA, *NKX3.1*, *ODC1*, *AMD1* and *ERG*, were log10-transformed and Z score normalized. Z score normalized expression intensities (I) representing the relative value of each gene was compared to the mean of expression intensities in prostate tumor cells. The androgen regulated function index was defined as the mean of expression intensities of ARGs with the integration of *ERG* expression into a cumulative intensity that was mathematically defined in the following formulas where (I) represents the relative value of expression compared to the mean of expression intensities for each ARG gene and cumulative intensity (CI, within a -3 to +3 range) of androgen regulated network intensity, that integrates *ERG* expression into the Androgen Receptor Function Index, ARFI.

$$ARGI = \Sigma (I_{PMEPA1} + I_{PSA} + I_{NKX3.1} + I_{ODC1} + I_{AMD1})$$

$$ARFI = CI = ARGI + I_{ERG}$$

In the high *ERG* expressing cluster where CI > 0, robust co-regulation of ARGs was revealed by the normalized mean expression intensity indexes (Fig. **2A**). Fusion junction types A, B and C (Table **3**) of *TMPRSS2-ERG* transcripts were detected in the entire CI> 0 cluster. By contrast, patients with low *ERG* expression in their CaPs cells exhibited attenuated ARFI (CI < 0) indicating compromised androgen receptor function in a substantial number of *ERG* expression negative tumors. Although less frequent, *TMPRSS2-ERG* fusion junction types A, B, or C were detectable in a subset of tumors in the CI < 0 cluster by using sensitive quantitative RT-PCR. We also analyzed the ARFI data in the context of tumor cell differentiation (Fig. **2B**). Intriguingly, PD tumor phenotype was associated with the low ARFI (CI < 0) cluster. Conversely, WD tumor cells clustered with high ARFI (CI >0).

Table 3. Relative Position of Amplification Primers on *ERG* and *TMPRSS2* Exons for Detecting A, B and C Fusion Junction Types (Solid Arrows). Red Arrow Represents Primers in the First Untranslated Exon of the *TMPRSS2* Gene. Green Arrow Indicates the Position of the Amplification Primer that may Detect Various Fusion Junctions



Low ARFI in Prostate Tumors Associates with PSA Recurrence

The observed association between tumor differentiation status and ARFI prompted us to examine the relation of ARFI with PSA recurrence (Fig. **3**). As expected from the observed clustering of PD tumors with the CI < 0 cases, PSA recurrence was observed in 55% of the low ARFI group. Intriguingly, the analysis revealed only 25% of PSA recurrence events in the CI > 0 ARFI group that occurred in tumor cells with PD morphology (Fig **2** and Fig. **3**). This

