Research Article

JOURNAL OF HEPATOLOGY

Elevated expression of Erbin destabilizes ERα protein and promotes tumorigenesis in hepatocellular carcinoma

Hua Wu[1,2,†], Su Yao[3,4,†], Shen Zhang[2], Jing-Ru Wang[2], Peng-Da Guo[2], Xiu-Ming Li[2], Wen-Juan Gan[5], Lin Mei[6], Tian-Ming Gao[7,*], Jian-Ming Li[1,2,3,*]

[1]*Department of Pathology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, China;* [2]*Department of Pathology, Soochow University, Suzhou 215123, China;* [3]*Department of Pathology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China;* [4]*Department of Pathology, Guangdong General Hospital and Guangdong Academy of Medical Sciences, Guangzhou 510080, China;* [5]*Department of Pathology, The First Affiliated Hospital of Soochow University, Suzhou 215006, China;* [6]*Department of Neuroscience and Regenerative Medicine, Georgia Regents University, Augusta, GA 30912, USA;* [7]*State Key Laboratory of Organ Failure Research, Key Laboratory of Psychiatric Disorders of Guangdong Province, Department of Neurobiology, School of Basic Medical Sciences, Southern Medical University, Guangzhou 510515, China*

Background & Aims: Aberrant estrogen receptor-α (ERα) expression and signaling are implicated in the development of hepatocellular carcinoma (HCC), but its regulation in HCC remains enigmatic. Herein, we aimed to identify a new mechanism by which ERα signaling is regulated in HCC, which may lead to a potential new strategy for HCC therapy.

Methods: Expression levels of Erbin and ERα in human HCC samples were evaluated by immunohistochemistry. *In vitro* and *in vivo* experiments were used to assess the effect of Erbin and ERα signaling on HCC cell growth. Crosstalk between Erbin and ERα signaling was analyzed by molecular methods. Animal models of diethylnitrosamine (DEN) or DEN/CCl_4-induced HCC in wild-type $Erbin^{+/+}$ and mutant $Erbin^{\Delta C/\Delta C}$ mice were observed. The regulatory effects of Erbin on tamoxifen treatment of HCC were evaluated *in vitro* and *in vivo*.

Results: Erbin inactivated ERα signaling to drive tumorigenesis of HCC, acting to enhance binding of Chip to ERα via its interaction with ERα and thereby promoting ubiquitination and degradation of ERα. Deletion of the PDZ domain of Erbin in $Erbin^{\Delta C/\Delta C}$ mice, disrupted the interaction of Chip and ERα, increased the stability of ERα protein, and thus inhibited tumorigenesis of HCC. Silencing of Erbin effectively sensitized the response of HCC after tamoxifen treatment *in vitro* and *in vivo*.

Conclusions: Our data uncovered an important role of Erbin in regulating HCC tumorigenesis through inactivating ERα-mediated tumor-suppressive signaling, suggesting a new strategy for tamoxifen therapy in HCC by targeting Erbin/ERα signaling axis.

Lay summary: Erbin expression is significantly elevated in human hepatocellular carcinoma (HCC) tissue. This elevated expression of Erbin contributes to tumorigenesis of HCC by negatively regulating ERα signaling. However, restoring ERα signaling by inhibiting Erbin expression enhances the sensitivity of HCC cells to tamoxifen treatment, providing a new approach for tamoxifen treatment in HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers in the world. Epidemiologically, the incidence of HCC in males is 2- to 11-fold higher than in females [1], and the male patients with HCC usually have a poorer prognosis than female patients with HCC [2], suggesting the roles of sex hormones and their receptors in HCC.

