RKIP-Derived Suppression of Prostate Cancer

Subjects: Oncology

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Despite an intensive research effort in the past few decades, prostate cancer (PC) remains a top cause of cancer death in men, particularly in the developed world. The major cause of fatality is the progression of local prostate cancer to metastasis disease. Treatment of patients with metastatic prostate cancer (mPC) is generally ineffective. Based on the discovery of mPC relying on androgen for growth, many patients with mPC show an initial response to the standard of care: androgen deprivation therapy (ADT). However, lethal castration resistant prostate cancers (CRPCs) commonly develop. It is widely accepted that intervention of metastatic progression of PC is a critical point of intervention to reduce PC death. Accumulative evidence reveals a role of RKIP in suppression of PC progression towards mPC.

prostate cancer metastasis RKIP signaling events

1. Introduction

In the developed world, prostate cancer (PC) is the most frequently diagnosed male malignancy and a major cause of cancer death in men [1]. The disease is initiated from prostate epithelial cells as high-grade prostatic intra-epithelial neoplasia (HGPIN) lesions that evolve to invasive prostate adenocarcinoma or PC which can progress to metastasis [2]. PCs are graded based on Gleason scores (GS) or GS-based World Health Organization (WHO) grading system which categorizes PC into WHO grade group 1–5 [3][4][5].

Prostate cancers are characterized with a high degree of disparity in terms of its prognosis. While tumors with GS ≤ 6 or WHO grade group 1 are generally indolent, others possess high-risk of progression. Local PCs are managed with watchful waiting (active surveillance) and curative therapies: radical prostatectomy (RP) or radiation therapy (RT) [6][7][8][9]. Approximately 30% of patients will experience disease relapse or biochemical recurrence (BCR) based on increases in serum prostate-specific antigen (PSA) [10]. BCR is defined with elevations of serum PSA > 0.2 ng/mL after RP or > 2 ng/mL above the nadir following RT [11]. Relapsed tumors elevated risks of metastasis; 24–34% of patients following BCR will develop metastatic PC (mPC) [12][13].

Built on the androgen-dependence nature of PC discovered in 1940s, current standard of care for mPCs remains androgen deprivation therapy (ADT) [14][15]. Despite remarkable initial response in more than 80% of patients with mPC, ADT is essentially a palliative care as metastatic castration-resistant PCs (mCRPCs) commonly develop [16][17]. Owing to extensive research efforts, multiple options are available to manage CRPCs, including taxane-based chemotherapy, anti-androgens targeted therapy involving either abiraterone or enzalutamide [17][18][19], and immunotherapy [20][21]. However, these therapies only offer modest survival benefits [17][22]. From this perspective,

interventions targeting early-stage progressions of BCR and metastasis are more desirable than treating CRPC or mCRPC.

Metastasis contributes to more than 90% of cancer deaths [23][24], and is regulated by complex networks. Epithelial–mesenchymal transition (EMT) is critical in promoting metastasis; EMT increases cancer cell's migratory and invasion capacities, which are essential properties in facilitating the establishment of cancer cells at the secondary organs from primary site [25][26]. EMT is a major contributor to cancer stem cells [27], including prostate cancer stem cells (PCSCs) [28]. PCSCs are a major source of PC metastasis [28]. Other processes contributing to PC metastasis include cell proliferation regulated by Raf-MEK-ERK and PI3K-AKT-mTOR pathways [29][30], the EZH2 polycomb protein [31][32][33], and NFκB [34][35]. Intriguingly, all these processes are connected to a suppressor of PC metastasis, Raf kinase inhibitor protein (RKIP) (Figure 1) [36][37].

2. RKIP as a Tumor Suppression of Prostate Cancer (PC)

Consistent with RIKP's potential role in molecular events relevant to tumor suppression, RKIP downregulations, and RKIP's associations with cancer progression, RKIP-derived tumor suppressive activities have been reported in multiple cancer types, including urogenital cancers (bladder cancer [38], clear cell renal cell carcinoma [39][40][41], and PC [42][43]), breast cancer [44], pancreatic cancer [45], hepatoma [46], non-small cell lung cancer [47], gastric cancer [48], and others [49].

