TIPE-2 suppresses growth and aggressiveness of hepatocellular carcinoma cells through downregulation of the phosphoinositide 3-kinase/AKT signaling pathway

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Abstract. Rapid proliferation and migration are the main features of hepatocellular carcinoma (HCC) cells, which serve an essential role in carcinogenesis and are a hallmark of cancer therapy resistance. Previous studies have reported that tumor necrosis factor-α-induced protein-8 like-2 (TIPE-2) is involved in cancer initiation and the progression of HCC. The present study aimed to clarify the role of TIPE-2 in HCC carcinogenesis, growth and aggressiveness. The effects of TIPE-2 on HCC were determined using colony forming and cell cycle analyses. Cell apoptosis, and growth and aggressiveness of HCC cells, were investigated following TIPE-2 treatment. Treatment with TIPE-2 markedly suppressed HCC cell proliferation and increased the number of cells in S phase of the cell cycle. The results demonstrated that TIPE-2 significantly inhibited growth, migration and invasion of HCC cells via the downregulation of tumor metastasis-associated genes. Flow cytometric analysis indicated that TIPE-2 promoted apoptosis of HCC cells via regulation of apoptosis-associated gene transcription. In addition, TIPE-2 administration downregulated the expression of phosphoinositide 3-kinase (PI3K) and protein kinase B (AKT) in HCC cells. In addition, TIPE-2 selectively decreased neuroblastoma Ras viral oncogene and p27 expression in HCC cells. *In vivo* assays revealed that TIPE-2 significantly inhibited tumor growth and prolonged animal survival by promoting apoptosis of tumor cells. The results of the present study indicated that TIPE-2 acts as an inhibitor of HCC cell growth and aggressiveness, and promotes apoptosis, thus suggesting that TIPE-2 may inhibit the metastasis-associated PI3K/AKT signaling cascade and may arrest the tumor cell cycle. These findings provide a potential molecular mechanism by which TIPE-2 promotes apoptosis of HCC cells.

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Introduction

Hepatocellular carcinoma (HCC) is the second most common type of cancer, which is characterized by high morbidity and mortality rates, and accounts for >90% of primary liver cancer cases (1,2). A previous systematic review and meta-analysis indicated that HCC is associated with a high level of recurrence and has the second highest cancer-associated mortality rate worldwide, even following radiotherapy, chemotherapy and/or surgery (3,4). At present, the available therapeutic strategies remain limited, particularly for patients with advanced HCC, which exhibit poor survival, according to a 5-year survival study (5-7). Previous studies have revealed that HCC is a genetically complex, multifactorial and heterogeneous tumor (8,9). Therefore, various anticancer therapies that target different signaling pathways have been investigated for HCC therapy (10,11).

The common clinical therapeutic strategies used to treat patients with cancer are currently surgery, chemotherapy and radiotherapy; however, efficacies remain poor and these treatments are associated with severe side effects during and after treatment (12). Numerous studies have focused on inhibition of the growth and aggressiveness of HCC, which may be associated with targeting proliferative signaling cascades (13,14). The anti-invasive and anti-migratory effects of drugs contribute to limit regional migration and long-distance tumor metastasis of HCC cells by targeting cellular signaling pathways, which are involved in key signal transduction for various extracellular growth factors, and receptors of HCC cells (15,16). In addition, the induction of human HCC cell apoptosis has been reported to stimulate anticancer activity via mitochondria-mediated activation of caspase-9 and caspase-8-mediated proteolysis of BH3 interacting-domain death agonist, which may warrant further investigation for application in cancer treatment (17,18).

Tumor necrosis factor (TNF)-α-induced protein 8-like-2 (TIPE-2) is a negative regulator of innate and adaptive immunity (19,20). TIPE-2 is downregulated in the majority of human cancer cells, including lung cancer, colon cancer, HCC and gastric cancer cells. Recently, Li *et al* (21) reported that TIPE-2 is a novel inflammatory regulator that may inhibit Toll-like receptor 4 (TLR4)-mediated development of colon

cancer via TLR4-mediated upregulation of caspase-8; this may be considered a novel therapeutic target for clinical treatment. Zhao *et al* (22) also indicated that TIPE-2 is associated with the pathogenesis of gastric cancer and acts as a novel negative regulator of the immune system, which has been systematically investigated in murine and human cancer. Furthermore, a previous study demonstrated that regulating T-cell apoptosis by directly targeting the tumor suppressor gene TIPE-2 enhances the apoptotic sensitivity of tumor cells (23).

