Research Article

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Inactivation of Spry2 accelerates AKT-driven hepatocarcinogenesis via activation of MAPK and PKM2 pathways

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Background & Aims: Aberrant activation of the AKT oncogenic pathway and downregulation of the Sprouty 2 (*Spry2*) tumor suppressor gene are frequently observed molecular events in human hepatocarcinogenesis. The goal of the present study was to investigate the eventual biochemical and genetic crosstalk between activated AKT and inactivation of Spry2 during liver cancer development by using *in vivo* and *in vitro* approaches.

Methods: Activated *AKT* and/or *Spry2Y55F*, a dominant negative form of *Spry2*, were overexpressed in the mouse liver via hydrodynamic gene delivery. Histological and biochemical assays were applied to characterize the molecular features of AKT and AKT/Spry2Y55F liver tumors. The human HLE hepatocellular carcinoma (HCC) cell line, stably overexpressing *AKT*, was transfected with *Spry2Y55F* to study the molecular mechanisms underlying hepatocarcinogenesis driven by Spry2 loss.

Results: *Spry2Y55F* overexpression significantly accelerated AKT-induced hepatocarcinogenesis in the mouse. AKT/Spry2Y55F liver lesions had increased proliferation and glycolysis and decreased lipogenesis when compared with AKT corresponding lesions. At the molecular level, AKT/Spry2Y55F HCCs exhibited a significantly stronger induction of activated mitogen-activated protein kinase (MAPK) and pyruvate kinase M2 (PKM2) pathways than in AKT corresponding lesions. This phenotype was reproduced in HLE cells overexpressing *AKT* following transfection with *Spry2Y55F*. Furthermore, we found that concomitant suppression of the MAPK cascade and PKM2 strongly inhibited the growth induced by Spry2Y55F in *AKT*-overexpressing cells.

Conclusions: Inactivation of Spry2 accelerates AKT-induced hepatocarcinogenesis via activation of MAPK and PKM2 pathways.
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Abbreviations: AKT, v-akt murine thymoma viral oncogene homolog; ERK, extracellular-related kinase; GLUT, glucose transporter; HCC, hepatocellular carcinoma; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; mTORC, mTOR complex; RPS6, ribosomal protein S6; Spry2, Sprouty2; PKM2, pyruvate kinase M2.

Introduction

Human hepatocellular carcinoma (HCC) is the third most common cause of cancer death worldwide [1]. Treatment options for HCC are limited. Sorafenib, a multikinase inhibitor, is the only available drug that significantly increases the survival of patients with advanced HCC [2–4]. To develop more effective therapies against HCC, a better understanding of the molecular mechanisms underlying HCC development is required [2,4,5].

HCC development is a multi-step process in which multiple pathways are deregulated [2,4,5]. Among them, the v-akt murine thymoma viral oncogene homolog (AKT)/mammalian target of rapamycin (mTOR) cascade is one of the most frequently activated pathways [5,6]. AKT exerts many of its cellular effects through its key downstream effector, the mTOR complex 1 (mTORC1) [6,7]. Accordingly, the mTORC1 axis is also frequently activated in human HCC [8]. Generally, the phosphorylation of the ribosomal protein S6 (RPS6) is used as a surrogate marker for mTORC1 activation [9]. The importance of the AKT pathway in hepatocarcinogenesis has been recently further substantiated by our group. Noticeably, we found that overexpression of an activated form of AKT in the mouse liver induces lipogenesis as well as hepatocyte proliferation, eventually leading to liver tumor formation within six months [10]. Similar results were previously obtained in mice depleted of the AKT specific inhibitor, phosphatase and tensin homolog [11].

The Ras/mitogen-activated protein kinase (MAPK) signaling is another aberrantly activated pathway in human HCC [12]. However, Ras or Raf mutations are extremely rare in HCC, implying that activation of the Ras/MAPK cascade occurs in a context of wild type Ras and Raf in this disease [13,14]. Sprouty 2 (Spry2), one of the Sprouty family members evolutionarily conserved inhibitors of receptor tyrosin kinases, negatively regulates the Ras/MAPK pathway [15]. Expression of Spry2 protein is frequently downregulated and its loss is significantly associated with activation of the Ras/MAPK pathway in HCC [16–18]. Furthermore, inactivation of Spry2 via Spry2Y55F, a dominant negative form of Spry2, cooperates with other activated oncogenic proteins, such as β-catenin or c-Met, to induce HCC development in mice [18,19].

The possible crosstalk between the AKT/mTOR and Ras/MAPK pathways during hepatocarcinogenesis is suggested by the recent

finding that co-expression of activated AKT and mutated N-Ras rapidly induces HCC development in mice [20]. To further investigate the interaction(s) between these two pathways during hepatocarcinogenesis and to better reproduce the human disease, in which the Ras genes are not mutated [13,14], we co-expressed an activated/myristoylated form of *AKT* (*myr-AKT*) and *Spry2Y55F* in the mouse liver by hydrodynamic injection. Our data show that loss of Spry2 synergizes with AKT activation to induce rapid hepatocarcinogenesis through the activation of MAPK and PKM2 pathways.

Materials and methods

Constructs and reagents

The constructs used for mouse injection, including pT3-EF1α-HA-myr-AKT, pT3-EF1α-Spry2Y55F-V5, and pCMV/sleeping beauty transposase (SB), were described previously [10,18,21]. Plasmids were purified using the Endotoxin-free Maxiprep kit (Sigma, St. Louis, MO).

