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Review

Signaling networks in TMPRSS2-ERG positive prostate cancers: Do we need a Pied Piper or sharpshooter to deal with "at large" fused oncoprotein

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Abstract: Overwhelmingly increasing scientific evidence has provided near complete resolution of prostate cancer landscape and it is now more understandable that wide ranging factors underlies its development and progression. Increasingly it is being realized that genetic/epigenetic factors, Intra-tumoral and inter-tumoral heterogeneity, loss of apoptosis, dysregulations of spatio-temporally controlled signaling cascades, Darwinian evolution in response to therapeutic pressures play instrumental role in prostate carcinogenesis. Moreover, multi-directional patterns of spread between primary tumors and metastatic sites have also been studied extensively in prostate cancer. Research over the years has gradually and systematically revealed closer association between tumor phenotype and type of gene fusion. Latest developments in deep sequencing technologies have shown that gene fusions originate in a non-random, cell type dependent manner and are much more frequent than previously surmised. These findings enabled sub-classification and categorization of seemingly identical diseases. Furthermore, research methodologies have shown that many gene fusions inform us about risk stratification and many chimeric proteins encoded by the fused genes are being studied as drug target/s. We partition this multi-component review into the molecular basis of formation of fusion transcripts, how protein network is regulated in fusion positive prostate cancer cells and therapeutic strategies which are currently being investigated to efficiently target fusion transcript and its protein product.

Key words: TMPRSS2-ERG; Prostate Cancer; Apoptosis; Signaling.

Introduction

Recent breakthroughs in computational approaches and next generation sequencing technologies have enabled us to study a number of cancer genome profiles by whole genome sequencing. We have witnessed tremendous advancements in information related to Cancer genomic alterations and variability in different cancers. Local and global cancer genome-sequencing projects, including The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium (ICGC), have helped researchers to analyze different cancer genomes particularly through exome sequencing (1,2). Data obtained through high-throughput technologies has considerably improved our understanding of somatic mutations in noncoding regions including untranslated region/s (UTRs), introns, non-coding RNAs and regulatory elements (1,2).

Prostate cancer (PCa) is multifaceted and genomically complex disease and treatment of dynamically evolving heterogeneous nature of prostate cancer is difficult. It is noteworthy that multiplatform sequencing technologies have markedly improved our understanding of the PCa biology and high-impact research has started to shed light on the well-defined and distinct molecular subtypes that have diverse passenger and driver genomic changes (1,2,3). Androgen receptor (AR), a nuclear steroid hormone receptor played a contributory role in prostate cancer progression and development. Structural studies had shown that it contained a ligand-binding domain (LBD), DNA binding domain (DBD), a hinge region and an N-terminal domain (NTD). Wealth of information suggested promiscuous activation of AR-LBD mutants by anti-androgens. Anti-androgens have been shown to activate AR-W741C and AR-T878A mutants, adrenal androgens and progesterones activate AR-V715M and glucocorticoids activate AR-L702H. Mutations also induced conformational changes in the AR. T878A mutation stereochemically altered LBD. There was a constitutive activation of AR splice variants that lacked LBD and a simultaneously upregulated expression of AR-target genes (1,2,3).

Substantial fraction of information has been added into the DNA damage repair biology and we have developed a sharper understanding of the intertwined nature of double stranded DNA which is characteristically unique and ensures the expression, transmission and storage of genetic information. However, almost all types of DNA transactions, such as chromatin compaction, gene transcription, replication, formation of higher order structures and recombination, lead to topologically entangled structures that must be resolved to maintain cellular functions. DNA topoisomerases are versatile regulators that have evolved to resolve these DNA entanglements.

Array-based platforms have revolutionized the research associated with copy number profiling and gene expression and opened new horizons to guide detection of fused genes. Array-based platforms had superior quality and offered higher resolution as compared to chromosomal banding analysis. PAX3– NCOA1, a fused gene formed by fusion of PAX3 (transcriptional factor) and nuclear receptor coactivator 1 (NCOA1) was the first novel gene fusion to be detected on the basis of the gene expression.

It was in 2005, when Tomlins et al, identified a fusion transcript in prostate cancer patients using fluorescence in situ hybridization (FISH) (4). Fusions juxtaposing the noncoding androgen regulated gene trans-membrane protease serine 2 (TMPRSS2) to ERG resulted in the formation of TMPRSS2– ERG. Three-color FISH was used by another research group to show that TMPRSS2-ERG fusion may be accompanied by a small hemizygous sequence deletion on chromosome 21 between TMPRSS2 and ERG genes (5). TMPRSS2–ERG interstitial region contained 16 protein-encoding genes, reportedly involved in tumor-suppression (6). ETS2 was also identified in this region and its ectopic expression markedly reduced proliferation and invasive potential of prostate cancer cells. ETS2 loss caused activation of MAPK pathway, which cooperated with Pten loss that consequently resulted in the development of advanced PCa (6).

