Received: 11 January 2018 | Accepted: 26 June 2018

DOI: 10.1002/jcp.27063

ORIGINAL RESEARCH ARTICLE

WILEY Journal of Cellular Physiology

Anticancer effects of echinacoside in hepatocellular carcinoma mouse model and HepG2 cells

Ying Ye | Yanan Song | Juhua Zhuang | Guoyu Wang | Jing Ni | Wei Xia

Department of Nuclear Medicine, The Seventh People's Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China

Correspondence
Wei Xia, Department of Nuclear Medicine, The Seventh People's Hospital, Shanghai University of Traditional Chinese Medicine, 358 Datong Rd, Shanghai 200137, China.
Email: awingxia@163.com

Funding information
Natural Science Foundation of China, Grant/Award Number: 81703791; Talents Training Program of Seventh People's Hospital of Shanghai University of TCM, Grant/Award Number: XX2017-04; Science and Technology Development Fund of Shanghai Pudong New Area, Grant/Award Number: PKJ2017-Y14; Science and Technology Development Fund, Grant/Award Number: 14DZ1940605; Key disciplines Group Construction Project of Pudong Health Burea of Shanghai, Grant/Award Number: PWZxk2017-06

Echinacoside (ECH) is a phenylethanoid glycoside extracted from a Chinese herbal medicine, *Cistanches salsa*. ECH possesses many biological properties, including anti-inflammation, neural protection, liver protection, and antitumor. In the current study, we aimed to explore the effects of ECH on hepatocellular carcinoma (HCC) and the underlying mechanisms. The results showed that ECH could attenuate diethylnitrosamine (DEN)-induced HCC in mice, and exerted antiproliferative and proapoptotic functions on HepG2 HCC cell line. ECH exposure in HepG2 cells dose-dependently reduced the phosphorylation of AKT (p-AKT) and enhanced the expression of p21 (a cell cycle inhibitor) and Bax (a proapoptotic protein). Furthermore, ECH significantly suppressed insulin-like growth factor-1-induced p-AKT and cell proliferation. These data indicated that phosphoinositide 3-kinase (PI3K)/AKT signaling was involved in the anti-HCC activity of ECH. Gene set enrichment analysis results revealed a positive correlation between the PI3K pathway and triggering receptors expressed on myeloid cells 2 (TREM2) expression in HCC tissues. ECH exposure significantly decreased TREM2 protein levels in HepG2 cells and DEN-induced HCC. Furthermore, ECH-mediated proliferation inhibition and AKT signaling inactivation were notably attenuated by TREM2 overexpression. In conclusion, ECH exerted its antitumor activity via decreasing TREM2 expression and PI3K/AKT signaling.

KEYWORDS
AKT, echinacoside (ECH), hepatocellular carcinoma (HCC), proliferation, triggering receptors expressed on myeloid cells 2 (TREM2)

1 | INTRODUCTION

Hepatocellular carcinoma (HCC), as the most frequent subtype of primary liver cancers, is one of the main causes of cancer-related death (EASL-EORTC, 2012; El-Serag, Marrero, Rudolph, & Reddy, 2008; Torre et al., 2015). The incidence and mortality rates for HCC have greatly increased during the past two decades, and the majority of HCC cases occur in Africa and eastern/south-eastern Asia (Bosetti, Turati, & La Vecchia, 2014). It is generally accepted that virus infection (hepatitis B and hepatitis C viruses), alcohol abuse, and nonalcoholic fatty liver disease are the most important risk factors for HCC (Bruix & Sherman, 2011). Currently, limited treatment options are available for HCC patients, such as surgery, liver transplant, radiotherapy, and chemotherapy (Bruix & Sherman, 2011). Thus, it is critically needed to explore for novel strategies for the control of HCC.

Traditional Chinese medicine (TCM) has been applied in cancer treatment. Many natural compounds derived from traditional Chinese herbs possess anticancer activity by inhibiting the proliferation, angiogenesis, and metastasis of HCC (Hu et al., 2013). Echinacoside (ECH) is a phenylethanoid glycoside extracted from *Cistanches salsa*, a Chinese herbal medicine. Numerous studies have elucidated the biological properties of ECH, such as anti-inflammation, neural protection, liver protection, and anticancer (Dong, Wang

et al., 2015; Dong, Yu et al., 2015; Li et al., 2014; Q. Zhao, Gao, Li, & Cai, 2010; W. Wang, Luo, Liang, & Li, 2016; Wu, Li, Wen, & Li, 2007). For example, ECH has shown its potentials in protecting against acute liver injury in animal models, which may be associated with the antioxidative, antiapoptotic, and anti-inflammatory activities of ECH (Li et al., 2014; Wu et al., 2007). Also, previous studies have shown that ECH could induce apoptosis in a wide spectrum of cancer cells, including HCC (Dong, Wang et al., 2015; Dong, Yu et al., 2015; W. Wang et al., 2016). Tumor-related pathways, including mitogen-activated protein kinase (W. Wang et al., 2016), phosphoinositide 3-kinase (PI3K)/AKT (S. Wang et al., 2015), and transforming growth factor β1 (Jia, Guan, Guo, and Du, 2012) pathways, are found to be regulated by ECH. However, direct evidence is lacking about the therapy effects of ECH on HCC.

