Expression of ERG protein in prostate cancer: variability and biological correlates

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Abstract

Prostate cancer is the second leading cause of cancer-related death of men in the USA. The *TMPRSS2/ERG* (*T/E*) fusion gene is present in approximately 50% of prostate cancers and promotes tumor progression *in vivo*. The presence of the *T/E* fusion gene is strongly associated with the expression of ERG protein, but emerging evidence indicates a significant interfocal and intrafocal variability in the levels of ERG protein expression. We therefore analyzed ERG protein expression by image analysis to objectively quantitate the extent of such heterogeneity, and confirmed significant interfocal and intrafocal variability of ERG protein expression levels in cancer expressing ERG. To define the pathways associated with ERG and its variable expression in prostate cancer, we have analyzed the correlations of ERG expression, as evaluated by immunohistochemistry, with 46 key proteins associated with signal transduction, transcriptional control, and other processes using a large tissue microarray with more than 500 prostate cancers. We found a significant correlation of ERG expression with the markers of activation of the PI3K, MYC, and NFκB pathways, which had previously been linked directly or indirectly to ERG expression. We have also identified significant correlations with novel proteins that have not been previously linked to ERG expression, including serum response factor, the p160 coactivator SRC1, and Sprouty1. Notably, SKP2 only correlated with a high level of ERG protein expression. Thus ERG expression is variable in prostate cancer and is associated with activation of multiple pathways and proteins including several potentially targetable pathways.

Key Words

- ▶ prostate cancer
- ▶ *TMPRSS2/ERG*
- ▶ MYC
- ▶ PTEN
- ▶ NFκB

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Introduction

Prostate cancer remains the most common malignancy affecting men, and the second leading cause of cancer-related death of men in the USA. It is a heterogeneous disease, and the biology of various subtypes is still poorly understood. The pathways that are altered at high

frequency in specific patient tumor types need to be better defined to optimize individually targeted therapy. Over the past 8 years, important progress has been made in the subclassification of prostate cancer, in particular the finding that the *TMPRSS2/ERG* (*T/E*) fusion gene is present

in approximately 50% of prostate cancers. Results of experiments carried out on prostate cancer cells containing the *T/E* fusion (Tomlins *et al.* 2005) indicate that the *TMPRSS2* promoter, which contains androgen receptor (AR)-responsive promoter elements (Lin *et al.* 1999), increases ERG expression in response to androgens. The ubiquitous activity of AR in prostate cancer cells thus results in the constitutive expression of *ERG* fusion transcripts and ERG protein. We have demonstrated that the *T/E* fusion gene can enhance proliferation, invasion, and motility of prostate epithelial cells (Wang *et al.* 2008). More importantly, stable knockdown of the *T/E* fusion mRNA in VCaP cells inhibits tumor growth *in vivo*, indicating that the *T/E* fusion gene is a potential therapeutic target which is present in the majority of prostate cancers (Wang *et al.* 2008). More recently we have shown that highly specific knockdown of the *T/E* fusion gene with siRNAs delivered via nanoliposomal vectors substantially decreases the growth of established tumors, confirming the importance of the *T/E* fusion gene in cancer progression *in vivo* (Shao *et al.* 2012).

The pathways and proteins implicated in the ability of the ERG oncoprotein to promote prostate cancer progression have been a focus of active investigation. Prostate cancers with ERG expression have been shown to activate multiple proteins and pathways such as Ezh2, Wnts, TGFB, and SOX9 (Sun *et al.* 2008, Tomlins *et al.* 2008, Gupta *et al.* 2010, Yu *et al.* 2010, Brase *et al.* 2011, Vainio *et al.* 2011, Magistroni *et al.* 2011, Cai *et al.* 2013, Massoner *et al.* 2013, Tian *et al.* 2013, Wu *et al.* 2013). We have shown that the *T/E* fusion gene increases NFκB-mediated transcription via increased phosphorylation of NFκB p65 on Ser536 (Wang *et al.* 2011). Results of correlative studies of human prostate cancers reveal a strong association of ERG expression with loss of *PTEN*, and results of studies using mouse models reveal that ERG expression and *PTEN* loss synergistically promote progression of prostate cancer (Han *et al.* 2009, King *et al.* 2009, Chen *et al.* 2013, Leinonen *et al.* 2013). Thus alterations in a number of key pathways and proteins important in tumor progression have been linked to the presence of ERG expression.