We have witnessed exponential growth in the field of genomic instability, genomic rearrangements and fused oncoproteins in different cancers. This review deals mainly with the underlying mechanisms of generation of TMPRSS2-ERG and how it modulates signaling machinery in prostate cancer. Before starting our discussion on the regulation of signaling networks in fusion positive prostate cancer, we summarize some of the landmark findings related to the generation of TMPRSS2-ERG in prostate cancer.

Generation of TMPRSS2-ERG

Since the discovery of TMPRSS2-ERG in prostate cancer, much attention has been given to the underlying mechanisms of generation of fusion transcripts in prostate cancer. We set spotlight on some of the most significant advancements made in our understanding of the mechanisms which underpin generation of fusion transcripts.

Topoisomerase II (TOP2) is an enzyme that transiently catalyzed DSBs to resolve topologically constrained segments of DNA (7). Dysregulation of TOP2 induced DSBs which were reported to be contributory in the generation of rearrangements in childhood leukemia and acute myeloid leukemia (t-AML). Estrogen receptor (ER) induced transduction cascade is reportedly involved in positioning TOP2B (TOP2 isoform) to regulatory sequences of target gene network, to induce TOP2B-modulated DSBs. LAPC4 and LNCaP prostate cancer cells have functionally active androgen induced transduction cascade. TOP2B and AR have previously been observed to co-exist at promoter and enhancer regions of TMPRSS2 and KLK2 (PSA encoding gene) in DHT-treated cells. Surprisingly, TOP2B mediated cleavage was notably higher in the presence of AR (7). Both proteins were present at the target sites in androgen treated cancer cells. In the absence of AR, TOP2B mediated cleavage was significantly impaired. For detection of DSBs at specified locations, these breaks were labelled with biotin-conjugated nucleotides. DSBs were induced by DHT at sites of TOP2B positioning and activity that accompanied binding of AR within TMPRSS2 and ERG genes (7). Shown in figure 1. Surprisingly, regions at ERG and PSA neither showed considerable TOP2B catalytic activity nor significantly detectable DSBs. DSBs formation and recruitment of Ataxia-telangiectasia mutated (ATM) kinase appeared within first 6 hours in DHT stimulated cells and was largely resolved after 24 hours. Information obtained from ChIP-re-ChIP experiments provided evidence that ATM was recruited to the biotinylated strands. Findings clearly suggested that these were double stranded breaks and not just single strand nicks (7). Furthermore, ligation-mediated PCR (LM-PCR) strategy was used to map single nucleotide breaks occurring at specified location showing higher DHT mediated loading of TOP2B and catalytic functions, loading of ATM and closely aligned TMPRSS2 genomic breakpoint (7).

Treatment of TMPRSS2-ERG negative LNCaP prostate cancer cells with TNF-α for 48 hours robustly triggered formation of the TMPRSS2-ERG gene fusion transcript. Markedly higher phosphorylated histone H2A.X (Ser139) levels were noted in TNF-α treated LNCaP cells within 12 hours of exposure (8). Breaks in the TMPRSS2 and ERG loci and RAD51 foci formation were also notable in TNF-α treated LNCaP cells. Murine model of in-vivo inflammation was used to study inflammation mediated generation of fusion gene. TMPRSS2-ERG was noted in LNCaP cells injected into the air pouch of C57BL/6 mice. Transcript levels were found to be upregulated upon treatment with lipopolysaccharide (8). Macrophages/neutrophils also played a role in the generation of TMPRSS2-ERG fusion transcript. As SCID mice also contained macrophages, researchers concluded that macrophages were necessary but not sufficient for formation of gene fusions. Data clearly suggested that a complex network of interactions between macrophages and other immune cell types (T and B cells) mediated formation of gene fusions (8). Bromodomain and extraterminal domain (BET) protein inhibitors (I-BET, JQ1) are reportedly effective against inflammation. BET inhibition significantly reduced production of pro-inflammatory cytokines in macrophages and ablated inflammatory responses in mouse models. Treatment with inhibitors (JQ1) blocked generation of TMPRSS2-ERG fusion transcript in the air-pouch mo-

