Research Article

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Salt-inducible Kinase (SIK1) regulates HCC progression and WNT/β-catenin activation

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Background & Aims: In this study, we investigated the role of salt-inducible kinase 1 (SIK1) and its possible mechanisms in human hepatocellular carcinoma (HCC).

Methods: Immunoprecipitation, immunohistochemistry, luciferase reporter, Chromatin immunoprecipitation, *in vitro* kinase assays and a mouse model were used to examine the role of SIK1 on the β-catenin signaling pathway.

Results: SIK1 was significantly downregulated in HCC compared with normal controls. Its introduction in HCC cells markedly suppresses epithelial-to-mesenchymal transition (EMT), tumor growth and lung metastasis in xenograft tumor models. The effect of SIK1 on tumor development occurs at least partially through regulation of β-catenin, as evidenced by the fact that SIK1 overexpression leads to repression of β-catenin transcriptional activity, while SIK1 depletion has the opposite effect. Mechanistically, SIK1 phosphorylates the silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) at threonine (T)1391, which promotes the association of nuclear receptor corepressor (NCoR)/SMRT with transducin-beta-like protein 1 (TBL1)/transducing-beta-like 1 X-linked receptor 1 (TBLR1) and disrupts the binding of β-catenin to the TBL1/TBLR1 complex, thereby inactivating the Wnt/β-catenin pathway. However, SMRT-T1391A reverses the phenotype of SIK1 and promotes β-catenin transactivation. Twist1 is identified as a critical factor downstream of SIK1/β-catenin axis, and Twist1 knockdown ($Twist1^{KD}$) reverses $SIK1^{KD}$-mediated changes, whereas $SIK1^{KD}$/$Twist1^{KD}$ double knockdown cells were less efficient in establishing tumor growth and metastasis than $SIK1^{KD}$ cells. The promoter activity of SIK1 were negatively regulated by Twist1, indicating that a double-negative feedback loop exists. Importantly, levels of SIK1 inversely correlate with Twist1 expression in human HCC specimens.

Conclusions: Our findings highlight the critical roles of SIK1 and its targets in the regulation of HCC development and provides potential new candidates for HCC therapy.

Cancer

Keywords: Salt-inducible kinase 1; Twist1; Epithelial-mesenchymal transition; β-catenin; Silencing mediator for retinoid; Thyroid receptors.

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Abbreviations: ChIP, chromatin immunoprecipitation; DFS, disease-free survival; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; GSK-3β, glycogen synthase kinase-3β; HCC, hepatocellular carcinoma; HDAC3, histone deacetylase 3; IF, immunofluorescence; IHC, immunohistochemistry; NCoR, nuclear receptor corepressor; OS, overall survival; PCR, polymerase chain reaction; TBL1, transducin-beta-like protein 1; TBLR1, transducing-beta-like 1 X-linked receptor 1; TF, transcriptional factor; shSIK1, small hairpin RNA targeting SIK1.

Introduction

Hepatocellular carcinoma (HCC) is the third most prevalent cause of cancer-related death globally [1]. High metastasis and recurrence rates have become the major obstacle in improving long-term survival of HCC. It has been indicated that dysregulation of oncogenes or anti-oncogenes is associated with metastasis and growth of HCC [2]. However, the mechanism for the development of HCC is still unclear.

The salt-inducible kinase 1 (SIK1) encodes a serine/threonine kinase of the AMP-activated protein kinase (AMPK) family, which plays major roles in regulating metabolism and cell growth [3]. Given that reduced levels of SIK1 are related to metastases and poor outcome in breast cancer, and SIK1-knockdown ($SIK1^{KD}$) cells escape anoikis to disseminate in distant organs [4], SIK1 has been associated with a tumor suppressor function [5]. However, the expression and function of SIK1 in HCC remains unclear and should be fully elucidated.