One aspect of the biology of the *T/E* fusion gene that has received limited attention to date is the substantial variability of ERG protein expression levels in cancers containing the *T/E* fusion. Detection of the presence of the protein product of the *T/E* fusion gene in a focus of prostate cancer by immunohistochemistry (IHC) is strongly associated with the presence of the *T/E* fusion gene in that focus, although less common variant translocations driving ERG expression have been identified. However, there can be

substantial intrafocal variability in the levels of ERG expression (Mertz *et al.* 2013, Minner *et al.* 2013). To evaluate objectively the variability of ERG protein expression in prostate cancers, we have carried out a multispectral image analysis of ERG expression in a cohort of more than 500 radical prostatectomies that we have studied extensively over the last 10 years. In addition, we have examined the correlation of 46 proteins and tumor-specific factors in this same cohort with ERG expression. The results of our studies confirm the extent of variability of ERG expression in prostate cancer. The results from correlative studies have confirmed the known or inferred associations with several important pathways/proteins that had previously been linked directly or indirectly to ERG expression, including the PI3K, MYC, and NFκB pathways. We have also identified significant correlations with novel proteins that have not, to our knowledge, been previously linked to ERG expression, including serum response factor (SRF), the p160 coactivator SRC1 and Sprouty1. Importantly, we have found differential correlations based on the level of ERG expression consistent with a significant biological effect of ERG expression levels. In particular, SKP2 only correlated with a high level of ERG protein expression. Thus ERG expression is a variable in prostate cancer and is associated with the activation of multiple pathways and proteins, including several potentially targetable pathways.

Materials and methods

IHC evaluation

Tissues required for analysis were collected with informed consent under and Institutional Review Board approved protocol. IHC for ERG expression in the tissue microarray (TMA) has been described previously (Wang *et al.* 2011). Briefly, antigen retrieval was carried out with heat using high pH Tris/borate/EDTA. The slides were incubated with a 1:100 dilution of rabbit anti-ERG MAB (clone EPR3864, Abcam, Cambridge, MA, USA). The anti-rabbit HRP antibody was applied for 16 min at room temperature and detected using a ChromoMap DAB Detection System (Ventana, Tucson, AZ, USA). The slides were then counterstained with hematoxylin. The same primary antibody has been used in other studies as well. In our initial study, ERG staining was scored visually as positive or negative, with any staining of cancer nuclei was classified as positive regardless of intensity or extent based on the finding that even weak expression was correlated with the presence of the *T/E* fusion gene (Park *et al.* 2010). To more

quantitatively evaluate ERG protein expression, each TMA slide was imaged using the Vectra-Image Analysis System (Cambridge Research & Instrumentation, Inc., Hopkinton, MA, USA), an automated multispectral slide analysis system. 4× scans were obtained to detect the TMA, and 20× multispectral images were acquired from each core for IHC scoring of ERG with inForm. The inForm Software supports image analysis projects by combining automated image processing with advanced object recognition and data analysis tools. Using image segmentation technology, we trained the system to recognize and distinguish significant histological features, including cancerous epithelial cells, stroma, and luminal spaces, in entire images. Cellular segmentation was used to detect cellular components such as nuclei and cytoplasm. After segmenting tissues and nuclei, we used the IHC scoring tool to score the intensity of ERG staining in cancer nuclei. We selected four bins, ranging from 0 to 3+, to score ERG in cancerous epithelium with ERG staining. This was then applied to all images, yielding, for each case, a 'distribution' of staining intensity represented as the fraction of cancer nuclei in each bin.

