Research Article

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A CCRK-EZH2 epigenetic circuitry drives hepatocarcinogenesis and associates with tumor recurrence and poor survival of patients

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Background & Aims: Aberrant chromatin modification is a key feature of hepatocellular carcinoma (HCC), which is characterized by strong sexual dimorphism. Both enhancer of zeste homolog 2 (EZH2) and cell cycle-related kinase (CCRK) contribute to hepatocarcinogenesis, yet whether the two oncogenic factors have functional crosstalk is unknown.

Methods: Cellular proliferation and tumorigenicity upon transgenic expression and RNA interference were determined by colony formation and soft agar assays, xenograft, orthotopic and diethylnitrosamine-induced HCC models. Gene regulation was assessed by chromatin immunoprecipitation, site-directed mutagenesis, luciferase reporter, co-immunoprecipitation and expression analyses. Protein levels in clinical specimens were correlated with clinicopathological parameters and patient survival rates.

Results: Ectopic CCRK expression in immortalized human liver cells increased EZH2 and histone H3 lysine 27 trimethylation (H3K27me3) to stimulate proliferation and tumor formation. Conversely, knockdown of CCRK reduced EZH2/H3K27me3 levels and decreased HCC cell growth, which could be rescued by EZH2 over-expression. Mechanistically, GSK-3β phosphorylation by CCRK activated a β-catenin/TCF/E2F1/EZH2 transcriptional feedback loop to epigenetically enhance androgen receptor (AR) signaling. Simultaneously, the phosphorylation of AKT/EZH2 by CCRK facilitated the co-occupancy of *CCRK* promoter by EZH2-AR and its subsequent transcriptional activation, thus forming a self-reinforcing circuitry. Lentiviral-mediated knockdown of *CCRK*, which abrogated the phosphorylation-transcriptional network, prevented diethylnitrosamine-induced tumorigenicity. More importantly, the hyperactivation of the CCRK-EZH2 circuitry in human HCCs correlated with tumor recurrence and poor survival.

Conclusions: These findings uncover an epigenetic vicious cycle in hepatocarcinogenesis that operates through reciprocal regulation of CCRK and EZH2, providing novel therapeutic strategy for HCC.

Keywords: Androgen receptor; Chromatin modifications; Hepatocellular carcinoma; Gender disparity; Kinase.

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Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; E2F1, E2F transcription factor 1; EZH2, enhancer of zeste homolog 2; PRC2, polycomb repressive complex 2; H3K27me3, trimethylation of lysine 27 at histone H3; AR, androgen receptor; CCRK, cell cycle-related kinase; GSK-3β, glycogen synthase kinase 3β; TCF, T-cell factor; DEN, diethylnitrosamine; RT, reverse transcription; ChIP, chromatin immunoprecipitation; shRNA, short-hairpin RNA; KD, kinase-defective; WT, wild-type; p-GSK3β^{Ser9}, GSK-3β phosphorylation at serine9; dn, dominant-negative; siRNA, small-interfering RNA; dp, dominant-positive; p-AR^{Ser81}, AR phosphorylation at serine81; p-AKT^{Ser473}, AKT phosphorylation at serine473; p-EZH2^{Ser21}, EZH2 phosphorylation at serine21; ARE, androgen-responsive element.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent malignancy worldwide with a strong sexual dimorphism. Male to female ratio averages between 2:1 and 7:1 in HCC associated with viral infection and non-alcoholic fatty liver diseases [1,2]. Hepatitis B virus (HBV) infection accounts for about 60% of the total liver cancer in developing countries. Unlike most other cancers, the incidence and mortality of HCC have increased in Western countries in the past decade due to hepatitis C virus infection and the obesity epidemic. Although the multikinase inhibitor Sorafenib can improve survival of HCC patients, the durability of treatment response is still far from satisfactory [3]. The alarmingly high failure rate of phase III molecular therapy trials in the past 5 years further underscores the compelling need for novel drug targets [4].

HCC pathogenesis is a complex process driven by accumulating genetic and epigenetic alterations [5,6]. Integrative oncogenomic analysis has uncovered prominent oncogenes such as *CTNNB1*, *AKT*, and *E2F transcription factor 1* (*E2F1*) in HCC [7]. While these oncogenic pathways are often concordantly activated in molecular subtypes of human HCCs [8,9], the mechanistic basis of the signaling connections is largely unknown.

Delineation of the signaling hubs that contribute to hepatocarcinogenesis may greatly advance therapeutic development [3,10].

One of the most remarkable discoveries in cancer genomics is the recurrent somatic mutations of epigenetic-modifying genes [11,12]. Although each HCC case has a unique mutational profile, up to 50% of cases are estimated to harbor mutations in different chromatin regulators [4,10,13]. Enhancer of zeste homolog 2 (EZH2), a subunit of Polycomb repressive complex 2 (PRC2) that catalyzes the repressive histone H3 lysine 27 trimethylation (H3K27me3) [14], is subject to genetic alteration in hematological malignancies and over-expression in a wide range of solid tumors including HCC [12,15]. Numerous evidence have suggested that aberrations of EZH2 represent early events that render cells susceptible to oncogenic transformation and refractory to differentiation program [15,16]. However, the mechanisms governing the over-expression and functions of this master chromatin regulator in HCC remain elusive.