The triggering receptors expressed on myeloid cells 2 (TREM2), a transmembrane protein, is a putative receptor of DAP12 (Campbell & Colonna, 1999), which can activate PI3K, phospholipase C, and Vav signaling cascades (Lanier, 2003; McVicar & Burshtyn, 2001; Vivier, Nunes, & Vely, 2004). Emerging evidence has implicated TREM2 in the regulation of bone remodeling (Cella et al., 2003; Paloneva et al., 2003) and immune responses (Sharif & Knapp, 2008; Turnbull et al., 2006). In addition, research proved that TREM2 mutation may be associated with Nasu-Hakola disease (Paloneva et al., 2002) and Alzheimer's disease (Niemitz, 2013). Moreover, recent studies have shown that TREM2 is involved in the pathogenesis of various human malignancies including glioma (X.-Q. Wang et al., 2016), gastric cancer (X. Zhang, Wang, Li, Wang, & Ni, 2017), renal carcinoma (H. Zhang et al., 2016), and HCC (S.-L. Zhang et al., 2016).

In the current study, ECH was administered to diethylnitrosamine (DEN)-induced HCC mice model, which was widely used to test the efficacy of anti-HCC agents (X. Zhao et al., 2015). In vitro experiments were then performed to explore the antiproliferative and proapoptotic activity of ECH on HepG2 HCC cells. Furthermore, we tried to investigate the molecular basis of how ECH elicited its function.

2 | MATERIALS AND METHODS

2.1 | Animal experiments

The animal study was approved by the Animal Care and Use Committee at The Seventh People's Hospital (Shanghai, China) and performed in accordance with the Guidelines on Animal Experimentation. Male C57BL/6J mice (age, 6–8 weeks old) were purchased from Shanghai Experimental Animal Center (Shanghai, China) and housed at 25 ± 2°C with a 12-hr light–dark cycle under specific pathogen-free conditions. Twenty-four mice were randomly divided into four groups with six animal in each group: Group 1 (Control), mice did not receive any treatment; Group 2 (DEN), mice were intraperitoneally (ip) administered with 100 mg/kg DEN (Sigma, St. Louis, MO) and then with 50 mg/kg DEN in the following week for three weeks; Group 3 (DEN+ECH [20 mg/kg]) and Group 4 (DEN +ECH [50 mg/kg]), mice were injected with DEN as Group 2, and then ip given ECH (≥98%; Aladdin, Shanghai, China) daily at a dose of 20 and 50 mg/kg, respectively, for 4 weeks. The mice were killed and liver tissues were collected. A portion of liver was fixed in 10% formalin and then subjected for paraffin embedding and sectioning, and the remained samples were immediately frozen in liquid nitrogen and kept at −80°C until analysis.

2.2 | Hematoxylin-eosin staining and immunohistochemical staining

The sections were deparaffinized, rehydration, and subjected to hematoxylin-eosin (HE) staining or immunohistochemical (IHC) staining. IHC staining with anti-Ki67 (Abcam, Cambridge, MA) and anti-α-fetoprotein (AFP; Proteintech, Chicago, IL) was performed as previously described (Yang et al., 2006).

2.3 | Cell culture

HepG2 cells obtained from Chinese Academy of Sciences (Shanghai, China) were grown in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 μg/ml streptomycin and 100 U/ml penicillin. The cells were maintained in a humidified incubator at 37°C with 5% CO_2/95% air

2.4 | Generation of lentivirus

The human TREM2 CDNA was cloned into a lentiviral vector pLVX-puro (Clontech, Palo Alto, CA). The small hairpin RNA (shRNA) oligonucleotides against human TREM2 (target region, 5′-CCAGGGTATCAGCTCCAAA-3′) and control shRNA non-specific control (NC) Oligonucleotides were annealed and inserted into AgeI-EcoRI site of pLKO.1-puro vector (Addgene, Cambridge, MA). Lentivirus expressing TREM2 overexpression (OE), pLVX-puro (Vector), TREM2 shRNA (RNAi), or control shRNA (NC) were generated by triple transfection of 293 cells with the corresponding lentiviral plasmids and two packaging vectors with lipofectamine 2000 (Invitrogen).

2.5 | Cell treatments

To examine the functions of ECH, various doses of ECH (0, 20, 50, and 100 μg/ml; Aladdin) were added to HepG2 cells. To explore the involvement of PI3K/AKT signaling on the function of ECH, HepG2 cells were exposed to insulin-like growth factor-1 (IGF-1, 10 ng/ml; PeproTeche, Rocky Hill, NJ) with or without ECH (50 μg/ml) treatment. To study the functions of TREM2, HepG2 cells were transduced with lentivirus expressing TREM2 (OE), pLVX-puro (Vector), TREM2 shRNA (RNAi), or control shRNA (NC). To investigate the involvement of TREM2 on the function of ECH, HepG2 cells were treated with lentivirus expressing TREM2 (OE) or pLVX-puro (Vector) with or without ECH (50 μg/ml) exposure.

2.6 | Cell counting kit-8 assay

HepG2 cells were seeded onto 96-well plates with 3×10^3 cells in each well. After cultured overnight, the cells were treated as indicated. At 24, 48, and 72 hr after treatment, the cultured media were replaced by cell counting kit-8 (CCK-8) solution (SAB biotech., College Park, MD; 10% in media), and the cells were incubated at 37° C for an additional 1 hr. The optical density at the wavelength of 450 nm (OD_{450}) was measured. OD_{450} for the cells before treatment were also measured and set as controls. The relative cell proliferation was evaluated by normalizing the OD_{450} of each group to the control OD_{450}. The experiments were performed three times with triplicates.

2.7 | Cell apoptosis assay

HepG2 cells plated in six-well plates (5×10^5 cells) and treated as indicated after overnight culture. At 48 hr after treatment, the cells were harvested, washed twice with phosphate buffered saline (PBS) buffer, and labeled with Annexin fluorescein isothiocyanate (V-FITC) and propidium iodide (PI; (Beyotime, Shanghai, China)) in the dark for 20 min. Cell apoptosis was analyzed by using flow cytometry (BD Biosciences, San Jose, CA).