2.1. RKIP-Mediated Suppression of PC Tumorigenesis and Metastasis

2.1.1. Facilitation of PC Initiation and Metastasis via Downregulation of RKIP at the Protein Level

The first evidence for RKIP as a metastatic suppressor of PC started with the identification of RKIP downregulation in LNCaP cells-derived metastatic C4-2B cells compared to their parental cells [50]; functionally, downregulation of RKIP elevated metastatic potential of C4-2B cells [42]. Specifically, restoration of RKIP expression in C4-2B cells to a comparable level in LNCaP cells reduced C4-2B cells' invasion ability in vitro and the cells' ability to produce lung metastasis in an orthotopic PC model without affecting its ability in forming primary tumors [42]. Downregulation of RKIP at the protein level was observed following PC progression from low grade (low Gleason score) to high grade and the downregulation was particularly evident in metastatic PCs (n = 22) [42]. In comparison to LNCaP cells, C4-2B cells displayed an increase in ERK activation, and inhibition of ERK activation with PD098059 decreased C4-2B cell invasion capacity in vitro.

Further analysis of RKIP expression in a tissue microarray containing non-tumor prostate tissues (n = 57), primary PCs (n = 79), and metastatic CRPC (n = 55), RKIP downregulation was detected in 48% of primary (or local) PCs and 89% of mCRPCs respectively [43]. RKIP downregulation in primary PCs stratifies the risk of PC recurrence (biochemical recurrence) following surgery and remains an independent risk factor of relapse after adjusting for

Gleason score, maximal tumor diameter, pathological stage, surgical margin status, digital rectal examination, PSA, and gland weight [43].

The RKIP downregulations in primary PC compared to non-cancerous prostate tissues and its further downregulation in mPC vs. primary PC also occurred following PC progression in TRAMP mice [51]. While systemic knockout of RKIP had minimal impact on mouse health [52], RKIP deficiency significantly enhanced PC formation and metastasis in TRAMP mice [51]. Collectively, clinical and transgenic mouse (functional) studies support RKIP's action in suppressing PC tumorigenesis and metastasis. However, whether decreases in PC metastasis in *RKIP*$^{-/-}$*;TRMAP* mice were a direct result of reductions in primary PC formation requires additional investigations. Major limitations of the above studies include lack of analyses of RKIP expression at the mRNA level and utilization of more targeted transgenic model such as prostate-specific *RKIP*$^{-/-}$ mice.

2.1.2. No Apparent Reduction of RKIP mRNA Expression Following PC Pathogenesis

The number of publications related to RKIP reductions following PC evolution remains limited. This might be in part attributed to the highly heterogenous nature of PC and challenges in studying RKIP downregulation at the protein level. With advances in DNA sequencing (Next-generation sequencing), an ever-increasing number of cancer genetics and gene expression data have been accumulated and made available. Using TCGA RNA-seq data on prostate tissue (n = 52) and PC samples (n = 497) available from the UALCAN platform (ualcan.path.uab.edu/home, accessed on 6 October 2021) [53], we did not find apparent downregulation of RKIP in PC, high grade PCs, and lymph node metastasis (**Figure 1**A–C). Similar observations could be obtained using the GEPIA2 platform [54] with more prostate tissues (n = 152) (**Figure 1**D). Furthermore, using the Sawyers microarray dataset [55] within the R2: Genomics Analysis and Visualization platform (http://r2.amc.nl, accessed on 3 December 2021), RKIP mRNA was not significantly expressed at reduced levels in mPCs compared to primary PCs. This strongly suggests the RKIP downregulation observed in PC occurs at least in part at post-mRNA levels. Future research should explore these mechanisms.