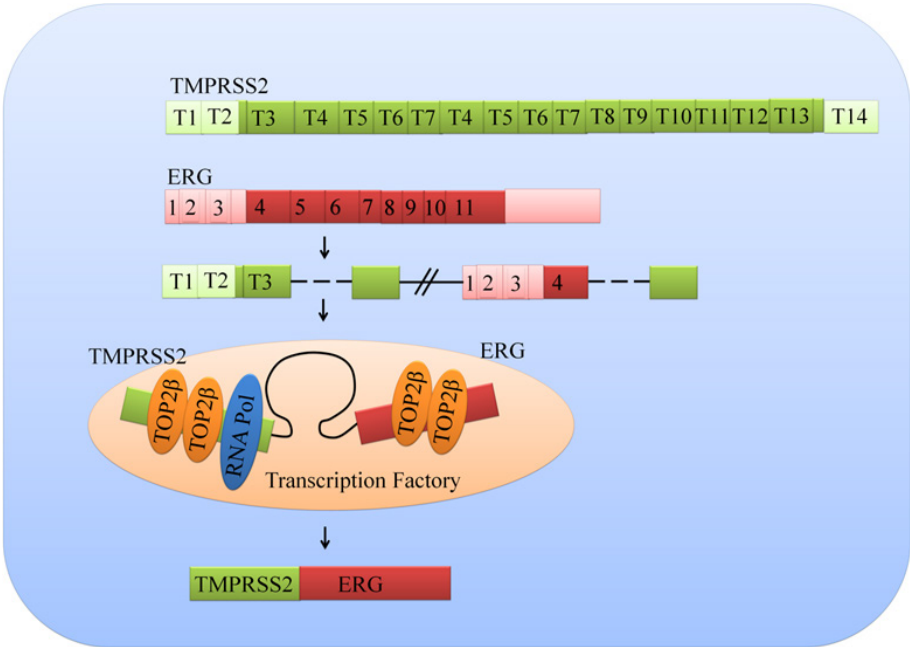

Figure 1. Androgen signaling induced juxtapositioning of *TMPRSS2* and ERG and triggered the association of these two genes within transcriptional factory. Androgen also induced the positioning of AR and Topoisomerase 2β to the sites of genomic breakpoints.

del for in vivo inflammation (8).

Upcoming section deals with how TMPRSS2-ERG interferes with DNA repair pathways particularly NHEJ in prostate cancer cells.

TMPRSS2-ERG gene fusion mediated inhibition of XRCC4-induced NHEJ repair

Non-homologous end-joining (NHEJ) and Homologous recombination (HR) represent two versatile, well co-ordinated DNA damage repair pathways. NHEJ is involved in repair of non-replication associated breaks, induced by ionizing radiations (IR). Recent report suggested that NHEJ was impaired in fusion gene expressing prostate cancer cells.

Fused gene expressing VCaP cells showed γH2AX and 53BP1 foci that was indicative of a basal level of DNA damage. IR exposed VCaP cells demonstrated markedly higher number of γH2AX and 53BP1 IR-induced foci (IRIFs) after 30 and 60 minutes of exposure (9) Shown in figure 2. Resolution of DNA damage foci was delayed in fused gene expressing VCaP cells as compared to TMPRSS2-ERG depleted cells. PC3 cells did not endogenously express TMPRSS2-ERG, however, enforced expression of fusion gene in PC3 cells triggered constitutive 53BP1 and γH2AX foci which further increased after IR exposure. Most of the γH2AX IRIFs were resolved in IR exposed parental PC3 cells by 6 hours. However, cells expressing TMPRSS2-ERG showed more persistent γH2AX IRIFs at 3 and 6 h, indicating that IR-induced DNA damage repair was impaired (9). Nevertheless, there was a significant reduction in the number of γH2AX IRIFs by 6 hours as compared to that found at 1 hour. Results provided evidence that DNA damage repair was not completely inhibited in the fusion expressing cells instead it proceeded with a slower kinetics. Regulation of c-NHEJ pathway has also been studied in TMPRSS2-ERG expressing cells. Chromatin recruitment of c-NHEJ factors has recently been investigated in fusion gene expressing PC3 parental and derivative cells. Recruitment of XRCC4 was not noticed in IR exposed TMPRSS2-ERG-expressing cells (9). However, there was a significant loading of XRCC4 to the chromatin of parental PC3 cells. Levels of Ligase IV, Ku70 and XLF that were recruited to the chromatin, increased after exposure of cells to IR. But these levels reduced dramatically in fused gene expressing cells as compared to parental PC3 cells (9). DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is the versatile modulator involved in regulation of c-NHEJ. DNA-PKcs phosphorylation in ABCDE cluster on Thr2609 played significant role in enabling the dissociation of DNA-PKcs from chromatin. Phosphorylated DNA-PKcs was present in chromatin-bound cellular fractions obtained from parental cells as early as 30 minutes after IR exposure whereas PC3 (TMPRSS2-ERG positive) cells had notably reduced phosphorylated DNA-PKcs (9). Dysregulated functionality of DNA-PKcs and XRCC4 was observed in fused gene expressing prostate cancer cells. Shown in figure 2.

