Published in final edited form as:

Hepatology. 2012 March ; 55(3): 970–974. doi:10.1002/hep.24774.

Cell cycle-related Kinase links androgen receptor & β -catenin signaling in HCC: Why men are at a loss?

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Abstract

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide. It is more prevalent in men than women. Related to this, recent genetic studies have revealed a causal role for androgen receptor (AR) in hepatocarcinogenesis, but the underlying molecular mechanism remains unclear. Here, we used genome-wide location and functional analyses to identify a critical mediator of AR signaling — cell cycle-related kinase (CCRK) — that drives hepatocarcinogenesis via a signaling pathway dependent on β -catenin and T cell factor (TCF). Ligand-bound AR activated CCRK transcription and protein expression via direct binding to the androgen-responsive element of the CCRK promoter in human HCC cell lines. In vitro analyses showed that CCRK was critical in human cell lines for AR-induced cell cycle progression, hepatocellular proliferation, and malignant transformation. Ectopic expression of CCRK in immortalized human liver cells activated β -catenin/TCF signaling to stimulate cell cycle progression and to induce tumor formation, as shown in both xenograft and orthotopic models. Conversely, knockdown of CCRK decreased HCC cell growth, and this could be rescued by constitutively active β -catenin or TCF. In primary human HCC tissue samples, AR, CCRK, and β -catenin were concordantly overexpressed in the tumor cells. Furthermore, CCRK overexpression correlated with the tumor staging and poor overall survival of patients. Our results reveal a direct AR transcriptional target, CCRK, that promotes hepatocarcinogenesis through the upregulation of β -catenin/TCF signaling.

COMMENTS

Hepatocellular Carcinoma (HCC) is the fifth most common cancer and the third cause of cancer-related death worldwide. HCC usually occurs in the backdrop of chronic liver disease due to hepatitis of various etiologies. Men are at higher risk to develop HCC and the role of male sex hormones as tumor promoters is highly relevant (1). The Androgen Receptor is critical for the development and maintenance of the male sexual phenotype (2). It is a type of nuclear receptor that regulates gene transcription when activated by androgens. Upon activation, AR translocates to the nucleus, binds to androgen response elements (ARE) on target genes and engages crosstalk with transcription factors to eventually induce transcription of certain genes. Overexpression of AR has been reported in 60%-80% of human HCCs (3) making it a formidable player in male HCC. In addition, liver specific

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JOURNAL CITATION:

Feng H, Cheng AS, Tsang DP, Li MS, Go MY, Cheung YS, Zhao GJ, et al. Cell cycle-related kinase is a direct androgen receptor-regulated gene that drives beta-catenin/T cell factor-dependent hepatocarcinogenesis. *J Clin Invest* 2011;121:3159-3175.

deletion of AR was shown to significantly reduce tumorigenicity in both carcinogen and HBV induced HCC mouse models (4, 5). The mechanism of AR in HCC remained unclear until a recent study established a novel crosstalk of this pathway through cell cycle-related kinase (CCRK) to the Wnt/ β -catenin signaling pathway, another important oncogenic pathway in HCC (6). Abnormal activation of the Wnt/ β -catenin signaling pathway due to multiple diverse mechanisms leads to stabilization and nuclear translocation of β -catenin, has been reported in a significant subset of HCC patients, *albeit* the downstream mechanisms of how β -catenin contributes to HCC are not completely understood (7). While β -catenin itself does not bind DNA, it can interact with other transcription factors especially the more-studied TCF/LEF family of transcription factors to induce target gene expression. Feng et al recently link the β -catenin and AR pathways in a feed forward loop through CCRK in HCC (6).