2.8 | Western blot analysis

Protein was extracted from frozen tissue powder and cells in a radioimmunoprecipitation assay buffer (Solarbio, Shanghai, China). Subsequently, the protein concentration was measured with the bicinchoninic acid assay kit (Thermo Fisher Scientific). Equal amount of protein (30 μg per well) was subjected to 10% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis and transferred to nitrocellulose membranes (Millipore, Bredford). After blocking with 5% skim milk, the membranes were probed with primary antibodies in accordance with the manufacturers' instructions. The sources of primary antibodies are as follows: anti-TREM2 (ab86491) was obtained from Abcam (Cambridge, MA); antibodies against p21 (#2947), phosphorylation status of AKT (p-AKT; #9271), AKT (#9272), and GAPDH (#5174) were from cell signaling (Danvers, MA); anti-Bax (sc-493) were purchased from Santa Cruz Biotech (Santa Cruz, CA). After incubation, the membranes with secondary antibodies, enhanced chemiluminescence system (Bio-Rad, Richmond, CA) was used to detect the target protein expression. The Band densitometry analysis was performed by using Image J software (http://rsb.info.nih.gov/ij/, Bethesda, MD) with GAPDH as a loading control.

2.9 | Bioinformatics analysis

Gene expression data of HCC and normal liver tissues were obtained from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/; GSE51401), and Student *t* test was used to compared the expression of TREM2 between tumor and normal tissues.

To elucidate the pathways and processes which related to TREM2 expression in HCC, the Gene set enrichment analysis (GSEA) was conducted based on GSE51401 data set as previously described (Cheng et al., 2015; Subramanian et al., 2005). Normalized enrichment score was calculated. The cutoff for statistical significance was set as the false discovery rate < 0.25 and $p < 0.05$.

2.10 | Statistical analysis

The GraphPad Prism software (version 6.0, San Diego, CA) was used for statistical analysis. The differences between the groups were analyzed by one-way analysis of variance. $P < 0.05$ was considered significant.

3 | RESULTS

3.1 | Echinacoside alleviates DEN-induced HCC in mice

To explore the possible function of ECH in HCC, HCC was induced by DEN injection in mice, and then ECH (20 or 50 mg/kg) was administrated. The histological changes within the livers were observed after HE staining. As shown in Figure 1a, HCC was formed in DEN group as manifested by significant fatty degeneration, liver fibrosis, and pseudolobule formation, while ECH administration alleviated such histological changes in a dose-dependent manner. The protein expression of AFP, the most commonly used marker for HCC (Baig et al., 2009), was significantly increased in DEN group, while alleviated by ECH administration. Ki67 expression was also detected by IHC staining to observe the changes of cell proliferation. Ki67 expression was weak and rare in the control livers and became strong and abundant in DEN group. ECH administration reduced the Ki67 expression. These results indicated that ECH possessed anticancer activity in vivo.

3.2 | Echinacoside suppresses cell proliferation and induces cell apoptosis in HepG2 cells

To confirm the anti-HCC effect of ECH in vitro, HepG2 cells were treated with a series doses of ECH (20, 50, and 100 μg/ml) for 24, 48, and 72 hr. The CCK-8 assay was then conducted to explore the roles of ECH on HCC cell proliferation. The cells before treatment were set as the control group. As illustrated in Figure 2a, exposure of HepG2 cells to ECH significantly suppressed HCC cell growth in a dose- and time-dependent manner.

Next, we elucidated whether ECH inhibited the proliferation of HepG2 cells by inducing cell apoptosis. Annexin V/PI staining and flow cytometric analysis (Figure 2b) showed that 48 hr exposure to ECH significantly increased HepG2 cells undergoing apoptosis in a dose-dependent manner (0 μg/ml, 3.30 ± 0.26%; 20 μg/ml, 9.57 ± 0.31%; 50 μg/ml, 26.67 ± 0.61%; 100 μg/ml, 37.20 ± 0.72%).

Then, we analyzed the effects of ECH on the protein expression of p21 (a cell cycle inhibitor [Zhou et al., 2001]) and Bax (a proapoptotic protein [Han et al., 2007]) by the western blot analysis