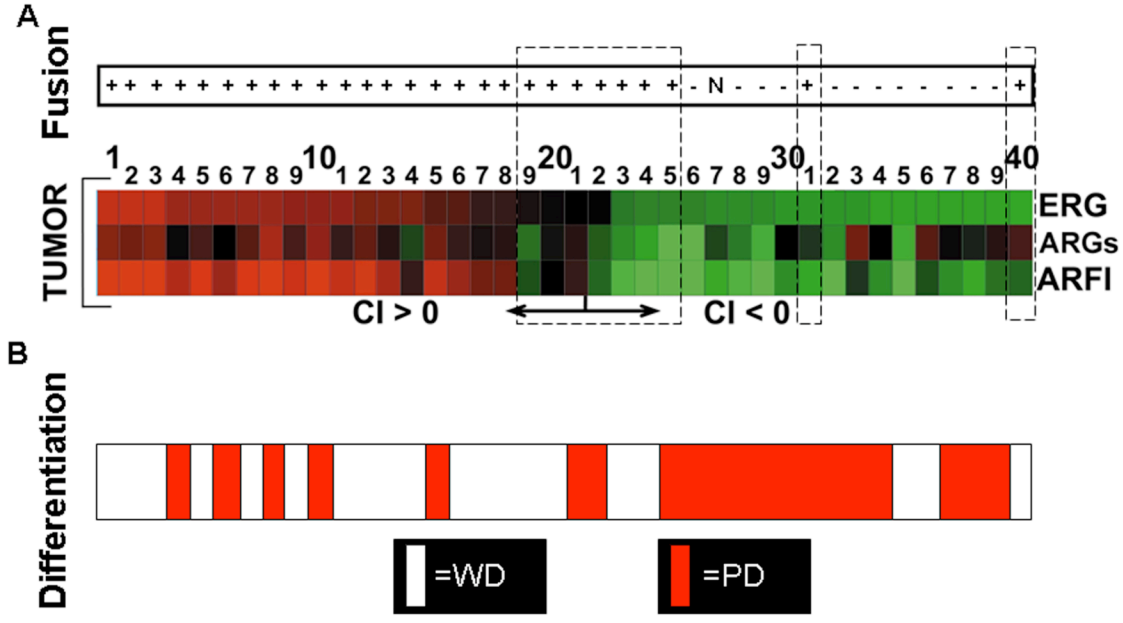


Fig. (2). Androgen Receptor Function Index (ARFI) that incorporates *ERG*, separates prostate tumor cells with functional androgen receptor from tumors with attenuated androgen receptor function. (**A**) Heat-map representation of the expression of ARGs (*PMEPA1, PSA, NKX3.1, ODC1*, *AMD1* and *ERG*) in prostate tumor cells. Cumulative intensity (CI) represents the cumulative values of ARFI. Z-score normalized expression intensities are depicted by heat map on a red (high)-to-green (low) scale. A cumulative intensity > 0 indicates functional AR signaling. By contrast, in tumor cells with attenuated AR functions CI < 0. Case numbers (N=40) are listed above the heat map. Arrows mark the separation of two patient groups by CI values. PCR detection of *TMPRSS2-ERG* fusion junction type A or B or C is marked by + above the patient numbers. Letter N indicates non-evaluated case. Dashed brackets mark tumors where both *TMPRSS2-ERG* fusion transcript and decreased AR function were observed. (**B**) LCM differentiation status of tumor cells are marked by red (PD: poorly differentiated phenotype) or by blank bars (WD: well/moderately differentiated morphology).

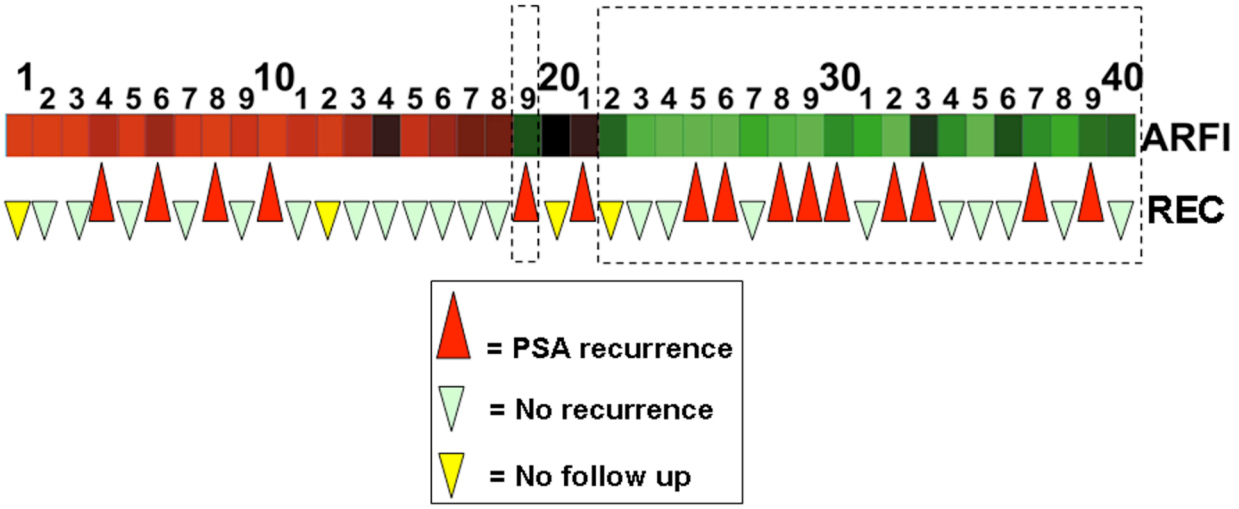


Fig. (3). Association of PSA Recurrence with Low ARFI in Prostate Tumors

Patient with Median follow up (range) = 43.2 (2.7-71.8) months were examined for PSA recurrence (REC) (two consecutive times of PSA $\geq$ 0.2 ng/ml) (red triangles) or no PSA recurrence (green triangles). Yellow triangles indicate no follow up data. Tumors with ARFI CI < 0 are marked by dashed boxes on the ARFI heat-map.

finding indicates that WD tumor cell phenotype and high ARFI (CI >0) together may indicate of favorable prognosis. ARFI in this study represents the expression signature of AR at the time of radical prostatectomy, thus, these findings indicate that ARF-index (ARGs+ERG) may indeed serves as an indicator of unfavorable tumor phenotype.