Over the last 10 years, we have carried out numerous studies of protein expression and biological alterations in prostate cancer using a large TMA of radical prostatectomy specimens containing more than 500 prostate cancers. Most of these studies have been published (Yang *et al.* 2002, Agoulnik *et al.* 2003, 2005, 2006, Ayala *et al.* 2003a,b, 2004, 2013a,b, Li *et al.* 2003, 2004a,b, 2006, 2007, 2009a,b, Kwabi-Addo *et al.* 2004, McAlhany *et al.* 2004, Dai *et al.* 2005, Haqq *et al.* 2005, Knudsen *et al.* 2005, Zhou *et al.* 2005, Cordon-Cardo *et al.* 2007, Hodgson *et al.* 2011, Yu *et al.* 2011, Ding *et al.* 2013, Qin *et al.* 2013) and the 46 biomarkers evaluated in this study are summarized in Table 1. For most markers, staining was quantitated in tumor cells in the appropriate subcellular compartment (nuclear/cytoplasmic) after IHC using a multiplicative index of staining intensity (0–3) and extent (0–3), yielding a ten-point staining index (0–9). For other markers, such as Ki67 and TUNEL, stained cancer cells in each core were counted while for CD34 and c-KIT positive cells in the stroma were counted. Not every tumor was scored on every marker, in part due to exhaustion of tumor, folds, other artifacts, etc.

Statistical analysis

ERG expression was summarized in three ways – by visual assessment as a dichotomous variable, as a distribution with four levels, and as the Mean Bin, computed as the

Table 1 Markers evaluated for association with ERG expression

Transcription	Signaling	Microenvironment	Others
AR	PTEN	CD34	ADRB2
SRC1	INPP4b	Semaphorin 4F	ASCT2
SRC2	AKT	c-KIT	CAS
SRC3	P-AKT		CAV1
COUPTFII	FKHR	Cell cycle	FGFR4
ER	P-FKHR	Cyclin D1	HAI1
DAX1	GSK3B	p27	VEGFR3
NCoR	P-GSK3B	SKP2	SPINK1
REGG	GGAP2		GLIPR1
SRF	NFκB p65	Proliferation/death	PIN1
MYC	NFκB p65 P-276	Ki67	WFDC1
p53	NFκB p65 P-536	TUNEL	
PIM2	PKC-E		
	Sprouty1		

frequency weighted average of the bin intensities. Distributional patterns of ERG expression were visualized using a Heatmap, with additional side bars to display corresponding values for visually assessed ERG status and Mean Bin ERG. Agglomerative hierarchical clustering with complete linkage was used to cluster cases with similar distributional patterns. The Kolmogorov–Smirnov (KS) statistic, which quantifies the distance between two empirical distribution functions and is sensitive to differences in both location and spread, was used as the distance metric to compute the 'distance' between each case and every other case. Clustering and heat mapping were performed using R. The Mann–Whitney *U* test was used to test for differences in the levels of studied markers between visually ERG-positive and -negative cancers. The association of average ERG expression (Mean Bin) with clinical and pathological variables as well as the panel of 46 previously studied TMA biomarkers was evaluated using Spearman's rank correlation. There was no formal adjustment for multiple comparisons, although a more stringent $\alpha=0.01$ was used. Kaplan–Meier survival curves and log rank tests were used to evaluate the effect of ERG positivity on biochemical recurrence-free survival (RFS) and the effect of other biomarkers on RFS within each ERG visual expression group model. The associations were summarized by hazard ratios (HRs) and 95% CIs, estimated by Cox proportional hazards regression. Candidate biomarkers were dichotomized as 'low' or 'high' using previously determined cutpoints. Unless otherwise specified, *P* values of ≤ 0.05 were considered statistically significant. Correlations and statistical comparisons were performed using the SPSS Software package (IBM SPSS Statistics, version 20.0, Armonk, NY, USA).