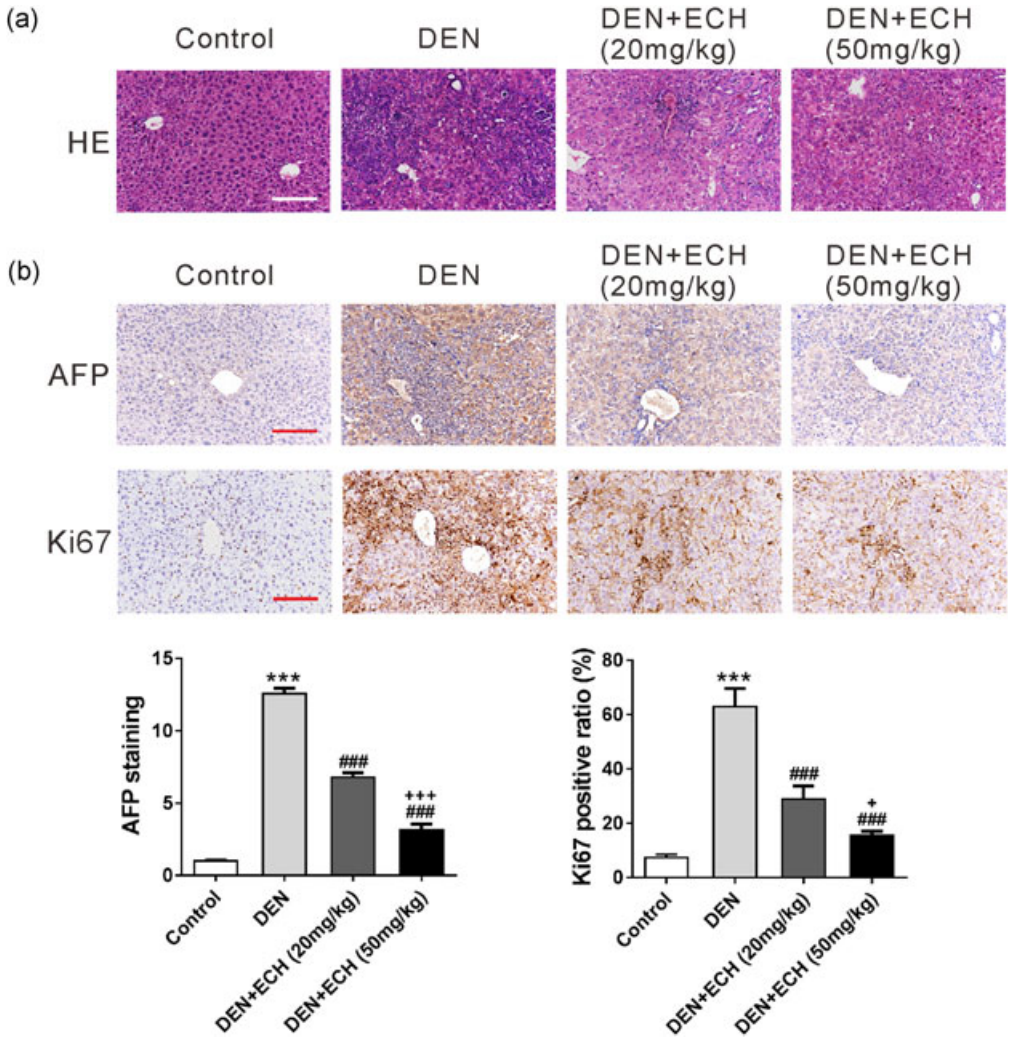

FIGURE 1 Hematoxylin-eosin (HE) staining and Ki67 immunohistochemical (IHC) staining of liver tissues. HCC was induced in mice by ip injection of DEN, and then echinacoside (ECH, 20 or 50 mg/kg) was administrated. Mice without any treatment were set as the control group. After 4 weeks of echinacoside treatment, the livers were collected for paraffin embedding and sectioning. (a) HE staining was performed to observe histological changes within the livers. (b) IHC staining was carried out with anti-AFP and anti-Ki67. The relative expression of AFP and Ki67 positive ratio was calculated. Each group had six animals, and HE and IHC staining were performed on liver samples from all animals. The representative images are shown. Scale bar: 100 μm. AFP: anti-α-fetoprotein; DEN: diethylnitrosamine; ip: intraperitoneal [Color figure can be viewed at wileyonlinelibrary.com]

(Figure 2c). As expected, ECH treatment significantly upregulated p21 and Bax, which was concomitant with the functional assays. The most obvious upregulation was observed at the dose of 100 μg/ml.

3.3 | Involvement of the AKT pathway in the antiproliferation role of echinacoside in HepG2 cells

The PI3K/AKT pathway is involved in the regulation of cancer cell proliferation and apoptosis (Martini, De Santis, Braccini, Gulluni, & Hirsch, 2014). To examine the effect of ECH on the PI3K/AKT pathway, we assessed the total and phosphorylated forms of AKT in ECH-treated HepG2 cells. ECH at the doses of 50 and 100 μg/ml significantly reduced the p-AKT but had no effects on the total protein of AKT (Figure 3a).

To further explore the involvement of PI3K/AKT signaling in the antiproliferation role of ECH, IGF-1, a known upstream activator for AKT (Mitsiades et al., 2002) was added together with ECH. As illustrated in Figure 3b, IGF-1 treatment (100 ng/ml) caused a remarkable increase in the level of p-AKT. Notably, ECH (50 μg/ml) significantly decreased p-AKT in IGF-1-treated HepG2 cells. The CCK-8 assay was then performed to study the changes of cell proliferation (Figure 3c). Treatment with IGF-1 promoted the proliferation of HepG2 cells, and ECH significantly suppressed IGF-1-induced cell proliferation. These results suggested that PI3K/AKT signaling was involved in the anti-HCC activity of ECH.

3.4 | Echinacoside exposure suppresses TREM2 expression

Our previous study has suggested that TREM2 is a probable oncogene in liver cancer (S.-L. Zhang et al., 2016). Bioinformatics analyses were performed on the microarray data from GEO