In the present study, TIPE-2-mediated phosphoinositide 3-kinase (PI3K)/protein kinas B (AKT) signaling was investigated in HCC cells. In addition, the inhibitory effects of TIPE-2 were analyzed on HCC cells; the results demonstrated that treatment with TIPE-2 significantly suppressed the growth and proliferation of HCC cells *in vitro*, and inhibited tumor formation *in vivo*. These findings suggested that TIPE-2 treatment may markedly inhibit tumor growth in HepG2-bearing mice, which further prolonged survival time for experimental mice. Mechanistic analysis indicated that TIPE-2 may induce apoptosis of HCC cells via the PI3K/AKT signaling pathway, which provides a potential therapeutic target for patients with HCC.

Materials and methods

Ethics statement. The present study was conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of Tianjin Medical University General Hospital (Tianjin, China). This study was approved by the Ethics Committee of Tianjin Medical University General Hospital. All surgery and euthanasia were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Cell culture. HepG2 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in an atmosphere containing 5% CO_2.

Endogenous overexpression of PI3K or TIPE-2. HepG2 cells were cultured in 6-well plates until they reached 90% confluence and the medium was then removed. Sequences were cloned into vectors as previously described (24). Subsequently, HepG2 cells ($1x10^6$) were transfected with pedue12.4-PI3K (100 nM), pedue12.4-TIPE-2 (100 nM) or pedue12.4-vector (100 nM) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h at 37˚C. Cells that exhibited stable PI3K or TIPE-2 overexpression were selected according to the dihydrofolate reductase/glutamine synthetase screening system (Invitrogen; Thermo Fisher Scientific, Inc.).

MTT cytotoxicity assays. HepG2 cells ($5x10^3$) were incubated with TIPE-2 (0.5-2.5 mg/ml; SinoGenoMax Co., Ltd., Beijing, China) in 96-well plates for 24, 48 and 72 h at 37˚C in triplicate for each condition. In the control group, cells were treated with 2 ml PBS instead of TIPE-2. Cell proliferation was determined using a MTT assay kit (Roche Diagnostics, Indianapolis, IN, USA). At each time point, 20 μl MTT (5 mg/ml) in PBS was added to each well and the plate was incubated for 4 h at 37˚C. Most of the medium was then removed and 100 μl dimethyl sulfoxide was added to the wells to solubilize the crystals. Optical density was measured using a Bio-Rad ELISA reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a wavelength of 450 nm.

Colony formation assay. Culture medium supplemented with TIPE-2 (2 mg/ml) or with PBS was added to 6-well plates (2 ml per well). HepG2 cells ($1x10^5$ cells/well) were seeded into 6-well plates. Medium was replaced at 48 h following cell attachment and subsequently every 48 h. Cells were trypsinized (1% trypsin), suspended in a 500 μl volume of medium and a hemacytometer was used to determine the total number of cells per well on day 4. The colony formation assays were performed in triplicate.

mRNA expression analysis by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total cellular RNA was extracted from PI3K-overexpressed (PI3KOR), TIPE-2-overexpressed (TIPE-2OR) or control HepG2 cells using the RNeasy mini kit (Qiagen Sciences, Inc., Gaithersburg, MD, USA) according to the manufacturer's protocol. A total of 1 μg total RNA was reverse transcribed into cDNA for 2 h at 42˚C. cDNA (0.1 μg) was subjected to qPCR using an iQ SYBR-Green system (Bio-Rad Laboratories, Inc.). All primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.) and the sequences used were as follows: PI3K forward, 5'-CTCATGCCAGGCACTGTGCTA-3' and reverse, 5'-GAATCAAGGCACACCTGTGGAA-3'; TIPE-2 forward, 5'-GGAACATCCAAGGCAAGACTG-3' and reverse, 5'-AGCACCTCACTGCTTGTCTCATC-3'; and β-actin forward, 5'-GACTACCTCATGAAGATCCTCACC-3' and reverse, 5'-TCTCCTTAATGTCACGCACGATT-3'. PCR was performed with the following thermocycling conditions: 45 amplification cycles of 96˚C for 30 sec, annealing at 63˚C for 20 sec, with touchdown to 54˚C for 20 sec and extension at 72˚C for 5 min. Results were expressed as a fold change by comparing levels of target mRNA expression to that of the reference gene using the $2^{-\Delta\Delta Cq}$ method (25).