In an effort to identify AR-dependent mechanisms and thus address male predominance of HCC, Feng and colleagues took advantage of ChIP-chip analysis and identified *CCRK* as a direct target of AR in two androgen-expressing HCC cell lines-Huh7 and PLC5. In fact, 212 target genes that were common between the two cell lines were identified and 21 of these 212 genes were cell cycle regulators and *CCRK* was identified to have the highest binding affinity to AR. Using multiple means it was shown that AR strongly bound and transactivated *CCRK* promoter. AR knockdown diminished the *CCRK* promoter activity and conversely AR agonist R1881 induced AR binding and transactivation of the *CCRK* reporter and additional validation provided through site-directed mutagenesis. The authors also transfected AR gene in two cell lines with low endogenous AR (SK-Hep1 and LO2) and identified a significant increase in CCRK expression, which was also substantiated with immunofluorescence (IF). The authors then proceeded to determine the functional relevance of CCRK expression control by the AR. While AR stimulation led to cell cycle progression, CCRK downregulation under such circumstances abrogated tumor cell proliferation. Conversely, ectopic expression of CCRK was able to significantly rescue proliferation and cell cycle arrest following AR inhibition. Similar relationship was also evident in focus assays and anchorage-independent soft agar assays and further corroborated a direct relationship between AR and CCRK in promoting cellular proliferation and transformation. To further test the biological relevance of these findings *in vivo*, the authors demonstrate that injection of PLC5 hepatoma cells expressing shRNA to CCRK had significantly less tumor volume and thus diminished tumor formation when compared to the controls in tumor xenograft studies. Conversely, stably transfected LO2 expressing CCRK displayed incredible tumor growth with 20-fold mean tumor volumes when compared to empty vector controls. These results undoubtedly demonstrate the oncogenic properties of CCRK.

To elucidate the mechanism of CCRK in hepatic tumorigenesis, and based on previously published data from a protein kinase-enriched shRNA library screen illustrating regulation of β -catenin activity by another cell cycle kinase (CDK8) in colorectal cancer (8), the authors postulated CCRK to be modulating β -catenin signaling. IF studies indeed showed redistribution of β -catenin from the nucleus to the cytoplasm after CCRK knockdown compared to control in two different cell lines. The authors further demonstrated decreased protein levels of active but not of total β -catenin in this situation. Mechanistically, they identify CCRK knockdown to decrease phosphorylation of GSK3 β and ensuing decrease in β -catenin activity. Conversely ectopic expression of CCRK led to increased phosphorylation of GSK3 β culminating into enhanced β -catenin signaling. Phosphorylation of GSK3 β has been shown to mediate β -catenin activation through inhibition of β -catenin degradation complex (9). In addition, the authors demonstrate that knockdown of CCRK abrogates some known β -catenin downstream targets such as EGFR and cyclin-D1, which was reversed by ectopic CCRK expression. These targets have independently been shown to regulate proliferation in HCC (10, 11). Knockdown of β -catenin despite ectopic expression of CCRK

led to decreased tumor cell proliferation and additional functional studies such as soft agar assays and tumor xenograft studies further validated these observations. The authors then ask if the modulation of β -catenin activity by CCRK had any impact on AR expression and function since β -catenin-AR crosstalk has been previously reported (12). Indeed the authors identified that ectopic CCRK expression led to increased total and Ser81-phosphorylated AR, which has independently been shown to induce AR promoter activity and in turn tumor cell growth (13). This effect was abrogated upon β -catenin silencing suggesting that CCRK-induced β -catenin activation indeed regulates AR expression and its biological effects. Lastly, knockdown of AR affected β -catenin activity, which could be rescued by CCRK overexpression and ectopic AR expression could increase active- β -catenin levels, which could be blocked by inhibition of CCRK. Thus, the authors establish a unique AR/CCRK/ β -catenin feed-forward circuitry (Figure 1), which was also evident in significant subset of HCC patients as concomitant upregulation of AR/CCRK/ and β -catenin with both western blot analysis and immunohistochemistry. Further examination also revealed a significant correlation between overexpression of *CCRK* and advanced tumor stage reflected by shorter overall all survival.