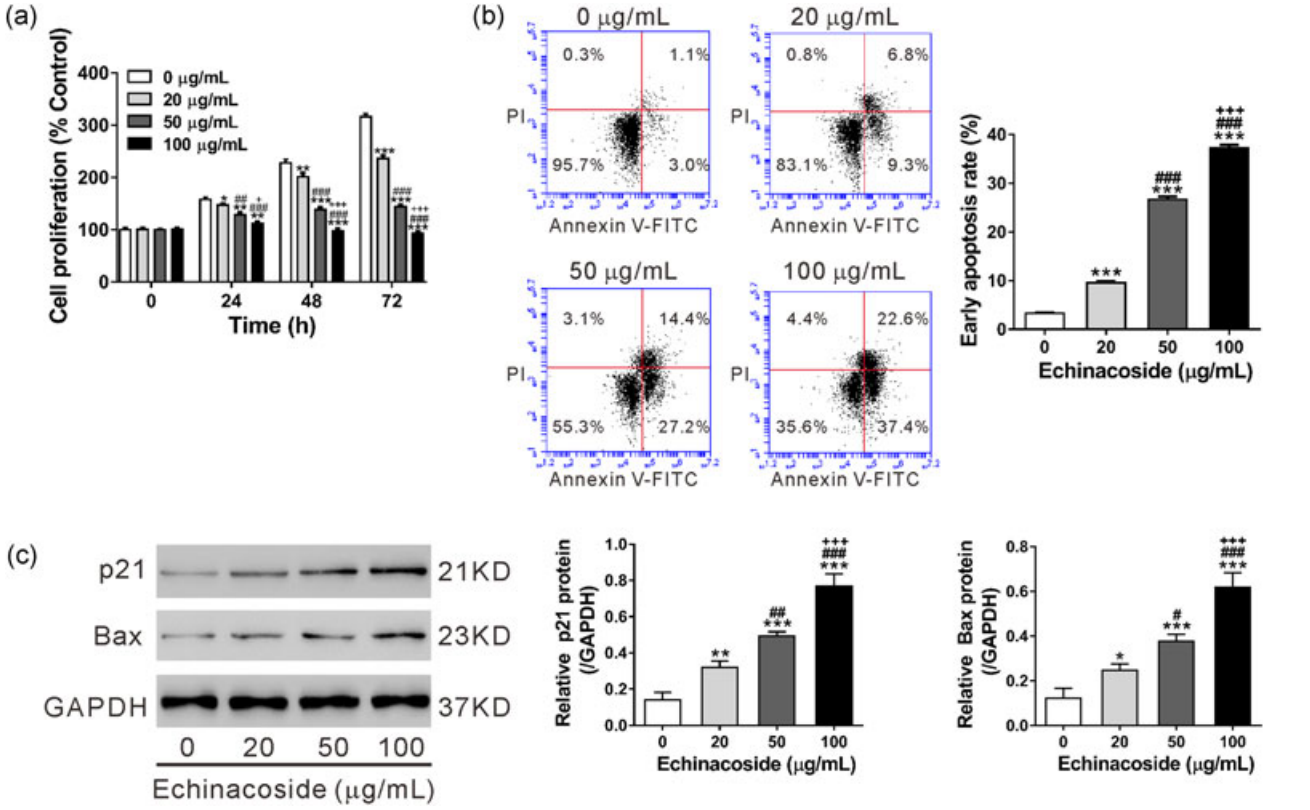

FIGURE 2 Effects of echinacoside on cell proliferation and apoptosis of HepG2 cells. HepG2 cells were exposed to various doses of echinacoside (0, 20, 50, and 100 μg/ml). (a) At 24, 48, and 72 hr after echinacoside exposure, CCK-8 assay was done to evaluate cell proliferation. The cells before treatment were set as the control group. The relative cell proliferation was evaluated by normalizing the OD_{450} of each group to the control OD_{450}. (b) At 48 hr after echinacoside exposure, cell apoptosis was assessed by double staining with annexin V-FITC and PI and flow cytometry analysis. The *x*-axis (FL1-A) and *y*-axis (FL2-A) represent annexin V and PI, respectively. (c) At 48 hr after echinacoside exposure, p21 and Bax expression were determined by the western blot analysis with GAPDH as a loading control. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with 0 μg/ml group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ as compared with the 20 μg/ml group; $+p < 0.05$, $+++p < 0.001$ as compared with 50 μg/ml group. All experiments shown were independently performed at least three times. CCK-8: cell counting Kit-8; ECH: echinacoside; FITC: fluorescein isothiocyanate; PI: propidium iodide [Color figure can be viewed at wileyonlinelibrary.com]

