**RESEARCH HIGHLIGHT**

**RKIP is commonly downregulated in clear cell renal cell carcinoma (ccRCC)**

Diane Ojo¹,²,³, Anil Kapoor⁴, Brianne Hill¹,²,³, Xiaozeng Lin¹,²,³, Nicholas Wong¹,²,³, Jehonathan Pinthus⁴, Damu Tang¹,²,³

¹ Division of Nephrology, Department of Medicine, McMaster University, Canada  
² Father Sean O’Sullivan Research Institute, Canada  
³ the Hamilton Center for Kidney Research, St. Joseph’s Hospital, Canada  
⁴ Department of Surgery, McMaster University, Hamilton, Ontario L8N 4A6, Canada

**Clear cell renal cell carcinoma (ccRCC) is characterized by VHL mutations.** More than 80% of ccRCCs are resulted from the loss of VHL, a remarkable prevalence that is unique for a tumor suppressor in ccRCC. Despite the required VHL loss, this event is not sufficient to result in ccRCC. The underlying mechanisms contributing to this insufficiency remain incompletely understood. Nonetheless, recent advances in whole genome sequencing have shed lights on these mechanisms. Whole genome sequencing a set of ccRCCs identified three tumor suppressors: BRCA1-associated protein 1 (BAP1), Polybromo-1 (PBRM1), and Set domain-containing 2 (SETD2) with their apparent mutation rates being 50% for PBRM1, 15% for BAP1, and 15% for SETD2. While concomitant mutations of VHL with these tumor suppressors will likely further the process of ccRCC pathogenesis, it is unlikely that these oncogenic events are inclusive. In supporting this possibility, we have recently reported reductions of the Raf kinase inhibitory protein (RKIP) in 80% of 600 ccRCCs examined. This is remarkable considering the tendency of ccRCC in predominantly using a single oncogenic factor, i.e. loss of the VHL tumor suppressor. RKIP inhibits Raf1-mediated activation of MEK/ERK, and also displays other tumor suppression activities. Downregulation of RKIP has been reported in many cancers particularly in those of metastasis, observations that support RKIP being a metastasis tumor suppressor. The current manuscript will provide insights regarding our ccRCC-related RKIP research, and suggest some future developments to delineate the contributions of RKIP to ccRCC tumorigenesis.

**Keywords:** RKIP; VHL; Raf; ccRCC; kidney cancer


Clear cell renal cell carcinoma (ccRCC) composes of 70% of renal cancer. The disease is the most aggressive and lethal form of kidney cancer [¹, ²]. Despite the recent advances, the etiology of the disease remains unclear and metastatic ccRCC is still incurable [³].

The dominant event occurring during ccRCC tumorigenesis is loss of the VHL tumor suppressor. Individuals with VHL mutations in the germline are associated with increasing risk of developing ccRCC [⁴]; somatic mutations were observed in 82.4% of sporadic ccRCC cases [⁵]. Despite such prevalence, inactivation of VHL is insufficient to cause ccRCC. Patients with loss of VHL function often develop pre-neoplastic renal cysts prior to the appearance of ccRCCs [⁶]; VHL deficiency specifically in the proximal tubule epithelium only yielded low grades of renal cysts in mice [⁷]. Some insights for this insufficiency lie in the manner in which allelic loss of VHL was achieved.
Typically, one allele is inactivated by intra-genetic events (point mutations and indel) and the second allele is lost by a large deletion leading to loss-of-heterozygosity (LOH). This two-hit process of VHL allelic loss is thus associated with deletions of other genetic materials that may facilitate ccRCC tumorigenesis. Indeed, this possibility is supported by the recent identification of three tumor suppressors in ccRCC through whole genome sequencing, PBRM, BAP1, and SETD2 which are located at the same chromosome arm 3p together with VHL [18-21]. Loss of PBRM occurred exclusively with 3p LOH [8], and mice deficient in VHL with BAP1+/− (mimicking 3p LOH in patients) developed small and organ-confined ccRCC [12]. While these results support the contributions of concomitant loss of other 3p tumor suppressors in VHL allelic loss-initiated ccRCC, the observations that allelic inactivation of VHL in patients initially produced pre-neoplastic renal cysts [6] and that VHL+/−;BAP1+/− mice developed small and non-metastatic ccRCC [13] highlight the needs for other oncogenic events.

Loss of VHL activates the HIF transcription factors, which transactivate a panel of target genes, including erythropoietin (EPO), transforming growth factor alpha (TGF-α), platelet-derived growth factor (PDGF-β), and vascular endothelial growth factor (VEGF) [13, 14]. These growth factors are well known to activate the Raf-MEK-ERK pathway via Ras [15, 16]. Likewise, activation of the Raf pathway occurs in RCC [17, 18] and is associated with advanced disease [19]. The Raf/MEK/ERK pathway plays an important role in promoting cell proliferation and survival [21, 23]. Sustained ERK activation is required for angiogenesis [22, 23]. Furthermore, VEGF-mediated survival of endothelial cells depends on Raf [24]. Collectively, the Raf pathway makes an important contribution to ccRCC tumorigenesis, a concept that is well supported by the elevated function of the Raf-MEK-ERK pathway in 30% of human malignancies [18].

The Raf pathway is inhibited by the Raf kinase inhibitory protein (RKIP) through RKIP-mediated association with Raf-1, an interaction that prevents Raf-1 to activate MEK [25-27]. Consistent with the Raf/MEK/ERK pathway being important in tumorigenesis, downregulation of RKIP has been observed in metastatic insulinomas, colon cancer, and hepatocarcinoma [27], suggesting a role of RKIP in metastasis suppression. Accumulative research reveals a RKIP reduction in almost every cancer [28], including ccRCC [29, 30].