Reduced or Absent *ERG* Expression Associates with Poorly Differentiated Tumor Phenotype

The GeneChip data that was obtained from the 40 patient included the expression features of tumor (T) and matching non-adjacent normal (N) epithelial cells with benign morphology. Together these data allowed us to calculate T/N ratios compensating for patient-to-patient variations and to evaluate the relation of *ERG* expression values to the LCM differentiation status of tumor cells. T/N normalized *ERG* expression values were plotted in a descending order and the tumor LCM differentiation status values were compared to the *ERG* expression levels. The analysis revealed two contrasting groups of *ERG* expressing tumors. *ERG* expression (T/N > 3) was observed in CaP cells in tumor cells from twenty-one patients (Fig. **4**). In contrast, loss of *ERG* expression (T/N < 1.5 folds) was observed in 19 cases.

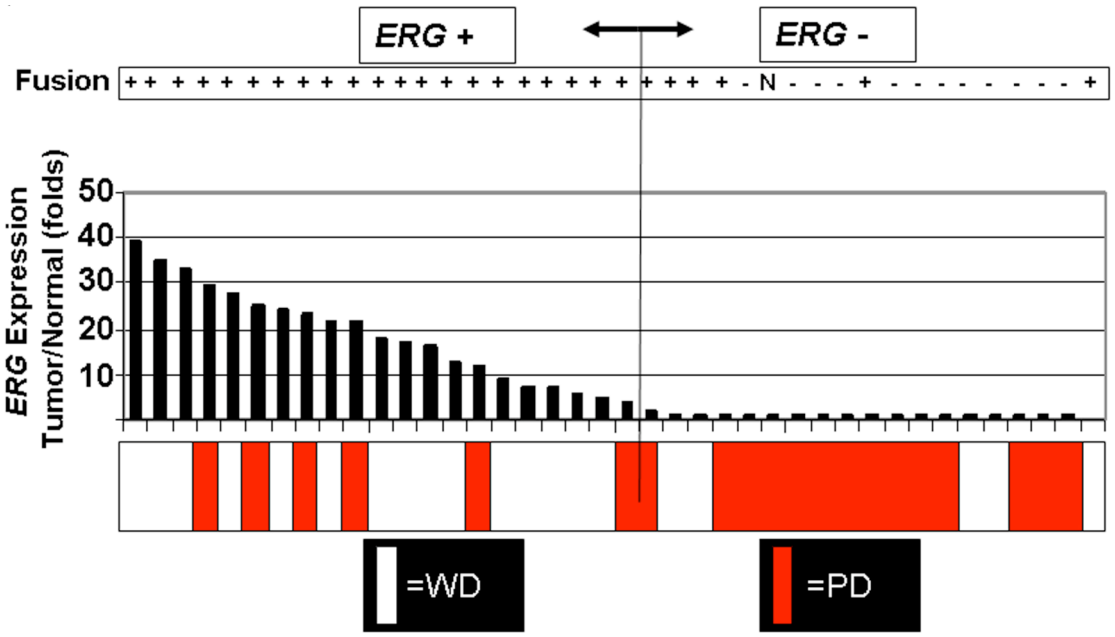


Fig. (4). Loss of *ERG* expression associates with poorly differentiated phenotype in prostate tumor epithelial cells. RMA normalized *ERG* gene expression values were expressed as tumor/normal ratios and are shown in descending order. *ERG* + and *ERG*- tumors are indicated by the arrows. *TMPRSS2-ERG* fusion status (fusion A or B or C) of tumor specimens are shown above the bar diagram. Tumor differentiation is marked by red (PD) or blank (WD) bars.

In all of these 19 cases the original RMA normalized tumor expression values were between 7-14 units that were similar to the detection limit/base-line GeneChip expression values of normal samples. In contrast RMA normalized expression values of the 21 *ERG* positive tumors were within the significant gene expression range of 40-350 units. This finding is in agreement with our previous study reporting a significant enrichment of PD tumors in cells with absent or reduced *ERG* expression [20]. Taken together, these data suggest that a higher fraction of poorly differentiated tumors cells either have low *ERG* due to low ARFI or lack *ERG* fusions.