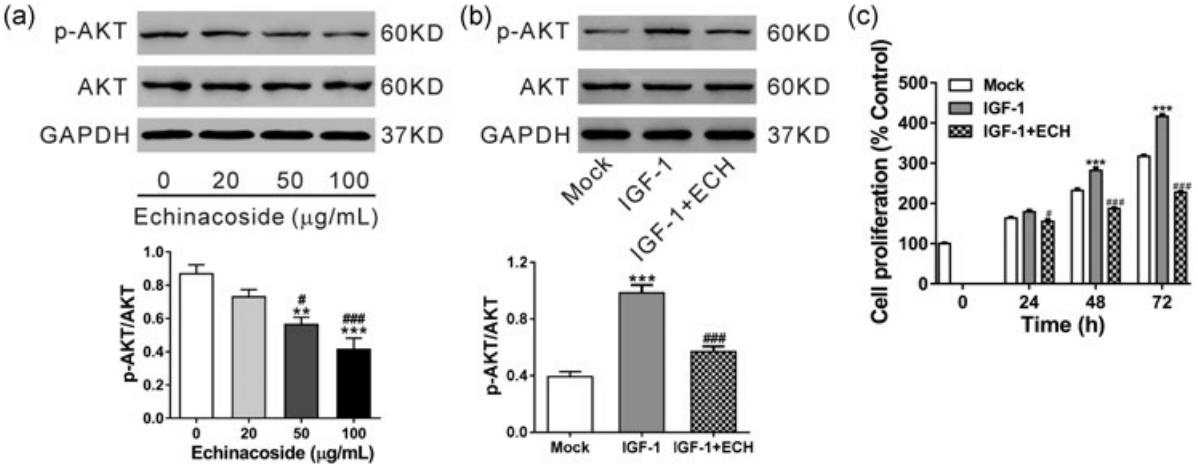

FIGURE 3 Effects of echinacoside on AKT activation. (a) HepG2 cells were exposed to various doses of echinacoside (0, 20, 50, and 100 μg/ml). After 48 hr, the western blot analysis was performed to assess the expression of p-AKT and AKT. $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with the 0 μg/ml group; $^{\#\#\#}p < 0.001$ as compared with 20 μg/ml group. (b,c) HepG2 cells were treated with 10 ng/ml IGF-1 with or without echinacoside (50 μg/ml). Cells without any treatment (Mock) were set as negative control. The western blot analysis (b) was performed to assess the expression of p-AKT and AKT at 48 hr after the treatment. The CCK-8 assay (c) was done to evaluate cell proliferation at 24, 48, and 72 hr after treatment. The OD_{450} was measured in cells before treatment and set as control OD_{450}. The relative cell proliferation was evaluated by normalizing the OD_{450} of each group to the control OD_{450}. $^{***}p < 0.001$ as compared with the Mock group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ as compared with the IGF-1 group. CCK-8: cell counting kit-8; ECH: echinacoside; IGF-1: insulin-like growth factor-1

(GSE51401). TREM2 expression was found significantly upregulated in HCC tissues in comparison to that in the normal liver tissues (Figure 4a). GSEA results showed that TREM2 expression was closely associated with the PI3K pathway (Figure 4b). In addition, the western blot analysis demonstrated that ECH exposure led to a notable reduction of TREM2 protein levels in HepG2 cells (Figure 4c) and liver tissues from DEN-treated mice (Figure 4d). From the above findings, we supposed that TREM2 may be a target of ECH in HCC.

3.5 | TREM2 expression affects HepG2 cell proliferation

In our previous study, we have shown that TREM2 knockdown inhibits HepG2 cell proliferation (S.-L. Zhang et al., 2016). Here, lentivirus expressing TREM2/control Vector or TREM2 shRNA/control shRNA were generated to transduce HepG2 cells. Western blot analysis demonstrated that lentivirus expressing TREM2 significantly increased the TREM2 expression, which was remarkably suppressed by lentivirus expressing TREM2 shRNA (Figure 5a). Not surprisingly, the CCK-8 assay indicated the growth-promoting and growth-inhibitory effects of TREM2 overexpression and knockdown in HepG2 cells, respectively (Figure 5b).

3.6 | Echinacoside inhibits HepG2 cell proliferation via TREM2

Next, to explore whether TREM2 was related to the growth-inhibitory function of ECH, HepG2 cells were treated with lentivirus expressing TREM2 and ECH (50 μg/ml). Figure 5c showed that ectopic expression of TREM2 could partially abolish the growth-suppression effects of ECH on HepG2 cells. We then detected the