Figure 1

Immunohistochemical analysis of ERG expression in prostate cancers. (A and B) ERG expression in a tissue microarray (TMA). Some cancers showed uniform strong expression (A, 40 $\times$) while other cancers on the same TMA slide showed distinctly lower staining overall, and in some cases substantial intrafocal variability (B, 100 $\times$). The arrowhead indicates a cribriform tumor nodule with variable ERG staining. The arrow indicates ERG staining in endothelial cells which is uniformly strong. (C, D, E, F, G and H)

ERG staining in cancer foci in radical prostatectomy specimens. (C, 40 $\times$) Heterogeneous staining in a cribriform nodule of cancer (arrowhead). Note strong uniform staining in adjacent tumor nodule. (D, 100 $\times$ and E, 400 $\times$) Heterogeneous staining with retained strong staining of endothelial cells (arrows). (F, G and H) Cancers with weaker staining in the center of the tumor (arrowheads).

Results

Variability of ERG expression levels in prostate cancer expressing the ERG oncoprotein

In previous studies, we have carried out IHC for ERG expression using a large TMA of cancers specimens from radical prostatectomies performed to treat clinically localized prostate cancer (Wang *et al.* 2011). As in most studies in the literature, prostate cancer cases were classified as positive for ERG expression if distinct staining was seen in cancer cell nuclei of any core, because even weak expression is correlated with the presence of the *T/E* fusion gene (Park *et al.* 2010). However, we noted differences in staining intensity between cases stained on the same TMA slide (Fig. 1A and B). It was also noted that in some cases, there was a substantial variability of expression between tumor nuclei within a single cancer (intrafocal variability; Fig. 1B). To better understand the extent of such variability, we evaluated ERG expression in 16 consecutive radical prostatectomy specimens by IHC. In each case, the section with the largest percentage of tumor from the radical prostatectomy specimen was used for IHC. Ten cases stained positively for ERG. As in the TMA, we noted substantial variability in staining intensity between different cancers and within a single cancer focus. We quantitated the expression of ERG protein in cancer nuclei visually as 0, 1+, 2+, or 3+ and estimated the

percentage of cancer cells with each staining intensity throughout the section. As shown in Fig. 2, there was substantial variability in staining intensity between the ten cases. Examples of variable staining are shown in Fig. 1C, D, E, F, G and H. We noted that in several cases, there was a tendency to stain more strongly at the

Figure 2

Analysis of ERG expression in prostate cancers by visual quantitation. Ten tumors from radical prostatectomy specimens with ERG staining by IHC were scored for staining intensity in cancer nuclei (0, 1+, 2+, or 3+). The percentage of nuclei with each staining intensity was estimated to the nearest 5%.

Figure 3

Immunohistochemistry with anti-ERG antibody using rapidly fixed prostatectomy tissues. Prostate cancer tissues were snap frozen in liquid nitrogen within 15 min of surgery and a thin slice removed was then fixed overnight in formalin, embedded, and ERG IHC performed. (A) Intrafocal variability. Weakly staining tumor is on the right with strongly staining tumor on the left. A blood vessel is indicated by the arrowhead. (B) Intrafocal variability. A tumor with variable staining within a single focus, ranging from negative to strong. An endothelial cell with strong staining is indicated by the arrowhead. (C, D, E and F) Interfocal variability. (C and D) Medium and high power views of a cancer with uniform strong staining similar to the staining intensity of endothelial cells. (E and F) Cancer with medium staining. Endothelial cells are indicated by the arrowheads. Magnifications are shown at top.

edge of tumors (Fig. 1F, G and H). This is unlikely to be an artifact of fixation because higher staining was seen both on the inner and outer (capsular) sides of the tumor, areas of strong staining often surrounded areas with weak or no staining, and staining of ERG in endothelial cells remained quite strong adjacent to cancer cells with decreased staining.