NKX3.1 suppressed gene rearrangements and mediated repair of AR-triggered DNA damage

NKX3.1, a tumor suppressor is reportedly involved in DNA damage repair. Certain hints have emerged suggesting that NKX3.1 inhibited juxtapositioning of TMPRSS2 and ERG loci in prostate cancer cells. Experimentally it had been verified that juxtapositioning of TMPRSS2 and ERG loci increased considerably in NKX3.1 knockdown cells (10). Frequency of TMPRSS2-ERG rearrangements induced by DNA damaging agents and DHT was remarkably enhanced in NKX3.1 knockdown LNCaP cells. Histone de-methylation and generation of peroxides are 2 of the important mechanisms during process of transcription by nuclear receptors. Newly-generated peroxides oxidized DNA that resulted in the formation of 8-oxoguanine adducts at the hormone-response elements (10). These were further modified by OGG1 and served as targets for base excision repair. Using chromatin Immunoprecipitation

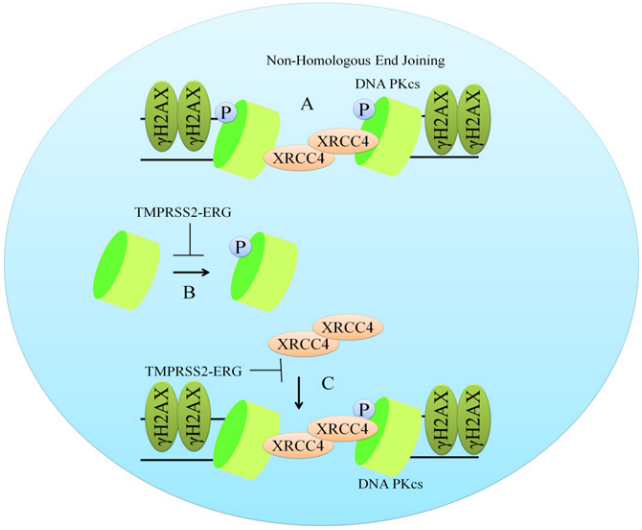

Figure 2. (A) NHEJ mediated repair. However, (B) TMPRSS2-ERG inhibited phosphorylation of DNA-PKcs. Furthermore, (C) loading of XRCC4 was also reduced.

technique it was shown that loss of NKX3.1 resulted in an increase in the recruitment of AR to both the ERG IV and ERG II, III break sites in NKX3.1 knockdown cells. In the absence of NKX3.1, there was a reduction in the loading of OGG1 to the ERG breakpoint sites that favored breakage of DNA and genetic recombination. It was concluded that reduction of NKX3.1 levels facilitated AR-mediated DNA breakage (10). Upcoming section deals with regulation of different proteins in fusion positive prostate cancer cells.

Regulation of protein network in fusion positive prostate cancer cells

Fusion status has been a matter of debate and circumstantial evidence suggested differential gene and protein network in fusion positive and negative prostate cancer cells. Here we restrict our discussion to protein network in fusion positive prostate cancer cells. We summarize how ERG interacts with different proteins, how it transcriptionally triggers expression of target genes and how different proteins are biochemically modified in fusion positive prostate cancer cells. In following section we will initially review different genes known to be controlled by ERG.

Transcriptional regulation of target genes

ERG T1-E4 (ERGΔ39), encoded by a fused gene formed between 1st exon of TMPRSS2 and 4th exon of ERG, interacted with bromodomain-1 of bromodomain containing protein 4 (BRD4), a member of BET family (11). Detailed mechanistic insights revealed that BRD4 and ERG co-occupied well-conserved binding sites present in the RHGDIA, ZBTB7B, WDR45B, TBRG4, YEATS4 and YWHAE (14-3-3ε). p300, an acetyltransferase acetylated ERG and promoted its binding with BRD4 in prostate cancer cells (11). Shown in figure 3.