Concordant with the male predominance of HCC, the sex hormone receptor androgen receptor (AR) plays a dominant role in hepatocarcinogenesis [17]. Our prior work underpinned cell cycle-related kinase (CCRK, also known as cyclin-dependent kinase 20) as an oncogenic effector of AR in HCC [18,19]. Despite its strong oncogenicity, no study has explored the epigenetic regulation by CCRK. Here we show that CCRK induces EZH2 up-regulation and phosphorylation in an epigenetic circuitry consisting of glycogen synthase kinase 3β (GSK-3β), β-catenin, T-cell factor (TCF), E2F1, AKT, and AR. This signaling network is activated during diethylnitrosamine (DEN)-induced hepatic tumorigenicity which can be dramatically suppressed by lentiviral-mediated knockdown of *CCRK*. As the self-reinforcing circuit hyperactivates in human hepatocarcinogenesis and associates with poor prognosis of patients, our data raise the possibility that CCRK is a promising molecular target for therapeutic intervention.

Materials and methods

Cell culture, transfection and functional assays

LO2, HuH7, PLC5, and SK-Hep1 cells were cultured in DMEM supplemented with 10% FBS (Hyclone). Cell transfection was conducted using X-tremeGene Transfection Reagent (Roche) or HiPerfect (Qiagen) according to the manufacturer's instructions. The information of small-interfering RNA (siRNA), short-hairpin RNA (shRNA) constructs, expression vectors, and luciferase reporter constructs are described in the Supplementary material. Anchorage-dependent and independent growth were analyzed by colony formation and soft agar assays, respectively, as previously described [18].

Quantitative RT-PCR, ChIP-PCR, immunoblotting, co-immunoprecipitation and immunohistochemistry

cDNA synthesis from RNA purified from cell lines and mouse samples was conducted using reverse transcription (RT) Master Kit (Invitrogen). Chromatin immunoprecipitation (ChIP) assays were performed as previously described [18,20]. Detailed protocols of quantitative RT-PCR, ChIP-PCR, cell lysis, immunoblotting and co-immunoprecipitation are described in the Supplementary material. Cell proliferation in mouse tissues was assayed by Ki67 immunohistochemistry as previously described [19].

In vivo tumorigenicity assays

The xenograft and orthotopic models were performed using athymic nude mice (n = 7 per group) as previously described [18]. The CCRK knockdown experiment in the DEN (2 mg/kg; Sigma-Aldrich) model was performed using male C57BL/6 mice (n = 12 per group) as previously described [19]. Lentiviruses encoding shRNA against *CCRK* or a control sequence were packaged according to the manufacturer's instructions (Dharmacon). All animal studies were reviewed and approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong.

Patients samples

Patients who underwent hepatectomy for HCC at the Prince of Wales Hospital (Hong Kong) were included in this study. All patients gave written informed consent on the use of clinical specimens for research purposes. Studies using human tissue were reviewed and approved by the Joint CUHK-NTEC Clinical Research Ethics Committee.

Statistical analysis

Unless otherwise indicated, data are presented as mean ± standard deviation of three independent experiments. Statistical analysis is described in the Supplementary material. A two tailed p value of less than 0.05 was considered statistically significant.

Results

EZH2 is required for CCRK-induced hepatocellular proliferation and transformation

To investigate the role and function of EZH2 in CCRK-induced hepatocarcinogenesis, we modulated gene expressions and performed colony formation and soft agar assays using both ectopic expression and knockdown approaches. Ectopic *CCRK* expression induced EZH2 and global H3K27me3 levels (Fig. 1A, left) and markedly increased focus formation (Fig. 1A, right) and anchorage-independent growth (Fig. 1B) of LO2 cells, an immortalized human liver cell line [18]. Notably, silencing of EZH2 by shRNA abrogated the induced proliferation and malignant transformation in CCRK-expressing cells (Fig. 1A–B). Conversely, shRNA-mediated knockdown of *CCRK* in PLC5 HCC cells dramatically reduced EZH2 and H3K27me3 levels (Fig. 1C, left) and decreased anchorage-dependent (Fig. 1C, right) and -independent growth (Fig. 1D), which could be rescued by ectopic *EZH2* expression (Fig. 1C–D). Consistently, downregulation of EZH2 in PLC5 and HuH7 HCC cells also resulted in significant growth inhibition (Supplementary Fig. 1A–B). We next performed *in vivo* experiments to validate the functional relationship between CCRK and EZH2. *CCRK* stably-transfected LO2 cells (CCRK-shCtrl-LO2) displayed significant increase in tumor volume and weight in the xenograft model when compared with empty vector-transfected cells (Vec-shCtrl-LO2), whereas knockdown of EZH2 completely attenuated the tumorigenicity of CCRK-shEZH2-LO2 cells (Fig. 1E). Concordantly, in an orthotopic model where the stable cell xenografts were implanted into the recipient livers, CCRK-induced intrahepatic tumorigenicity was abolished by EZH2 knockdown (Fig. 1F). These results demonstrate that EZH2 upregulation is required for CCRK-induced hepatocarcinogenesis.

CCRK activates a GSK-3β/β-catenin/TCF/E2F1 cascade to upregulate EZH2

We have recently identified GSK-3β as a CCRK kinase target which mediates β-catenin activation in liver and HCC cells [18,19]. To elucidate whether the GSK-3β/β-catenin signaling is involved in CCRK-induced EZH2 upregulation, we first employed a kinase-defective (KD) *CCRK* mutant whose Thr161 active site was replaced by alanine (T161A). Ectopic expression of wild-type