To confirm that the variable protein levels were not an artifact of delayed or poor fixation, we took prostate cancer tissues that had been snap frozen in liquid nitrogen within 15 min of surgery and took a thin slice that was then fixed overnight in formalin, embedded, and ERG IHC performed. As shown in Fig. 3, we still observed both interfocal and intrafocal variability of ERG expression in these rapidly fixed tissues. It should be noted that if IHC protocols are optimized to give a maximal ERG signal, as

is desirable for clinical use, the variable expression may be far less apparent than reported in this study.

Image analysis of ERG expression in prostate cancer

Given the substantial variability in staining, we carried out image analysis using multispectral imaging to quantify the extent of variable staining objectively. This analysis allowed us to determine the intensity of staining in cancer nuclei on a cell-by-cell basis, with each nucleus binned into one of four expression levels (0, 1+, 2+, or 3+). The mean intensity of ERG staining was then determined for each cancer (ERG Mean Bin) based on the mean of all cancer nuclei. This analysis is shown in Fig. 4 in the Mean Bin row, with the lowest mean intensity shown in blue and the highest in red. The visual image quantitation data for each case is also shown. There was a very strong, although not perfect, correlation between the visual classification and the Mean Bin scores ($r=0.797$, $P<0.001$). We then carried out hierarchical clustering of the distributions of staining, using the KS statistic as the

Figure 4

Examination of ERG expression in prostate cancers by image analysis. ERG expression was evaluated by immunohistochemistry of a prostate cancer tissue microarray as described previously. Expression was evaluated visually and classified as positive if any staining was seen in cancer nuclei. Expression was also quantitated by image analysis of all cancer nuclei. We selected four bins from 0–3+ to score ERG in cancerous epithelia with ERG staining, which was then applied to all images. Among cases with any visually positive staining there was substantial variability between tumors and within each tumor. The mean staining (Mean Bin) was calculated for each case. Cluster analysis of quantitative expression was then carried out for identification of four cluster groups indicated by green (negative), blue (low) orange (medium), and red (strong).

Table 2 Markers associated with ERG expression

Group	Protein or factor	Binary ERG	Mean Bin ERG	
		M–W <i>P</i> value ^a	<i>r</i>	<i>P</i> value ^b
MYC	MYC	<0.001	0.437	<0.001
	PIM2	0.001	0.231	0.002
PI3K/AKT	PTEN	0.004	−0.224	0.007
	INPP4B	0.002	−0.140	0.011
	P-AKT	0.005	0.195	0.003
	P-GSK3B	0.022	0.244	<0.001
NFκB	NFκB p65	0.001	0.303	<0.001
	NFκB P-S536	<0.001	0.316	<0.001
Transcription	p53	0.013	0.257	<0.001
	SRF	<0.001	0.344	<0.001
	SRC1	0.008	0.207	0.004
Signal transduction	Sprouty1	0.002	0.244	0.001

^aMann–Whitney *U* test comparing expression in ERG negative vs ERG positive.

^bSpearman's rank correlation and associated *P* value.

distance metric. The dendrogram is shown in Fig. 4 with cases as columns showing the proportion of nuclei for each staining intensity (white, 0 and dark blue, 1.0). The largest cluster (negative; green) corresponds perfectly with the cases evaluated as negative by visual inspection. The visually positive staining cases are clustered into three groups, corresponding to low (blue), intermediate (orange), and strong (red) average staining. Most cases showed variable levels of staining within the tumor as well as between tumors when analyzed by image analysis. No strong association with pathological variables or biochemical recurrence was noted based on either the visual presence or absence of ERG, Mean Bin values, or cluster group (data not shown).