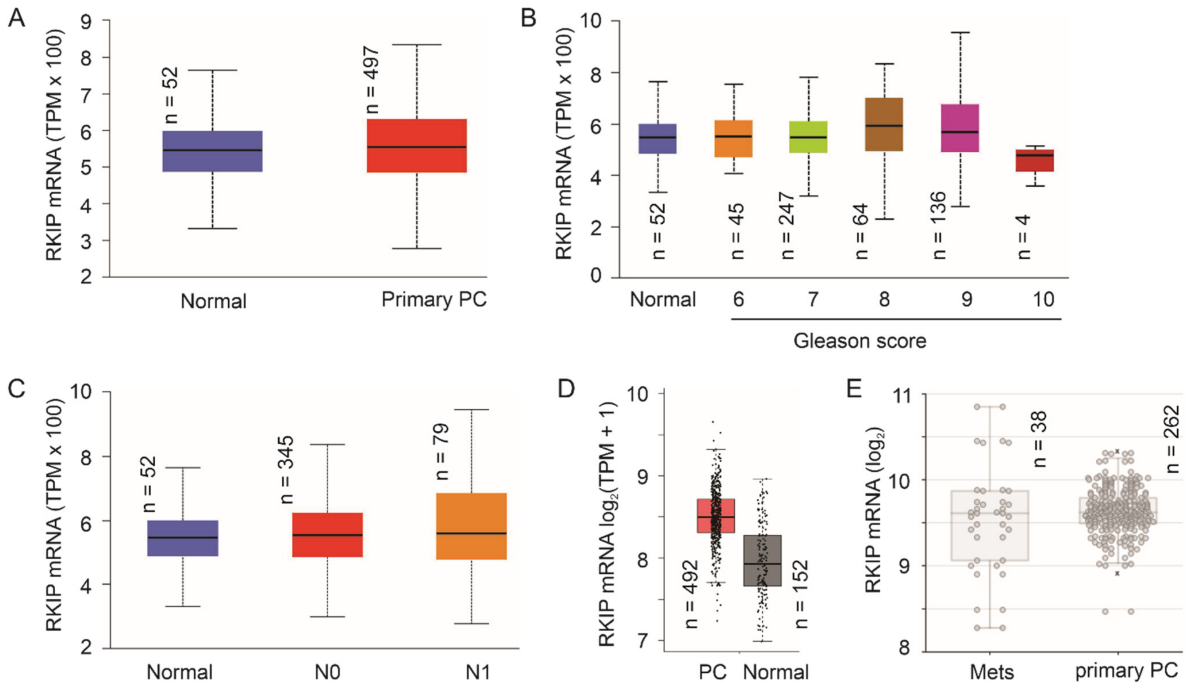

Figure 1. No apparent downregulation of RKIP mRNA expression in PC. (**A**–**C**) Images were generated using the UALCAN platform. (**D**) The graph was produced using the GEPIA2 platform. TPM: transcript per million. (**E**) RKIP mRNA expression in distant metastases (Mets) and primary PCs. The image was generated using the Sawyers dataset within the R2: Genomics Analysis and Visualization platform.

2.2. Regulation of RKIP Expression in PC Cells

2.2.1. RKIP as a Target of Androgen Receptor (AR)

Androgen signaling plays dominant roles in PC initiation, progression, and CRPC development [28][56][57]. This knowledge implies a relationship between AR and RKIP, which is supported by experimental evidence. In immortalized and non-tumorigenic human prostate epithelial RWPE-1 cells [58], dihydrotestosterone (DHT) upregulated RKIP transcription which was blocked by antiandrogen bicalutamide [59]. Androgen response element (ARF), an AR-binding DNA motif, was identified in the RKIP promoter region between nucleotide −571 and −548. A RKIP promoter fragment (−2206 to −26) encompassing this region mediated reporter expression in response to DHT in RWPE-1 cells [59]. Castration of C57BL/6 mice significantly reduced RKIP mRNA expression in prostate, providing a physiological relevance of RKIP as an AR target.

The relationship between AR and RKIP in PC pathogenesis and progression might be complex. AR signaling is required for prostate development, evident by the lack of prostate in *AR*$^{-/-}$ mice [60] and humans with AR mutations being completely insensitive to androgen [61][62]. In adults, AR function is essential for the maintenance of luminal secretory epithelial cells [62]. Alterations in AR signaling are the major mechanism underlying all aspects of PC pathogenesis, including CRPC development [28][56][57][62]. These alterations may lead to changes in AR targets in normal prostate epithelial cells, prostate cancer, and following PC progression. In this regard, RKIP expression is reversely correlated with PSA expression, a typical AR target [63], in PCs [64]; serum PSA is the only clinically used

biomarker assessing PC relapse and disease severity [10][65][66]. The selective expression of PSA over RKIP in PC is consistent with the concept that loss of AR targets with tumor suppression activities contributes to AR-promoted PC initiation and progression. This concept is consistent with AR's complex actions in PC: inhibiting c-Met and AKT activation (both promoting PC progression) in PC [67][68][69] and attenuation of PC3 cell proliferation [70]. It thus would be interesting to determine the impact on PC by enforcing RKIP as an AR target in PC, for instance placing RKIP under the control of PSA promoter.