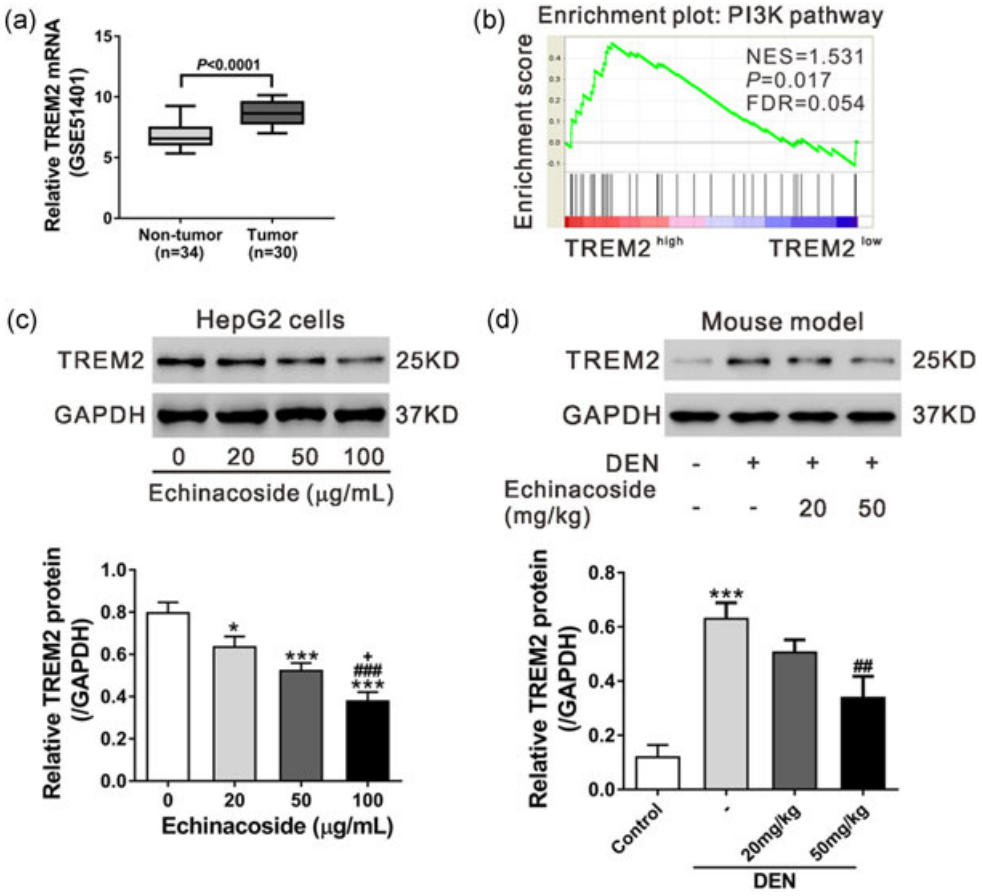

FIGURE 4 Effects of echinacoside on TREM2 expression. (a) TREM2 expression was compared between HCC tissues and normal tissues based on GSE51401 data set. (b) GSEA on GSE51401 revealed that the PI3K pathway was closely related to TREM2 expression. (c) HepG2 cells were treated with echinacoside (0, 20, 50, and 100 μg/ml) for 48 hr. The protein level of TREM2 was dose-dependently suppressed by echinacoside treatment. $^{*}p < 0.05$, $^{***}p < 0.001$ as compared with 0 μg/ml group; $^{\#\#\#}p < 0.001$ as compared with 20 μg/ml group; $+p < 0.05$ as compared with 50 μg/ml group. (d) HCC was induced in mice by DEN injection and then echinacoside (ECH, 20 mg/kg or 50 mg/kg) was administrated. After 4 weeks of echinacoside treatment, the protein level of TREM2 was determined by the western blot analysis. Mice without any treatment were set as the control group. $^{***}p < 0.001$ as compared with the control. $^{\#\#}p < 0.01$ as compared with 20 mg/kg group. DEN: diethylnitrosamine; FDR, false discovery rate; GSEA: gene set enrichment analysis; HCC: hepatocellular carcinoma; NES, normalized enrichment score; PI3K: phosphoinositide 3-kinase; TREM2: triggering receptors expressed on myeloid cells 2 [Color figure can be viewed at wileyonlinelibrary.com]

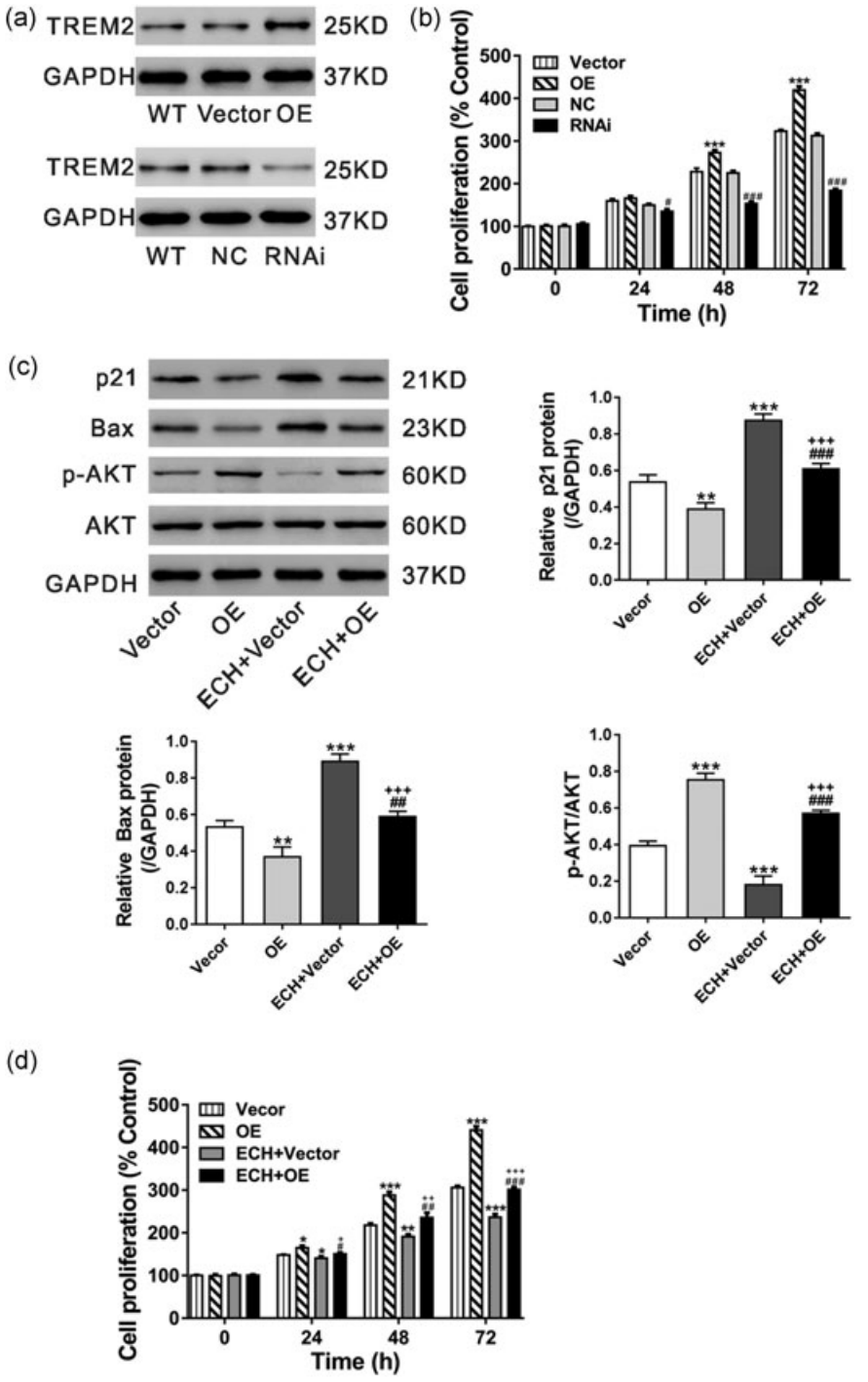

FIGURE 5 The involvement of TREM2 on the function of echinacoside. (a,b) HepG2 were transduced with TREM2 (OE)/vector expressing virus, or TREM2 shRNA (RNAi) or control shRNA (NC) virus. (a) The protein level of TREM2 was determined by the western blot analysis at 48 hr after treatment. The CCK-8 assay (b) was done to evaluate cell proliferation at indicated time points. The OD_{450} was measured in cells before treatment and set as control OD_{450}. The relative cell proliferation was evaluated by normalizing the OD_{450} of each group to the control OD_{450}. $^{***}p < 0.001$ as compared with vector. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ as compared with NC. (c,d) HepG2 were transduced with TREM2 (OE)/vector expressing virus with or without echinacoside treatment (50 μg/ml). The CCK-8 assay (c) was done to evaluate cell proliferation at indicated time points. Western blot analysis (d) was conducted at 48 hr after treatment. ECH: echinacoside; CCK-8: cell counting kit-8; NC: non-specific control; OE: overexpression; TREM2: triggering receptors expressed on myeloid cells 2

expression of p21, Bax, p-AKT, and AKT (Figure 5d). The expression of p21, Bax, and p-AKT that induced by ECH exposure was notably decreased by TREM2 overexpression. These data evidently indicated that TREM2 was involved in the antiproliferative function of ECH.