The Wnt/β-catenin signaling pathway plays a critical role in cell fate determination and is associated with a variety of human cancers, including HCC [6]. Recently, the dissociation of the corepressors containing silencing mediator of retinoic acid and thyroid hormone receptor (SMRT)/nuclear receptor corepressor (NCoR)/ histone deacetylase 3 (HDAC3) from β-catenin and recruitment of coactivators transducin-beta-like protein 1 (TBL1)/transducing-beta-like 1 X-linked receptor 1 (TBLR1) are shown to be the final step for the activation of Wnt/β-catenin signaling [7]. However, there are no previous reports defining a role for SIK1 in the regulation of Wnt/β-catenin signaling.

Thus, this study was aimed to elucidate the function of SIK1 in HCC and its action mechanisms. We show for the first time that SIK1 is a negative upstream regulator of the Wnt/β-catenin pathway and is significantly correlated with clinical outcome in human HCC.

Materials and methods

Cell culture, establishment of stable cell lines and tissue samples

Human HCC cell lines were routinely cultured, the stable, overexpressed and knockdown HCC cell lines were generated by transient transfection followed by antibiotics selection. In addition, 188 human tissue samples were obtained and analyzed in this study.

Real-time PCR analysis, Western blot, immunofluorescence and immunohistochemistry

RNA extraction, cDNA synthesis and final real-time PCR and Western blots were performed according to general protocols. HCC cells were processed for immunofluorescence using relative antibodies with optimized condition. Additionally, human samples were processed for immunohistochemistry staining to evaluate the expression of target proteins.

Plasmid constructs, site-directed mutagenesis and luciferase reporter assays

The complementary DNA clones of target genes were inserted into different backbones by PCR. The Quickchange® site-directed mutagenesis kit (Stratagene) was used for plasmid mutagenesis according to the manufacturer's protocol. Luciferase activity was determined using the Dual Luciferase Assay System (Promega).

Chromatin immunoprecipitation (ChIP), immunoprecipitation and in vitro kinase assays

A ChIP assay was performed to evaluate the binding ability of target transcription factors according to the manufacturer's protocol. *In vivo* protein interaction was assessed by immunoprecipitation. Identification of *in vitro* phosphorylation events was performed using a [γ-^{32}P] ATP kinase assay.

Soft agar, XTT, transwell migration and wound healing assays

In vitro and *in vivo* cell proliferation ability was measured according to general protocols. Transwell migration assays and a wound healing assay were performed to assess cell migration and invasion ability and images were captured by microscope.

Animal studies

A nude mouse orthotopic HCC model and a lung metastasis model were used to assess *in vivo* tumor growth and metastasis.

Statistical analysis

Different statistical analysis methods were used to compare different groups or different categories of data. Extended details on materials and methods can be found in the Supplementary material.

Results

SIK1 is significantly downregulated in HCC and low SIK1 expression predicts poor prognosis

To explore the role of SIK1 in HCC development, we first investigated the expression of SIK1 protein in 6 human HCC and 3 normal liver cell lines. We observed a drastic reduction of SIK1 expression in all HCC cell lines (Fig. 1A). We also confirmed the presence of aberrant downregulation of *SIK1* expression in HCC specimens (Fig. 1B). Typically, SIK1-negative or -weak staining was observed in the cancer cells, but SIK1-positive staining was shown in adjacent normal liver cells (Fig. 1C).

To investigate the clinical significance of SIK1 downregulation in HCC, we further analyzed the relationship between clinicopathologic features and SIK1 expression levels in HCC cases. SIK1 expression was negatively correlated with tumor differentiation (p <0.001) (Supplementary Table 1). Importantly, downregulation of SIK1 expression was associated with larger tumor size or distant metastasis (Fig. 1D). Furthermore, patients in the low SIK1 expression group exhibited shorter overall survival (OS) and worse disease-free survival (DFS) than those in the high expression group (Fig. 1E, F). Multivariate analysis revealed that tumor size, pathological differentiation and SIK1 were independent predictors of OS (Supplementary Table 2). These findings suggest that SIK1 expression might play a critical role in HCC progression and be a valuable biomarker of this disease.

SIK1 suppresses HCC cell growth in vitro and in vivo

Next, we stably expressed SIK1 in human HCC cells (Supplementary Fig. 1A). Strikingly, SIK1 not only dramatically inhibited anchorage-dependent or anchorage-independent growth, as