2.2.2. Mutual Regulation of RKIP and the EMT Machinery

As a suppressor of metastasis, RKIP preferentially inhibits LNCaP, C4-2B, and PC-3M cells invasion but not proliferation in vitro [42][71]. This is not unique in PC cells; similar observations were also reported in clear cell renal cell carcinoma A498 cells [40]. EMT plays a major role in enhancing cancer cell invasion capacity in vitro and metastasis in vivo [25][26]. In a HEK-293 cells-based study, RKIP downregulates Snail and Slug expression via stabilization of GSK3β [72]. Both Snail and Slug are major transcription factors of EMT [73][74]. Snail increases LNCaP cell migration in vitro through facilitating degradation of the SPOP tumor suppressor [75]; EMT plays a major role in the generation of prostate cancer stem cells which contribute to PC metastasis and CRPC development [17][28]. In accordance with this evidence, Snail inhibits RKIP expression in metastatic and AR-negative PC3 and DU145 PC cells [76]; this inhibition occurs at the transcription level through an E-box located in the RKIP promoter [76]. A reverse correlation between Snail and RKIP mRNA expressions was observed in primary PCs [76]. The connection between Snail and RKIP likely has a functional impact on PC. Downregulation of Snail sensitized DU145 PC cells to TRAIL- and CDDP (Cisplatin)-induced apoptosis via upregulation of RKIP and knockdown of RKIP reversed the sensitization [77].

Evidence presented above suggests a mutual inhibition between RKIP and Snail. While RKIP can downregulate Snail in HEK-499 (a derivative of HEK-293 cells) cells [72], whether this occurs in PC cells remains to be demonstrated. Nonetheless, RKIP may inhibits PC cell invasion and migration independent of the core transcriptional factors of EMT. RKIP inhibits PC-3M cell migration and invasion in vitro via modulating extracellular matrix by downregulating MMP-2 and MMP-9 (matrix metalloproteinases) [71].

2.2.3. Non-Coding RNA-Mediated Downregulation of RKIP in PC Cells

It is an emerging concept that long non-coding RNAs (lncRNAs) sponge microRNAs (miRNAs or miRs) and thus facilitate mRNA expression [78][79]. In this regard, miR-543 was reported to downregulate RKIP in PC and thus promote PC cell proliferation and EMT [80]. In LNCaP and C4-2B cells, downregulation of RKIP and upregulation of miR-543 occur concurrently in C4-2B cells [80]. The presence of miR-543 target sequence was detected in the 3′UTR (untranslated region) of RKIP mRNA and expression of miR-543 but not its negative control reduces RKIP mRNA expression in LNCaP cells, supporting RKIP as a direct target of miR-543 [80]. Ectopic expression of miR-543 enhances LNCaP cell proliferation, invasion, and xenograft formation along with evidence of EMT; conversely, these events were reduced upon knockdown of miR-543 in C4-2B cells [80], supporting a likely functional impact of miR-543 in inhibition of RKIP. Additionally, a reverse correlation between miR-543 and RKIP expression was demonstrated in a PC cohort consisting of n = 28 local tumors and n = 14 metastatic PCs [80].

The same group also reported a regulation between lncRNA XIST and RKIP expression in PC. Specifically, XIST sustains RKIP expression through binding to miR-23a. In clinical samples, concurrent downregulation of XIST and RKIP occurs in primary PC vs. normal prostate and mPC vs. primary PCs [81]. Ectopic expression of XIST in DU145 cells increases RKIP expression, decreases cell proliferation, and attenuates xenograft formation [81]. In a reverse manner, knockdown of XIST in LNCaP cells, which express a high level of endogenous XIST, downregulates RKIP along with an enhancement in cell proliferation [81]. In support of this investigation, miR-23c, a close relative of miR-23a, was suggested to target RKIP [82].

MiRNA likely has many targeted genes or mRNAs; for instance, miR-130b has approximately 600 target genes [83]. MiRNA thus affects complex network alterations. In this regard, miR-543 and XIST-miR-23a likely impact PC progression with multiplex mechanisms in addition to regulating RKIP expression. This inference is supported by miR-543-derived tumor-promotion in lung cancer [84][85] and tumor-inhibition in colorectal and cervical cancers [86][87]. Even in PC, downregulation of miR-543 was reported in primary PC with bone metastasis (n = 20) compared to primary PCs without bone metastasis (n = 15), and in bone mPCs compared to the paired primary PCs; the reported target of miR-543 in this investigation was endothelial nitric oxidase (eNOS) [88]. In PC3 cells, miR-543 downregulates eNOS and inhibits PC3 migration [88]. On the other hand, miR-543 can also enhance PC cell oncogenic properties via stimulating the AKT/mTOR pathway [89] and enhancing prostate cancer stem cell traits [90]. While modulations of miR-543 [80] and XIST [81] leading to RKIP downregulation were associated with increases in PC cell proliferation, direct downregulation of RKIP did not affect PC cell proliferation [42][71]. Collectively, miR-543 and XIST likely impact PC cell oncogenic properties via multiple targets, including RKIP.