Estrogen receptor-α (ERα), a member of the nuclear hormone receptor subfamily, has been implicated in the development of HCC. The elevation in the activity of the ERα signaling contributes to the lower viral loads and reduces the risk of HCC by repressing transcription of hepatitis B virus genes [3,4]. It is also noteworthy that administration of estradiol (E2) in wild-type (WT) male mice, but not in ERα-deficient ($ER\alpha^{-/-}$) male mice, exerts significant protection against diethylnitrosamine (DEN)-induced hepatocarcinogenesis [5], indicating that the ERα signaling plays a protective role in attenuating HCC development. The anti-estrogenic compound tamoxifen has been shown to inhibit hepatocyte proliferation *in vitro* and *in vivo* [6], and several clinical trials have shown that tamoxifen therapy can improve the

Keywords: Hepatocellular carcinoma; Tumorigenesis; ERα signaling; Tamoxifen therapy; Mouse model.

Received 14 October 2016; received in revised form 23 January 2017; accepted 29 January 2017; available online 10 February 2017

* Corresponding authors. Address: Department of Pathology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, China. Tel.: +86 512 65882673; fax: +86 512 65882673 (J.-M. Li) or State Key Laboratory of Organ Failure Research, Key Laboratory of Psychiatric Disorders of Guangdong Province, Department of Neurobiology, School of Basic Medical Sciences, Southern Medical University, Guangzhou 510515, China. Tel.: +86 20 62789362; fax: 86 20 62789362 (T.-M. Gao).

E-mail addresses: tgao@fimmu.com (T.-M. Gao), jianmingli@suda.edu.cn (J.-M. Li).

† These authors contributed equally to this work.

survival in advanced HCC patients [7,8]. Unfortunately, in a large clinical study, tamoxifen therapy did not improve the survival of patients with advanced HCC [9]. All these controversial studies suggest that better understanding of the key regulator and mechanisms responsible for ERα signaling in HCC is necessary.

Erbin, a member of the leucine-rich repeat and PDZ domain protein, was originally described as Erbb2-interacting protein (ERBB2IP) [10]. Accumulating studies have suggested that Erbin plays a critical role in regulating cancer cell proliferation, apoptosis, and inflammatory response [11–15]. The role of Erbin in cancer is very complex, and is dependent on specific cell types and contexts. On the one hand, Erbin can function as a tumor suppressor by negatively regulating TGFβ or ERK signaling [16–18]. Loss of Erbin expression in cervical cancer contributes to anoikis resistance of cervical cancer cells by inhibiting STAT3 signaling [19]. On the other hand, Erbin can act as a tumor promoter. Silencing of Erbin expression inhibits the formation of multicellular tumor spheroids in HT-29 colon carcinoma cells [20]. Notably, our recent data revealed that Erbin was required for ErbB2-dependent breast tumor formation and progression [21] or EGFR-dependent colorectal tumorigenesis [13]. Nevertheless, the role of Erbin in the development of HCC remains unknown, and it is further unclear whether Erbin is involved in regulating ERα signaling in HCC.

Herein, we demonstrate that Erbin exerts oncogenic effects in the tumorigenesis process of HCC. Mechanistically, Erbin inactivates ERα-mediated tumor-suppressive signaling via its interaction with ERα, which facilitates Chip (Carboxyl terminus of Hsc70-interacting protein) binding, and ubiquitination of ERα, and thereby promoting ubiquitination and degradation of ERα. Importantly, our study suggests a new strategy by targeting Erbin/ERα signaling axis and sensitizing the response of tamoxifen treatment in HCC.

Cancer

Materials and methods

Animal maintenance and treatments

All mice were housed in a pathogen-free facility under 12 h light-dark cycle with free access to food and water. To evaluate *in vivo* tumor growth, 1.5×10^6 cells were subcutaneously injected into nude mice (BALB/c, male, 16–18 g, 4–5-week old). Body weight and tumor sizes were measured weekly. The mice were sacrificed after 6 weeks, and their tumors were removed for assessments. For drug treatment, mice (n = 6 per group) were treated intraperitoneally after 1 week of xenografts with olive oil or tamoxifen (10 mg/kg) once every other day (eleven injections in total). Tumor sizes of mice were measured every four days. One month later, mice were killed and the tumors removed for assessments. To evaluate hepatocarcinogenesis, wild-type (WT; $Erbin^{+/+}$) and $Erbin^{\Delta C/\Delta C}$ mice (male, n = 8 per group) were injected intraperitoneally with 25 mg/kg of diethylnitrosamine (DEN, Sigma) at 14 days of age. The mice were killed at 9 months of age, and liver tissues were harvested for tumor evaluation and standard histopathological studies. For DEN/CCl_4-induced hepatocarcinogenesis, WT ($Erbin^{+/+}$) and $Erbin^{\Delta C/\Delta C}$ mice (male, n = 8 per group) as indicated were injected intraperitoneally with either DEN (25 mg/kg) or CCl_4 (1.2 mg/kg), and killed at the indicated time. All animal experiments were approved by the Animal Care and Use Committee of Soochow University.