Mir-200b/a/429 gene cluster

Mir-200b/a/429 gene cluster and miR-205HG gene are controlled by ERG binding sites located proximally to the transcriptional start site. miR-200b/200a/429 promoter region contained an ERG binding site which had 2 potential ERG binding sequences, ETS-1 and ETS-2 (12). miR-200b/a/429 primary transcript was also considerably downregulated in ERG silenced VCaP cells. However, surprisingly, ERG did not trigger expression of these miRNAs in prostates of TMPRSS2/ERG transgenic mice (12). Shown in figure 3.

TRIM25

Tripartite Motif Containing 25 (TRIM25) is frequently overexpressed in PCa. ERG binding site was present in promoter region of TRIM25. Both C-terminal activation domain (CAD) of ERG and RING domain of TRIM25 were noted to be essential for facilitation of the structural association between these proteins (13). Mechanistically it was shown that levels of ubiquitinated ERG increased dramatically in TRIM25 overexpressing cancer cells. Data clearly suggested that TRIM25 mediated poly-ubiquitination of truncated and full-length ERG variants (13). Shown in figure 3.

Prostaglandin E receptor

IL-6 production was markedly higher in ERG-overexpressing DU145 cells (14). There was a 2.8-fold increase in Prostaglandin E Receptor 4 (PTGER4) in ERG overexpressing prostate cancer cells. 2 ERG binding sites (EBS) have recently been reported at 4.4 kb and 6.4 kb in PTGER1 promoter region and 2 EBS at 6.3 kb and 6.8 kb in promoter region of PTGER2 (14). Contrastingly, no EBS was identified in promoter region of PTGER3 or PTGER4. Moreover, IL-1b promoter also contained 4 EBS within a distance of 4.2 kb from the transcriptional start site (TSS) (14). Shown in figure 3.

Neurotransmitters and their receptors

ERG$^+$ LNCaP cells had significantly higher levels of neurotransmitters, quinolinate, choline and glutamate. Moreover, 2 important molecules of acetylcholine synthesis pathway, glycerophosphocholine and CDP-choline were found to be upregulated (15). Data suggested that ERG induced an upregulation of receptors for neurotransmitters and production of neurotrans-

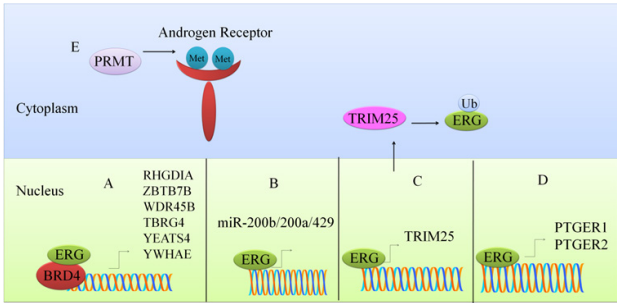

Figure 3. (A) ERG mediated regulation of different genes. ERG interacted with BRD4 and regulated different target genes. (B) ERG also regulated expression of miR-200b/200a/429. (C) TRIM25 was regulated by ERG. In the cytoplasm, TRIM25 ubiquitinylated ERG. (D) PTGER1 and PTGER2 are also regulated by ERG. PRMT added methyl groups to androgen Receptor.

mitters.

Insulin-like Growth Factor Receptor (IGF1R)

The T1/E4 variant is most commonly noted rearrangement which includes UTR of the TMPRSS2 gene fused to 4th exon of ERG leading to an amino-terminally truncated ERG protein having an intact ERG DNA-binding domain. Truncated ERG (tERG) expression induced a 23-fold increase in insulin-like growth factor receptor (IGF1R) promoter activity (16). There was a 47% decrease in Sp1 levels in tERG knockdown cells. Markedly reduced tERG levels correlated with a 50% decrease in mRNA levels of Sp1 at 72 hours post-transfection. IGF1R was internalized through clathrin/caveolin-dependent pathway. There was a blockade of IGF1R internalization in clathrin or caveolin-1 silenced cells. tERG, AP-2, IGF1R and Sp1 co-immunoprecipitated with caveolin. However, role of this multicomponent machinery required detailed research. Nuclear IGF1R levels were markedly decreased in tERG overexpressing cancer cells (16).

It is clear that gene network is differentially regulated in fusion positive PCa cells. Fusion transcript encoded proteins not only transcriptionally regulate wide array of genes but different proteins also undergo biochemical modifications in fusion positive PCa cells, which is the topic of our discussion in next section.