While Moon et al reported RKIP reductions in 42.4% of 252 ccRCC cases, we observed RKIP downregulation in 80% of more than 600 ccRCC cases obtained from several cohorts. The different rates of RKIP reduction were likely attributed to different methods used to quantify immunohistochemistry (IHC) staining. Moon et al quantified IHC by adding the respective intensity scores (0, 1, 2, and 3) with those of percentage scores (0, 1, 2, and 3); we used a more defined quantification system: H-Score = (% weak x 1 + % medium x 2 + % strong x 3 +1) x 100 [31]. Additionally, the rate of RKIP reduction obtained by our IHC scores was supported by western blot analysis of 50 ccRCC cases, in which 45/50 (90%) displayed RKIP reduction and 44/50 (88%) ccRCCs exhibited VHL downregulation [30]. Furthermore, in our analysis of 7 data sets involving 381 ccRCCs, suppression of RKIP and VHL mRNAs was ranked #582 and #2343, respectively, among the genes that were downregulated in ccRCC [30]. Collectively, evidence supports the prevalence of RKIP reduction in ccRCC approaching that of VHL. While a prevalent reduction of RKIP was observed in local endometrial and hepatocellular carcinomas (Table 1), based on our best knowledge RKIP is the second tumor suppressor with a rate of reduction matching that of VHL in ccRCC.

This knowledge is intriguing considering ccRCC has the tendency to use a specific oncogenic event to a large extent; this knowledge also indicates downregulation of both VHL and RKIP being early events during ccRCC tumorigenesis. It will be interesting to examine the relationship between these two events. In the familial cases of ccRCCs with germline VHL mutations, reduction of RKIP will support a possible sequential RKIP reduction following VHL inactivation. Will mice deficient in both VHL and RKIP develop ccRCC? How about mice deficient in VHL and RKIP in the BAP1+/− genetic background, a situation that mimics 3p LOH in patients?

Although RKIP expression is reduced in a variety of cancers at different rates (Table 1) and despite RKIP is most thoroughly investigated for its inhibitory role in Raf-mediated activation of MEK, surprisingly the underlying mechanisms responsible for RKIP-derived inhibition of tumorigenesis remain essentially unclear [20]. While the same situation also applies to ccRCC, it was apparent that the Raf pathway was not a major component contributing to RKIP-mediated suppression of ccRCC pathogenesis. In three ccRCC cell lines and 50 pairs of ccRCCs and their adjacent non-tumor kidney tissues, RKIP levels did not correlate with ERK activation [30].

In addition to the Raf pathway, RKIP also suppresses NF-κB and Snail, and activates GSK3β. These actions are consistent with RKIP's role in tumor suppression [29]. Snail is a major transcription factor inducing epithelial-mesenchymal transition (EMT), a process that is inhibited by GSK3β [32, 33]. EMT empowers cell migration and invasion, and is an essential process promoting cancer metastasis [34]. In line with this knowledge, modulations of RKIP had no effects on...
the proliferation of 786-0 and A498 ccRCC cells, but affected ccRCC invasion and EMT [30]. These in vitro observations were supported by in vivo results that modulation of RKIP did not change A498 cell-derived xenograft tumor formation in NOD/SCID mice [30]. Collectively, we prefer the possibility that RKIP suppresses ccRCC pathogenesis at least in part via inhibiting EMT, a situation that mirrors RKIP-mediated inhibition of prostate cancer metastasis but not the formation of xenograft tumors [35, 36].

Besides suppression of tumorigenesis, RKIP may also facilitate the process. Upon phosphorylation at Serine 153 by protein kinase C, RKIP is able to inactivate GSK3β, and activate NF-κB and G-protein coupled receptor (GPCR) signaling, a collective set of activities that favor tumorigenesis [28]. Although these tumor-promoting activities have been observed in vitro, their relevance in vivo has yet to be demonstrated. It seems that tumors remove RKIP's tumor suppression activities via downregulation of the protein rather than altering its function via post-translational modifications. However, the later possibility cannot be excluded particularly in the proportion of individual tumor types in which RKIP was not reduced. This possibility may not be likely in ccRCC, as RKIP is reduced in 80% of cases.

While it remains to be demonstrated whether reduction of RKIP occurs at the translational levels, transcriptional regulations play a role in tumor-associated RKIP downregulation [28]. The same situation also applies to RKIP reduction in ccRCC [30]. Our observed transcription-mediated regulation of RKIP abundance is supported by a recent report that a single nucleotide polymorphism (rs17512051) in the promoter region of the RKIP gene enhanced RKIP expression in the kidney [37]. Interestingly, this RKIP upregulation was associated with a decrease of ccRCC risk, which was presumably resulted from the polymorphism-caused interference of RKIP reduction [37] in ccRCC. This observation is in accordance with our publication suggesting RKIP being a tumor suppressor of ccRCC [30].

Regardless how RKIP may suppress ccRCC tumorigenesis, its common reduction in ccRCC seems to be in accordance with the tumor's characteristics of widely downregulating the VHL tumor suppressor. It thus warrens further investigations into the impact of RKIP downregulation on ccRCC tumorigenesis and the underlying mechanisms.

### Conflicting interests

The authors have declared that no competing interests exist.

### Acknowledgments

This research was supported by a grant from Kidney Foundation of Canada (KFOC110017, 2011 - 2013) to D. Tang.

### References