Hydrodynamic injection and mouse monitoring

Wild type FVB/N mice were obtained from Charles River (Wilmington, MA). Hydrodynamic injections were performed as described previously [10,18,21]. Briefly, ten micrograms of the plasmids encoding *myr-AKT* and/or *Spry2Y55F* along with sleeping beauty transposase in a ratio of 25:1 were diluted in 2 ml saline (0.9% NaCl) for each mouse. Saline solution was filtered through a 0.22 μm filter and injected into the lateral tail vein of 6- to 8-week-old FVB/N mice in 5–7 s. Mice were housed, fed, and monitored in accordance with protocols approved by the committee for animal research at the University of California, San Francisco.

Histology and immunohistochemistry

Livers were fixed in 4% paraformaldehyde and processed for paraffin embedding. Preneoplastic and neoplastic liver lesions were assessed by two board-certified pathologists (M.E. and F.D.) in accordance with the criteria by Frith *et al.* [22]. Immunohistochemistry was performed, and proliferation and apoptotic indices were determined, as described [20].

Metabolic parameters measurement

Fatty acid synthesis was measured by incorporation of [U–^{14}C] acetate into lipids. Liver lysates were labeled with [U–^{14}C] acetate. Lipids were Folch extracted and counted for ^{14}C. Hepatic cholesterol and lactate content was assessed with the Cholesterol Quantification and the Lactate Assay Kit II (BioVision Inc., Mountain View, CA), respectively, following the manufacturer's protocol.

Immunoblotting and kinase assay

Murine hepatic tissues were processed as described in Supplementary Materials and methods. Nitrocellulose membranes were probed with specific primary antibodies (Supplementary Table 1). AKT and MAPK kinase activities were assessed with the AKT and p44/42 MAPK kinase assay kits (Cell Signaling Technology, Danvers, MA), respectively, following the manufacturer's protocol.

Cell line

The human HCC cell line HLE was used for the *in vitro* experiments. This cell line expresses low AKT levels and does not harbor β-catenin mutations. Transfection with cDNA and siRNAs and treatment with inhibitors were performed as described in Supplementary Materials and methods.

Statistical analysis

Tukey–Kramer test was used to evaluate statistical significance. Values of $p < 0.05$ were considered significant. Data are expressed as mean ± SD.

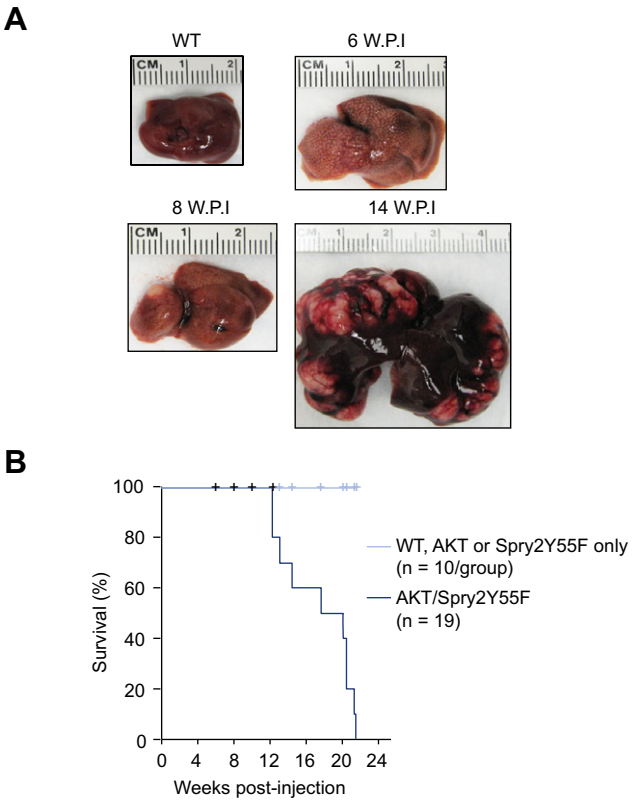

Fig. 1. Co-expression of Spry2Y55F and activated AKT induces liver tumor development in mice. (A) Macroscopic pictures of wild type (WT) and AKT/Spry2Y55F-injected mice livers at different time points. W.P.I, weeks post-injection. (B) Survival curve of the wild type (WT), AKT only-, Spry2Y55F only- and AKT/Spry2Y55F-injected mice. (This figure appears in colour on the web).

See Supplementary Materials and methods for more detailed descriptions of Materials and methods.

Results

Spry2Y55F accelerates AKT-induced liver tumor development in mice

To determine whether downregulation of Spry2 cooperates with activated AKT to induce hepatocarcinogenesis, we co-injected HA-tagged *myr-AKT* and V5-tagged *Spry2Y55F*, a dominant negative form of Spry2 [18,19], along with the sleepy beauty transposase, into the mouse liver by hydrodynamic injection. In accordance with our previous studies, we found that overexpression of *Spry2Y55F* alone (n = 10) did not lead to histological abnormalities 6 months post-injection [18,19], whereas overexpression of *myr-AKT* resulted in hepatocellular adenoma (HCA) and HCC development by 3 and 6 months post-injection, respectively [10]. Noticeably, following co-injection of *myr-AKT* and *Spry2Y55F* (which will be referred to as AKT/Spry2Y55F mouse in this paper), AKT/Spry2Y55F mouse livers became larger, spotted and paler around 6 weeks post-injection (Fig. 1A). Eight weeks after hydrodynamic injection, liver nodules developed in AKT/Spry2Y55F mice (Fig. 1A). Large, palpable liver tumors were observed in 4 of 5 AKT/Spry2Y55F mice after 14 weeks post-injection, while AKT mice did not develop any nodule at this time point (Fig. 1A and Supplementary Fig. 1) [10]. AKT/Spry2Y55F

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