Correlation of ERG expression with other proteins and biological processes in prostate cancer

To better understand the biological influence of ERG expression and its variability in prostate cancer, we examined the correlation of ERG expression with the expression of proteins and biological factors in this same TMA given in Table 1. We compared marker levels between visually graded ERG groups and evaluated the correlations of these markers with Mean Bin ERG expression. The proteins that showed highly significant associations with ERG expression by both visual and quantitative methods of analysis are listed in Table 2. To be included in this table, associations detected using both methods of analysis had to be statistically significant ($P \leq 0.05$) with at least one method being significant at

$P \leq 0.01$. Scatter plots demonstrating some of these associations are shown in Fig. 5.

The strongest correlation is with MYC expression ($r=0.437$; Fig. 5). Notably, MYC has been shown to be a direct transcriptional target of ERG (Sun *et al.* 2008) and

Figure 5

Relationship between expression of selected proteins and ERG. Scatter plots with conditional uniform jitter and Loess fit curve (75% of points) to illustrate the relationship between MYC, SRF, SRC1 and ERG. The correlation coefficient *r* is Spearman's rank.

our results are consistent with a significant role for ERG in driving MYC expression in primary prostate cancers. PIM2 is also correlated with ERG expression. While PIM2 has pleiotrophic effects on tumor progression, it can synergize with MYC overexpression (Allen *et al.* 1997), and this may be at least one of its important roles in prostate cancer, where MYC overexpression is common (Antonarakis *et al.* 2012).

As described previously, there is a significant positive correlation of ERG with increased levels of NFκB p65 phospho-Ser536, which is an indirect target of the ERG oncoprotein via its induction of TLR4. In addition, nuclear NFκB p65 is correlated with ERG expression. This may be in part via increased PIM1 kinase, which has been reported to be increased by ERG (Magistrini *et al.* 2011), and is known to activate the NFκB pathway. PIM2 has also been linked to NFκB pathway activation (Dai *et al.* 2005).

ERG is negatively correlated with both PTEN and INPP4B (Table 2). Loss of the *INPP4B* phosphatase also activates the AKT pathway and is an alternative mode of activating this pathway in prostate cancer (Agoulunik *et al.* 2011). The association of ERG expression with loss of *PTEN* at the genomic level has been described previously, and loss of *PTEN* and expression of ERG synergize in prostate cancer induction in genetically engineered mouse models of prostate cancer. Our results confirm this association and indicate that there is also correlation with activation of the AKT pathway by alternative genomic alterations, and thus the observed association with *PTEN* loss is unlikely to be due to loss of other activities of *PTEN* (Bassi *et al.* 2013). Notably, in those cancers with ERG expression, low INPP4B levels were associated with a significantly decreased time to biochemical recurrence ($P=0.023$, HR = 2.01, 95% CI (1.09, 3.70), Fig. 6). There was no difference in ERG negative cases ($P=0.31$). We did not see a similar association with *PTEN* levels (results not shown). Loss of *PTEN* has been associated with worse outcome in T/E-fusion-positive prostate cancer in some but not all studies (Reid *et al.* 2010, Leinonen *et al.* 2013). Consistent with these findings, ERG is positively associated with phosphorylated AKT (Ser473) and phosphorylated GSK3B. Taken together, these results are mutually consistent and indicate that ERG expression is broadly associated with activation of the PI3K pathway.

We have previously shown that SRF is increased in prostate cancer, and higher expression is associated with biochemical recurrence after radical prostatectomy (Yu *et al.* 2011). Results of recent studies have indicated that SRF is associated with aggressive metastatic disease as well (O'Hurley *et al.* 2014). Unexpectedly, we have found that SRF is significantly positively associated with ERG

Figure 6

INPP4B loss decreases time to biochemical recurrence in prostate cancers expressing the *TMPRSS2/ERG* fusion gene. Kaplan-Meier analysis of time to biochemical recurrence following radical prostatectomy of ERG-positive cancers with low ('0-5', $n=65$) or high ('6-9', $n=103$) *INPP4B* index. The log-rank test for P value is 0.023.

expression and is the second strongest association ($r=0.344$; Fig. 5) we identified (after MYC).