DISCUSSION

The cornerstone for therapy for late stage CaP is systemic androgen ablation, which eventually fails in most patients. Therefore, the knowledge of AR pathway dysfunctions which are predictive of poor outcome or androgen ablation therapy failure would significantly impact the patient stratification for new emerging therapeutic strategies. In contrast to the care of breast cancer, where estrogen receptor protein status in primary tumor is effectively used in making therapeutic and prognostic decisions [26] AR protein expression status does not appear to assist in treatment decisions in CaP. Although, AR expression can be detected throughout the continuum of CaP progression, it is heterogeneous and changes over time. Studies have indicated reduced, increased or inconclusive AR expressions with poorly differentiated areas with higher Gleason score and with decreased PSA recurrence-free survival [27-31]. The reasons for the discordance in part may be explained by the plasticity of AR expression and/or by the function in clonal selection and progression of CaP and potential technical limitations of AR related read-out in clinical specimens. During the past decade, significant efforts from several prostate research laboratories, including ours, have provided novel insights into the androgen regulated transcriptome. These endeavors identified AR targets which have promise in defining the role of AR dysfunctions in CaP, as well as, in providing novel, functionally relevant biomarkers and potential therapeutic targets for CaP [18,22,23,32-38]. Recent data obtained from prostate cancer cell culture models highlighted a distinct AR regulated transcription program in androgen blockade resistant derivatives of LNCaP cells [37]. Although, AR can be altered by numerous mechanisms, the net effect of these changes is reflected in defective transcription factor functions of the AR. Therefore, measurement of the expression of carefully selected AR downstream targets provides information on the *in vivo* functional status of AR in CaP cells. Over the past years, numerous studies have focused on defining cell type specific gene expression from microdissected matched tumor and benign prostate epithelial cells. We recently reported a general decrease in androgen regulated gene expression with CaP progression [39]. Other reports have also noted a signature of attenuated AR function in late stage, especially in metastatic CaP in human specimens [40-43], as well as in a xenograft model system [44]. As part of a 12-gene panel, PSA was found to be underexpressed in aggressive CaP [45].

In summary, using *in vivo* AR function read-out, we have defined a set of six androgen inducible/co-regulated genes, such as, *PSA/KLK3, PMEPA1, NKX3.1, ODC1, AMD1,* and *ERG* in gene fusion context to define AR function by the androgen receptor function index (ARFI), in CaP specimens. These genes are either direct targets of AR or are tightly regulated by AR, and cover major biological functions regulated by AR in CaP. Several of these genes (*NKX3.1, PMEPA1 and ERG*) may also be causally associated with CaP [32,46-51]. Taken together, the ARFI approach reported here, if developed further has potential to stratify prostate tumors on the basis of *in vivo* functional status of AR which could lead to development of new paradigms in the treatment selection of patients for androgen ablation or other therapies. For example patients with ARFI positive *versus* ARFI negative/attenuated tumors may be identified in early stages of disease and latter may be more responsive to non androgen ablation focused strategies. Along similar lines patients with *ERG* gene fusion but not expressing *ERG* may not benefit from a potential *ERG* targeted therapy. Alternatively patients with varying degree of ARFI positivity may need different androgen ablation therapy strategies. Finally, association of low or no *ERG* in a large percentage of poorly differentiated tumors appears to be either reflection of attenuated AR signaling in tumors harboring *ERG* fusions or a distinct class of tumors with out *ERG* alterations. These data also may help us to better understand the association of higher *ERG* expression in less aggressive CaP as noted in our original study [20] and recent reports focusing on quantitative expression of *ERG* and/or *TMPRSS2-ERG* expression and fusion rearrangement in CaP [52-56]. Taken together, the emerging data on the quantitative expression of *ERG* may help in defining the tumor biologic mechanisms under these intriguing observations.

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ABBREVIATIONS

ARGs = Androgen Regulated Genes

ARFI = Androgen Receptor Function Index

CaP = Cancer of the Prostate

CI = Cumulative Intensity

ERG = ETS Related Gene

LCM = Laser Capture Microdissection

N = Normal

PD = Poorly Differentiated Tumor

RP = Radical Prostatectomy

RMA = Robust Multi-Chip Average

T = Tumor

WD = Well-to-moderately Differentiated Tumor

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