4 | DISCUSSION

Recently, TCM has gained more attention as an effective strategy for cancer prevention. It has been recognized that natural compounds from traditional Chinese herbs, such as Curcumin, Berberine, and Celastrol, have antitumor potential in HCC (Hu et al., 2013). A better understanding of the underlying mechanisms of how the natural products action will facilitate the development of efficient and safe anticancer medicines. In the current study, the anti-HCC activity of ECH, a natural compound, was investigated. In vivo protective and antiproliferative effects of ECH on HCC were demonstrated in mice model of HCC induced by DEN (Figure 1). Whereas previous studies have shown that ECH protected against acute liver injury induced by carbon tetrachloride and D-galactosamine plus lipopolysaccharide,

which may be associated with the antioxidative, antiapoptotic and anti-inflammatory activities of ECH (Li et al., 2014; Wu et al., 2007). The discrepancy may reflect differences in the acute and chronic injury caused by different toxicants. Moreover, in vitro experiments on HepG2 cells further confirmed that ECH exerted antiproliferative and proapoptotic functions on HCC cells (Figure 2), which was consistent with a previous report on SK-Hep-1 cells (Dong, Wang et al., 2015). These findings suggest that ECH may be a candidate drug for HCC therapy. In this study, the antitumor activity of ECH was elucidated in the HepG2 HCC cell line. Further studies using more HCC cell lines are required.

Recent studies have indicated that the PI3K/AKT signaling pathway plays critical roles in promoting tumorigenesis. Following activation of PI3K, AKT is phosphorylated and activated fully. p-AKT participates in the regulation of multiple cellular processes, including cell proliferation, apoptosis, and metastasis (S. Wang et al., 2015). p-AKT could directly phosphorylate p21 (a cell cycle inhibitor) at threonine 145, resulting in the cytoplasmic localization and degradation of p21 (Zhou et al., 2001). p-AKT could also downregulate the expression of Bax (a proapoptotic protein; Han et al., 2007). p-AKT has been suggested as a significant poor prognosis factor for HCC and the level of p-AKT was associated with the invasiveness of HCC (Nakanishi, Sakamoto, Yamasaki, Todo, & Hirohashi, 2005). Considering the previous report that ECH may function in bone marrow cells through activating the PI3K/AKT signaling pathway (S. Wang et al., 2015), we hypothesized that ECH could regulate p-AKT in HCC cells. As expected, ECH treatment in HepG2 cells dose-dependently reduced the level of p-AKT (Figure 3a) and enhanced the expression of p21 and Bax (Figure 2c), which was in concordance with the biological functional data (Figure 2a,b). Furthermore, ECH significantly suppressed IGF-1-induced p-AKT (Figure 3b) and cell proliferation (Figure 3c). These data suggest that the PI3K/AKT signaling pathway may participate in the molecular mechanisms of ECH-suppressed cell proliferation.

Previous research have implied the important role of TREM2 in the regulation of bone remodeling (Cella et al., 2003; Paloneva et al., 2003), immune responses (Sharif & Knapp, 2008; Turnbull et al., 2006), and cancer progression (H. Zhang et al., 2016; S.-L. Zhang et al., 2016; X.-Q. Wang et al., 2016). In this study, GSEA results revealed a positive correlation between the PI3K pathway and TREM2 expression in HCC tissues (Figure 4b), which prompted us to investigate the association between ECH, PI3K pathway, and TREM2. ECH exposure significantly decreased TREM2 protein levels in HepG2 cells (Figure 4c) and DEN-induced HCC (Figure 4d), indicating that TREM2 might be a target for ECH in HCC. In line with our previous study (S.-L. Zhang et al., 2016), TREM2 knockdown suppressed the proliferation of HepG2 cells, whereas ectopic expression of TREM2 had inverse effects (Figure 5b). Further, HepG2 cells were overexpressed with TREM2 and treated with ECH. The antiproliferative effect of ECH was notably attenuated by TREM2 overexpression (Figure 5c). Moreover, we found that TREM2 overexpression remarkably repressed ECH-mediated inactivation of AKT signaling (Figure 5d). These findings clearly indicated that ECH exerted its antitumor activity via decreasing TREM2 expression although further study should be performed to elucidate the detailed mechanism.

In conclusion, ECH exerted antiproliferative and proapoptotic functions on HepG2 cells via decreasing TREM2 expression and inactivating AKT pathway. This study suggested the potential clinical application of ECH in the therapy of HCC.

ORCID

Wei Xia http://orcid.org/0000-0002-1490-6060

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How to cite this article: Ye Y, Song Y, Zhuang J, Wang G, Ni J, Xia W. Anticancer effects of echinacoside in hepatocellular carcinoma mouse model and HepG2 cells. *J Cell Physiol*. 2018;1–9. https://doi.org/10.1002/jcp.27063