Expression of the p160 steroid receptor co-activator SRC1 is also positively correlated with ERG protein levels (Table 2 and Fig. 5). This positive association is most probably due the known role of SRC1 in promoting *TMPRSS2* (and presumably *T/E* fusion gene) expression (Nakka *et al.* 2013).

The p53 pathway is among the most commonly altered pathways in cancer and although p53 mutation is uncommon in clinically localized cancer, we noted a positive correlation between ERG and p53. An increase in the expression of p53 protein is generally seen when this pathway is inactivated by mutation or some other mechanisms. Others have previously noted a correlation between the presence of the *T/E* fusion gene and p53 protein expression (Minner *et al.* 2011, Kluth *et al.* 2014).

An increase in the expression of Sprouty1 is associated with ERG expression. We have previously shown significant heterogeneity of Sprouty1 expression in prostate cancer with some cases being negative and other cases retaining staining or having increased staining compared with benign epithelium (Kwabi-Addo *et al.* 2004). The reason why ERG-expressing cancers would express Sprouty1 is not known, but *ETS* transcription factors are known to play an important role in *Sprouty* gene expression (Tsang & Dawid 2004) and therefore it may be a direct target for ERG. *Sprouty1* is known to inhibit FGF

Table 3 Markers associated with ERG expression levels

Protein or P-protein	<i>r</i>	<i>P</i> value ^a
SKP2	0.291	0.003
NFκB P-S536	0.328	<0.001
Sprouty1	0.244	0.001
SRF	0.274	0.002

^aSpearman's rank correlation and associated *P* value.

receptor signaling, but can enhance EGFR signaling (Tsang & Dawid 2004), therefore some ERG-expressing cancers may have enhanced EGFR signaling via this mechanism.

Associations with ERG protein levels

To determine whether there is a unique biology associated with different levels of ERG protein as determined by image analysis, we examined the association of ERG protein levels with the 46 proteins/phosphoproteins and biological processes given in Table 1, excluding all cases that were ERG-negative (green cluster, Fig. 4). Thus all associations are driven by differences in ERG protein level between positive cases. Results of this analysis are given in Table 3, which lists the factors strongly associated with higher ERG expression ($P < 0.01$). Of note, most of the factors in Table 2 are no longer strongly significant except for NFκB p65 phospho-Ser536, SRF, and Sprouty1. SKP2 is identified as a novel factor associated with ERG protein levels. Thus differential ERG protein expression levels are associated with the activation of distinct pathways and proteins that are distinct from the pathways linked to the presence of ERG *per se*.

Discussion

Prostate cancer expressing the *TMPRSS2/ERG* fusion gene is by far the most commonly identified prostate cancer variant, and understanding how this fusion affects carcinogenesis is critical in developing novel approaches for therapy in men with prostate cancers bearing this fusion gene. We have analyzed associations 46 proteins/phosphoproteins or biological process with the presence and level of ERG protein in clinically localized prostate cancer and identified multiple key proteins and pathways that are associated with the expression and/or level of expression of ERG. Such associations may reflect different types of underlying biology. One potential cause of such association is if the identified protein drives the expression of the fusion gene, as is probably the case for SRC1 (Nakka et al. 2013). Alternatively, the *ERG* fusion gene may

directly drive transcription of a protein (such as MYC) or indirectly drive increased expression (NFκB p65 phospho-Ser536 via TLR4). In addition, such associations may be due to selection of strong biological complementation of a pathway/protein with ERG expression, as is apparently the case for the PI3K/AKT pathway. Finally, it is possible that some observed associations may be due to chance, as may occur with examination of multiple associations, although our stringent *P* values make this less likely. At present the exact mechanisms underlying many of the associations we have identified will require further study. However, we have confirmed using human tissues some previously inferred associations based on mechanistic data (MYC), identified new associations related to prior identified associations (INPP4B, NFκB, and PIM2) and also identified novel associations (SRF, Sprouty1). These associations in human cancers provide a strong rationale for further mechanistic studies.