Biochemical modifications

PRMT5, an arginine methyl-transferase regulated signal transduction cascades through mono- and symmetric dimethylation of arginines of its target proteins. PRMT5 methyl-transferase activity was essential for transcriptional repression of target genes of AR (17). AR was immuno-precipitated from ERG positive prostate cancer cells and noted to contain mono-methylated or symmetrically di-methylated arginine. More importantly, ligand binding domain of AR was noted to undergo mono- and symmetrical dimethylation in an ERG- and PRMT5-dependent manner (17).

KDM1A, a histone demethylase is involved in removal of monomethyl and dimethyl marks from either 4th or 9th Lysine of histone 3. KDM1A removed repressive methyl marks from H3K9 and enhanced transcriptional activation of androgen receptor target genes. Euchromatic histone-lysine N-methyltransferase 2 (EHMT2) methylated KDM1A at 114th lysine (18). There was an increase in KDM1A K114me2 methylation in EHMT2 overexpressing LNCaP cells. Chromodomain-helicase DNA-binding protein 1 (CHD1) interacted with KDM1A K114me2 and co-occupied androgen receptor binding sites. Treatment of cells with dihydrotestosterone induced an increase in the levels of KDM1A K114me2 and simultaneously enhanced loading of AR and CHD1 at enhancer regions of TMPRSS2. KDM1A K114me2 androgen-dependently controlled formation of TMPRSS2 enhancer-breakpoint loop. There was a robust impairment of androgen-induced looping in EHMT2, KDM1A or CHD1 knockdown prostate cancer cells. Mutant CHD1 failed to interact with KDM1A K114me2 peptide and consequently androgen-dependent chromatin looping was impaired (18).

Cul3-based Cullin-RING ligases structurally associate with BTB adaptors to form a BTB-CUL3 E3 ubiquitin ligase complex. SPOP, a substrate binding subunit of this complex had tumor suppressive role in prostate cancer. ERG protein levels were notably enhanced in SPOP silenced prostate cancer cells. RBX1 and CUL3 are essential components of the BTB^{SPOP}-CUL3-RBX1 multi-protein nano-machinery. There was also an increase in the ERG protein levels in CUL3 and RBX1 silenced LNCaP cells. Different mutations have been reported in the MATH domain present in the substrate-binding motif of SPOP. Protein structural studies revealed important sequence of amino acids present within lining of substrate binding pocket of Meprin and TRAF-C homology (MATH) domain which was frequently disturbed because of mutations. ERG levels increased dramatically in mutant SPOP expressing cells (19). Co-immunoprecipitation assay revealed that SPOP did not interact with T1-E4 fusion in VCaP cells. However, it interacted with endogenous ERG (full-length) in PCa cells. Anchorage-independent growth of Benign prostatic hyperplasia (BPH) epithelial cells (PTEN-deficient) that ectopically expressed T1-E4 and T1-E5 was markedly higher as compared to T1-E1/E2 expressing cells (19).

Androgen biosynthetic enzymes

ERG regulation of androgen biosynthetic enzymes (ABEs) had recently been studied in (PCa). Expression levels of Aldo-keto reductase family 1 member C3 (AKR1C3), Hydroxysteroid 17-Beta Dehydrogenase 4 (HSD17B4) and HSD17B6 were highly reduced in ERG silenced VCaP cells (20). ERG regulated AKR1C3 expression by directly binding to the AKR1C3 gene. DHT production from 5α-Adione and androsterone was significantly reduced in ERG knockdown cells. HSD17B3 and AKR1C3 catalyzed the biochemical reduction of 5α-Adione to DHT. ERG enhanced 5α-Adione mediated activation of AR by directly upregulating AKR1C3 expression (20).

TGF signaling

ERG interacted with Smad3 and phosphorylated Smad3 proteins. SB431542 is a small molecule that inhibits TGF-β type I receptor and a potent anticancer agent (21). Cells co-expressing ERG and Smad3 were treated with SB431542. Expectedly, SB431542 exerted inhibitory effect on transcriptional activity of TGF-β/Smad3. However, SB431542 mediated inhibitory effect on transcriptional activity of TGF-β/Smad3 were relieved by increasing expression of ERG. Results suggested that ERG counteracted inhibitory effects of SB431542. It was further suggested that ERG interacted with TGF-β type I receptor and interfered with the interaction of SB431542 and TGF-β receptor (21).

Differential expression of genes in fusion positive prostate cancers

Expression levels of ERG, PLXNB1 and MMP-9 were upregulated in metastatic PCa and the mRNA expression of these genes correlated positively with fusion

positive PCa (22).