The current study has identified a novel circuitry that consists of AR-CCRK- β -catenin axis that may provide at least one major mechanism of hepatocarcinogenesis in this male-predominant tumor type, thus presenting unique molecular interactions that may be exploited for therapeutic intervention. This study also suggests at least one additional mechanism by which Wnt/ β -catenin signaling may in fact cause tumor progression in males. It is plausible that β -catenin activation in HCC in males would lead to higher expression and activation of AR that in turn would result in higher CCRK expression and thus incite a vicious cycle of cell growth and proliferation. Traditionally, many modes of β -catenin activation have been reported in HCC (14). It is unclear though, if various mechanisms of β -catenin activation in HCC will have similar and robust growth promoting effects on tumor cells. Contrary to expectations, a study found that β -catenin-TCF-activation was not equivalent when β -catenin stabilization was a consequence of *CTNNB1* versus *AXIN1* mutations (15). It would have been useful to determine the cause of β -catenin activation in patients examined in the current study. Would β -catenin activation due to AR/CCRK axis be even more pronounced irrespective of preexisting mutations in *CTNNB1*? Similar studies *in vitro* to examine effect of AR/CCRK on tumor cells expressing β -catenin with point mutations affecting key serine and threonine residues in exon-3 would be relevant in future as such mutations may be predicted to be autonomous of any upstream feed-forward regulation. In other words, mutations in *CTNNB1* that are observed in 20-40% of all HCC patients, may be free of GSK3 β -dependent β -catenin phosphorylation and degradation and may in fact introduce a break in the proposed positive regulatory circuit.

The authors elegantly unveil one of the mechanisms of sex-related disparity of HCC and extend on the existing findings that AR and testosterone contribute to HCC predominance in males. The major conclusion drawn from the study is that the presence of androgens in males engage the AR to stimulate the CCRK expression, which activates β -catenin signaling and that in turn would enhance expression of EGFR and cyclin-D1 thus promoting cell proliferation and at the same time would upregulate AR expression and activity and thus establish a positive regulatory cycle (Figure 1). CCRK belongs to mammalian cyclin-dependent kinase (CDK) family and although it has been shown to be upregulated in several cancers, its role and regulation is not fully understood. In fact, it has been reported elsewhere that CCRK does not have an intrinsic CDK-activating kinase (CAK) activity, but it does enhance cell proliferation (16). It is likely that through additional yet uncharacterized interactions, CCRK may be influencing Thr390 phosphorylation of GSK3 β . Significant studies will be necessary to extrapolate these interactions especially since p38 MAPK is known to induce GSK3 β inactivation through this specific event (17).

Eventually, AR signaling has been attributed to induction of cellular oxidative stress both in vivo and in vitro. Intriguingly, β -catenin has been shown to also regulate the redox state of the cell. In fact recent studies have shown that β -catenin can switch from binding to TCF4 to other transcription factors like HIF1 α or FOXO and mount an anti-oxidant response (18, 19). It was also recently shown that loss of β -catenin in hepatocytes led to an enhanced liver tumorigenesis when exposed to chemical carcinogen such as diethylnitrosamine with or without phenobarbital and this was accompanied by enhanced oxidative stress, fibrosis, inflammation and regeneration (20, 21). Thus it will be critical to determine in the context of AR signaling whether enhanced CCRK-driven β -catenin activation is globally contributing to tumorigenesis or in some cases may in fact be an oxidative stress-driven response promoting cell proliferation and regeneration in the background of chronic liver injury and fibrosis (10).

Acknowledgments

Funding support: This study was funded by NIH grants 1R01DK62277 and 1R01CA124414 to SPSM and by Rango's Fund for the Enhancement of Pathology Research.

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Figure 1.

Feed-forward circuitry in male HCC demonstrates androgen (A) dependent androgen receptor (AR) activation that induces CCRK expression that results in GSK3 β phosphorylation and inhibition to cause nuclear translocation of β -catenin thus bypassing the upstream Wnt signaling. The end result is stimulation of β -catenin-TCF4-dependent target gene expression of cyclin-D1 (Cnnd1), epidermal growth factor receptor (EGFR), glutamine synthetase (GS) and last but not least the AR itself, which then positively engages back into this vicious signaling cascade.