On the basis of the results of quantitative image analysis, our studies confirm that there is interfocal and intrafocal variability in ERG protein levels in prostate cancer. Early studies had shown that different cancer foci in radical prostatectomy specimens were discordant for the presence of the fusion gene in some cases (Mehra et al. 2007). This is well established and we have not examined this question in the current studies. In contrast, our studies of both the radical prostatectomy specimens and in the TMAs examine only a single cancer focus in each radical prostatectomy. We now show objectively that there is heterogeneity in overall levels of ERG protein expression in different patients. Such heterogeneity may arise due to germline variability between patients and/or genomic variability between cancers. For example, germline variants of the *TMPRSS2* enhancer affecting AR binding (Clinckemalie et al. 2013), germline AR variants, and/or germline variants affecting androgen synthesis and/or metabolism may all potentially affect T/E fusion protein levels. Vitamin D can also increase transcription of the T/E fusion gene (Washington & Weigel 2010) and individual variation in vitamin D metabolism and/or intake may also potentially influence fusion gene expression. The variable levels of coactivators (such as SRC1 and potentially others) may also affect transcription. Differential miRNA expression between different cancers may also affect ERG levels because miRNAs targeting the T/E fusion have been identified (Hart et al. 2013). In any case, we have previously shown marked variability in levels of fusion gene mRNA in different fusion-gene-expressing cancers (Wang et al. 2006), so some of the variability between different patient's cancers is almost certainly due to differential levels of

transcription and/or mRNA stability in those tumors. We have also described significant differences in alternative splicing in different cancers, which can affect translational efficiency (Zammarchi *et al.* 2013); therefore, translational alterations must also be considered. Differences in protein stability or degradation based on differences in alternatively spliced isoforms or associated tumor alterations may also play a role.

We also observed significant differences in ERG levels within a single cancer lesion. The finding that expression was stronger at the edge of some tumors indicates that invasion, proliferation, or local microenvironmental factors may modulate ERG expression.

Alternatively, genomic heterogeneity within cancers, including prostate cancer, is well known, and other genomic alterations may be heterogeneous within a given cancer focus and this could affect ERG protein levels. Further studies are needed to determine the basis of intrafocal heterogeneity in fusion gene protein levels in prostate cancer.

The influence of different levels of ERG protein on the biology of fusion-gene-expressing cancers is not completely clear. The core proteins/pathways identified in our study (Table 2) appear to be associated with any level of ERG protein expression, because the associations are present even when tumors are classified based on visual detection of any ERG protein in cancer cells. However, the associations noted with variable ERG expression are different and only partially overlapping. Thus there is probably significant similarity in the biology of all ERG-expressing prostate cancers but also some differences. The differences may be important in some contexts. For example, NFκB p65 phospho-Ser536 levels are positively correlated with the levels of ERG protein, which indicates that cancers with high ERG protein may be more sensitive to therapies targeting the NFκB pathway. We did not observe any differences in clinical outcome related to the levels of ERG expression. However, results from a recent study of a radical prostatectomy cohort enriched for highly aggressive disease have indicated that higher levels of ERG protein are significantly associated with distant metastasis and death of prostate cancer cells (Spencer *et al.* 2013). Notably, in this study, the presence of ERG protein *per se* was not associated with aggressive prostate cancer, concordant with the results of most, but not all, studies of radical prostatectomy cohorts to date. It is possible that our study is underpowered for detecting such associations because the majority of our cases were not aggressive. Additional studies of larger cohorts with larger numbers of aggressive cancers will be therefore

needed to determine whether the levels of ERG expression influence clinical outcome.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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