Alpha-methylacyl-CoA racemase (AMACR), a biomarker extensively utilized in diagnosis of prostate cancer was noted to be correlated with ERG expression in PCa patients with adverse clinical outcome (23). Stronger expression of DNA ligase IV was noted in TMPRSS2-ERG expressing and PTEN deleted tumors (24).

Dualistic roleplay of miR-204 in PCa

miR-204 has most recently been investigated to show dualistic activity in PCa. It had diametrically opposed roles in the regulation of AR mediated signaling. There was a markedly downregulated TMPRRS2/ERG transcript in miR-204 overexpressing VCaP cells. DNA methyltransferase inhibitors abrogated miR-204 induced inhibitory effects on TMPRSS2/ERG mRNA in VCaP cells (25). Significantly downregulated TMPRSS2/ERG transcripts were noted in RUNX2, MYB and ETS1 silenced VCaP cells. There was a significant increase in DNA hypermethylation in promoter region of fusion gene upto 91% in RUNX2 silenced VCaP cells. AR mRNA and protein levels were noted to be upregulated in miR-204 overexpressing cells (25). Inhibition of RUNX2 triggered an increase in methylation of AR promoter in LNCaP (20 to 57%) and VCaP (30 to 47%) cells. Whereas, inhibition of ETS1 resulted in an increase in methylation of AR promoter in LNCaP (20 to 81%) and VCaP (30 to 67%) cells. Data provided comprehensive evidence that there was a paradigm shift in the activity of miR-204 from a tumor suppressor to an oncogenic miRNA via modulation of chromatin organization, epigenetically reprogrammed cellular differentiation, regulation of histone and DNA methylation. This change was also accompanied by upregulation of differentiation disrupting AR interacting transcription factors like ETS1, c-MYB and RUNX2 (25).

Characterization of molecular details of ERG^+ tumors in castration resistant and primary PCa

Calcium signaling associated proteins (CACNA1D and NCALD), an inflammation associated protein (HLA-DMB), an ERG associated protein (DCLK1) and CD3 positive immune cells have recently been studied in primary PCa and CRPC metastasis (26). In ERG^+ PCa (primary) weaker correlations were noted with CACNA1D and NCALD. There was a decrease in the association of HLA-DMB with ERG. Moreover, association of CD3 cell number with ERG shifted to negative from positive in CRPC metastasis. DCLK1 was found to be considerably enhanced in unpaired ERG^+ PCa (primary origin) and CRPC metastasis. In PCa of primary origin, expression of targeted proteins or ERG status did not show any association with biochemical recurrence -free survival (26). However, in case of primary PCa, patients who had ERG^+DCLK1^- revealed longer duration of time to biochemical recurrence as compared to ERG^+DCLK1^+ patients. Data suggested that ERG^+ may partially drive DCLK1 induced progression of PCa (26).

Therapeutics

TMPRSS2-ERG has recently been shown to be versatile regulator of bone metastasis. TMPRSS2-ERG expressing cells were inoculated into the right flank of male SCID mice and results revealed that fusion positive PCa cells induced formation of subcutaneous tumors in experimental mice. Fusion positive PCa cells were injected in the left ventricle of male SCID mice (27). PCa cells colonized mandibles and nose in tested mice from 4^{th} day. Luciferase was later detected in hind limbs and the spine around 14^{th} day. 24 days after injection, mice had developed bone metastatic foci, however, fusion status played contributory role as evidenced by 57% more bone metastases in mice injected with fusion positive PCa cells as compared to the control group (27). Therefore efficient targeting of fusion transcript encoded protein is very important. Surprisingly, some morphologically homogeneous malignancies were noted to be heterogeneous with respect to status of fused genes. Status of the fused genes contributed to the patient′s clinical outcome.

50 nM concentration of short interfering RNAs (siRNAs) directed against variants III and IV of TMPRSS2-ERG reduced both mRNA and protein levels of fused gene in VCaP cells. There was a notable downregulation of negative regulators of apoptosis in siRNA treated VCaP cells but inhibition was more pronounced in VCaP cells treated with siRNA designed against variant IV (28). Furthermore, oxidative stress associated proteins (HO-1/Hsp32 and claspin) were also inhibited in VCaP cells treated with siRNA designed against variant IV. Nanoparticles were used to ensure protection and delivery of siRNAs to the target sites in xenografted mice. Tumor growth was markedly reduced in SCID mice intravenously injected with nanoparticle conjugated siRNAs (28).