2.3. RKIP-Derived Sensitization of PC Cells to Treatment In Vitro

Accumulative investigations present a consistent message for RKIP as tumor suppressor and/or metastatic suppressor of PC. This concept is further supported by RKIP's action in sensitization of PC cells to multiple cytotoxic treatments in vitro.

RKIP contributes to DU145 cell response to TRAIL- and cisplatin-induced apoptosis; this sensitization was reversed upon inhibition of RKIP expression by Snail [77]. Similarly, nitric oxide (NO) inhibits EMT in PC3 and DU145 cells via RKIP upregulation and Snail downregulation. RKIP upregulation in this setting makes a major contribution to EMT inhibition caused by Snail downregulation [91].

RKIP plays a role in PC cell's sensitivity to photodynamic therapy (PDT) in response to NO levels produced during PDT. In PC3 cells treated with PDT, optimized treatment condition led to the production of a high level of NO, which inhibits NFκB and YY1 (Yin Yang 1) transcription factor. As a result, RKIP is upregulated and resulted in cytotoxicity [92][93]. Sub-optimal PDT treatment produces low levels of NO, a condition that activates NFκB-mediated YY1 expression. YY1 subsequently inhibits RKIP, contributing to EMT and the activation of PI3/AKT [92][93]. YY1 promotes PC via EMT development and contributes to therapy resistance [94]. In addition to the NFκB-YY1-RKIP connection, high and low NO levels can also modulate EMT and drug resistance in PC3 cells via NFκB-RKIP-GSK3β-NRF2, where high NO inhibits NFκB, leading to RKIP upregulation, GSK3β stabilization and NRF2

downregulation along with inhibition of EMT and sensitization to drug treatment. Low NO produces the opposite actions [95].

Evidence suggests a contribution of RKIP to genotoxic agent 9-nitrocamptothecin (9NC)-induced apoptosis in PC cells [96]. 9NC triggers DNA damage response and apoptosis along with RKIP upregulation in DU145 cells but not in 9NC-resistant RC1 cells which were derived from DU145 cells. Sensitivity of PC cells to 9NC-induced apoptosis was reduced or increases with RKIP downregulation and overexpression respectively [96]. It was indicated that NFκB contributed to RKIP expression alteration and RKIP sensitized PC cells to DNA damage-induced apoptosis [96]. This investigation was supported by a report that radiation upregulated RKIP in C4-2B cells [97]. RKIP overexpression and knockdown sensitized and reduced C4-2B cell apoptosis in vitro in response to radiation [97]. In mice bearing tumors produced by C4-2B cells with RKIP knockdown, radiation was substantially less effective in inhibiting tumor growth compared to tumors generated by wild type C4-2B cells [97]. Collectively, evidence supports RKIP playing a role in PC cell response to genotoxic treatment. As radiation is clinically used in treating PC, whether RKIP is a major contributor to the therapy response needs further investigation.

Docetaxel is commonly used in treating CRPC [98][99]. It was observed that in PC3 cells, a PC cell line that does not require androgen for survival, RKIP overexpression sensitized the cells to docetaxel-induced inhibition of cell proliferation [100]. While this report shows a potential for clinical implication, more work is required to further explore this potential.

3. Conclusions

The research activities in the past 20 years collectively demonstrated RKIP as a tumor suppressor of PC tumorigenesis and metastasis. This knowledge is supported by (1) functional evidence derived from in vitro, in vivo (xenograft and transgenic mouse models), and clinical studies as well as (2) mechanistic pathways contributing to RKIP-derived suppression of PC. Future research should explore the functionality and underlying mechanisms for RKIP mediated suppression of PC using more refined transgenic models, including mice with prostate-specific expression of RKIP and its mutants. The latter may help to define the regulations relevant to RKIP tumor suppressive actions; this is important, as RKIP can be switched to promote tumorigenesis following its phosphorylation at S153. Additionally, mechanisms leading to RKIP downregulation in PC and RKIP's involvement in other aspects of PC progression should also be investigated (see Section 5 for details).

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