Human HCC samples

Human HCC samples were obtained from Outdo Biotech Co., Ltd (Shanghai, China). These samples were collected from 2002 to 2009. The clinical characteristics of all patients are listed in Table S1. This study was approved by Soochow University for Biomedical Research Ethics Committee, and all of the patients provided informed consent.

Statistical analysis

The relationships between Erbin expression and clinicopathological factors were analyzed using Pearson's chi-square test, and the correlations between the expression levels of Erbin and ERα, C-myc were calculated using Spearman's rank correlation test. The Kaplan-Meier survival analysis was used to illustrate the prognostic relevance of Erbin in univariate analysis. Each assay was performed in three independent experiments. All of the data were presented as mean plus standard error of the mean. Statistical analyzes were conducted using SPSS package (version 18.0). Statistical comparisons between two groups were performed using a Student's *t* test. One-way ANOVA was used to compare the differences among more than two groups. $p < 0.05$ was considered statistically significant.

Protocols for other procedures are provided in the Supplementary methods. For further details regarding the materials used, please refer to the CTAT table.

Results

Erbin is highly expressed in HCC and correlates with tumor size, differentiation and poor survival of HCC patients

To determine the role of Erbin in the development of HCC, we first investigated the expression profiles of Erbin in human HCC cell lines and clinical HCC samples. Western blot analysis showed that Erbin displayed a high expression level in most HCC cell lines (Fig. 1A). In the human HCC samples, we found a higher level of Erbin transcripts and protein expression in primary HCC tumors than matched surrounding tissues (Fig. S1A, B). Immunohistochemistry (IHC) analysis further confirmed that Erbin was highly expressed in most HCC tissues compared with the matched surrounding tissues of HCC (Fig. 1B, C). Moreover, Erbin expression was increased with advanced clinical grades of HCC (Fig. 1D, E). In line with these findings, increased Erbin protein levels in HCC tumors were significantly correlated with the clinicopathological parameters such as tumor size, AJCC stage, and differentiation (Table S1), thereby indicating a potential critical role of Erbin in the development of HCC.

We also assessed the correlation between Erbin expression and HCC patients' prognosis. Tissue microarrays from 131 patients with HCC were examined by IHC with Erbin antibody, and the results revealed that patients with high Erbin expression had a worse overall survival (OS) than those with low Erbin expression (Fig. 1F). The median OS time of HCC patients with high Erbin expression was approximately 7 months, which was markedly shorter than those with low Erbin expression (~24 months). These results suggested that Erbin expression may be a valuable prediction factor for HCC prognosis.

Erbin promotes HCC cell proliferation in vitro and tumor growth in vivo

To determine the significance of the above clinical findings, we sought to generate cells with stable knockdown of Erbin protein using a lentiviral shRNA technique in SMMC-7721 and BEL-7402 cells which express high levels of Erbin protein. The knockdown efficiency was confirmed by Western blot (Fig. S2). The ability of cell proliferation was firstly monitored by Cell Counting Kit-8 (CCK-8) assay, and the results showed that silencing of *Erbin* expression greatly inhibited the proliferative ability of HCC cells (Fig. 2A). In agreement with this result, colony formation assays demonstrated that knockdown of *Erbin* expression dramatically repressed the ability of SMMC-7721 and BEL-7402 cells to form colonies (Fig. 2B). We also injected the same number of