ATF3 is reportedly involved in transcriptional repression of AR target genes by directly binding to AR. Edelfosine, a synthetic alkyl-lysophospholipid worked effectively in androgen deprived prostate cancer cells (29). Edelfosine dose dependently inhibited ARv7 and ERG in androgen deprived prostate cancer cells. Edelfosine considerably inhibited tumor growth in androgen deprived mice xenografted with LNCaP cells (29).

For the preservation of efficacy of siRNA TMPRSS2-ERG, it was covalently attached to the squalene (SQ) to form an amphiphilic bioconjugate having an ability to undergo self-assembly and consequently form nanoparticles. siRNA TMPRSS2-ERG-SQ nanoparticles in combination with flutamide strikingly inhibited tumor growth in xenografted mice (30).

Pyrrole-imidazole polyamides are highly effective molecules and have strong DNA-binding affinity. Polyamide 1 remarkably reduced dihydrotestosterone induced expression of the TMPRSS2-ERG, PSA and FKBP5 in VCaP cells (Hargrove). Polyamide 1 dose-dependently retarded tumor growth in xenografted SCID mice. After 5 weeks, tumor growth was approximately 6-folds as compared to initial volume of vehicle treated group while growth of the tumors in xenografted mice treated with polyamide 1 at 5.0 mg/kg was approximately 1.6-fold as compared to initial volume (31).

PIM (Proviral Integration site of mouse Moloney leukemia virus) is a Ser/Thr kinase frequently overexpressed in different cancers. Genomic instability and development of resistance against taxane based treatment

strategies are commonly noted in PIM overexpressing cancer cells. Because of Ser/Thr kinase nature of PIM1, STAT3 could not be directly phosphorylated by PIM1, thus suggesting the presence of additional effectors which regulate phosphorylation of STAT3 (32). MIG6 (an EGFR inhibitor) was significantly down-regulated in PIM1 over-expressing cancer cells. PIM1 was upregulated in immortalized prostate cells (RWPE-1) stably transfected with the TMPRSS2-ERG. NMS-P645, a PIM1 inhibitor was effective against TMPRSS2-ERG expressing RWPE-1 cells and reversed taxol induced aneuploidy that resulted in reduction of >4n population to basal level after 36 hours of treatment with taxol. Vandetanib, an EGFR inhibitor exerted repressive effects on STAT3 activity in PIM1 expressing cells. Pharmacological inhibition of EGFR has also been shown to interfere with activation of SRC, a known downstream effector of EGFR signaling axis and STAT3 activator. NMS-P645 worked with effective synergy when combined with PI3K inhibitor GDC-0941 (32). Both drugs inhibited PI3K/AKT/mTOR signaling axis, as evidenced by decrease in phosphorylated levels of p70S6K. Results suggested that both drugs synergistically exerted superior effects on phosphorylation of the ribosomal protein S6 (RPS6), an important downstream effector of the PI3K/AKT/mTOR pathway (32).

Data clearly suggested that GDC-0941 and NMS-P645 were found to be effective against both fusion- negative and positive PCa cells. Although it is seemingly surprising, however PIM1 expression may be controlled via different mechanisms, including activation of JAK/STAT mediated signaling cascade. Keeping in view the fact that though expression level of PIM1 in the fusion negative 22Rv1 cells was lower as compared to fusion positive (VCaP cells), but sufficient enough to fuel the process of tumorigenesis.

Identification of fused oncogenes through the use of state-of-the-art, high-throughput, genomic platforms will prove to be useful in stratification of patients and facilitate the optimal use of clinically effective therapeutics.

Conclusion

It is now clear that genetic/epigenetic mutations, dysregulation of intracellular signaling cascades and loss of apoptosis in TMPRSS2-ERG positive PCa have been shown to significantly influence the response to therapeutic agents that were designed under the 'one gene, one drug' paradigm. However, our rapidly evolving concepts related to multifaceted nature of PCa, pharmacodynamic effects of target inhibition and clonal evolution patterns under treatment pressure have urged basic and clinical biologists to shift from a one gene, one drug approach to a 'multi-gene, multi-drug' model to improve clinical outcome.

Detailed understanding of how signaling cascades are rewired in TMPRSS2-ERG positive PCa cells and which downstream targets are regulated has helped us to differentiate between fusion positive and fusion negative PCa cancers. It is now clear that fusion positive PCa cells rewire intracellular signaling cascades and modulate gene and protein network. Targeting of fusion positive prostate cancers needs extensive research and better understanding of the underlying mechanisms will be helpful in the design and development of synthetic and natural products for treatment of prostate cancer.

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