Western blot analysis. HepG2 cells were treated with TIPE-2 (2.0 mg/ml) for 24 h and were then homogenized with lysis buffer containing protease-inhibitor (cat. no. P3480; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Protein concentration was measured with a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.), protein (10 μg/lane) was separated by 12.5% SDS-PAGE and subsequently transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% milk for 2 h at 37˚C prior to incubation with the following primary antibodies overnight at 4˚C: PI3K (1:1,000; cat. no. ab191606), AKT (1:1,000; cat. no. ab8805), neuroblastoma Ras viral oncogene (N-ras; 1:1,000; cat. no. ab206969), P27 (1:1,000; cat. no. ab191606), phosphorylated (p)AKT (1:1,000; cat. no. ab81283), binding immunoglobulin protein (BIP; 1:1,000; cat. no. ab108615), P53 (1:1,000; cat. no. ab1431), B-cell lymphoma 2 (Bcl-2; 1:1,000; cat. no. ab59348), cyclin D1 (1:1,000; cat. no. ab134175), cyclin-dependent kinase (CDK)1 (1:1,000; cat. no. ab131450), CDK2 (1:1,000; cat. no. ab32147), vimentin (VIM;

1:1,000; cat. no. ab92547), collagen type I (CT-I; 1:1,000; cat. no. ab34710), Slug (1:1,000; cat. no. ab27568), c-Jun N-terminal kinase (JNK; 1:1,000; cat. no. ab124956), nuclear factor (NF)-κB (1:1,000; cat. no. ab28849), nuclear factor (erythroid-derived 2)-like 2 (NRF2) (1:1,000; cat. no. ab62352), caspase-3 (1:1,000; cat. no. ab13847), caspase-8 (1:1,000; cat. no. ab25901), glucose-regulated protein 78 (GRP78; 1:1,000; cat. no. ab21685), CCAAT-enhancer-binding protein homologous protein (CHOP; 1:1,000; cat. no. ab179823), eukaryotic initiation factor 2 (eIF2α; 1:1,000; cat. no. ab32713), peIF2α (1:1,000; cat. no. ab214434) and β-actin (1:1,000; cat. no. ab827; Abcam, Shanghai, China) for 12 h at 4˚C. Membranes were then incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated immunoglobulin G secondary antibody (1:2,000; cat. no. PV-6001; OriGene Technologies, Inc., Beijing, China) for 24 h at 4˚C. The blots were visualized using a chemiluminescence detection system (GE Healthcare, Chicago, IL, USA). Relative protein expression levels were calculated using Quantity-One software (version 3.0; Bio-Rad Laboratories, Inc.) and are presented as n-fold of β-actin expression levels.

Cell invasion and migration assays. HepG2 cells were treated with TIPE-2 (2.0 mg/ml) for 24 h; untreated cells were used as a control. Migration and invasion assays of HepG2 cells were conducted in 6-well culture plates with chamber inserts (BD Biosciences, San Diego, CA, USA). For migration assays, $1x10^6$/well HepG2 cells were placed into the upper chamber with DMEM medium. The lower chamber contained DMEM medium with 0.1% FBS. For invasion assays, cells ($1x10^6$/well) were placed into the upper chamber with a Matrigel-coated insert. After 24 h culture at 37˚C, migratory and invasive cells were fixed with 1.4% paraformaldehyde for 30 min at 37˚C and were stained for 30 min in 0.1% crystal violet solution in PBS. Migration and invasion of HepG2 cells was counted in at least three randomly selected fields per membrane using a light microscope (Olympus BX51; Olympus Corporation, Tokyo, Japan).

Flow cytometric analysis. HepG2 cells were cultured until they reached 85% confluence. Apoptotic rate was assessed following incubation with TIPE-2 (2.0 mg/ml) for 48 h. Briefly, HepG2 cells were trypsinized and collected, the cells were then washed in cold PBS, adjusted to $1x10^8$ cells/ml with PBS, and labeled with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI; Annexin V-FITC kit; BD Biosciences) for 2 h at 4˚C. Apoptotic rate was analyzed using FCS Express™ IVD software (version 4; De Novo Software, Glendale, CA, USA).

Viability assay. HepG2 cells were cultured in 6-well plates until they reached 85% confluence and the medium was then removed. Subsequently, HepG2 cells were washed three times and were incubated with DMEM supplemented with 10% FBS and TIPE-2 (2.0 mg/ml) for 24 h. Subsequently, the supernatant was removed and cells were incubated with Triton X-100 (1%) for 30 min. Lactate dehydrogenase activity in cell lysates was used to analyze cell viability using the Promega CytoTox 96 assay kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol.

Analysis of the cell cycle. To analyze the effects of TIPE-2 (2 mg/ml) on the HepG2 cell cycle, flow cytometry was performed. HepG2 cells in the exponential phase of growth were treated with TIPE-2 (2 mg/ml) for 48 h at 37˚C. Cells were subsequently washed with PBS, trypsinized (1% trypsin) for 5 min at 37˚C and rinsed with PBS. Cells were fixed in 75% ice-cold ethanol for 15 min and washed with PBS three times. RNaseA (20 μg ml/l; Fermentas; Thermo Fisher Scientific, Inc.) was added to fixed cells, which were subsequently stained with PI (20 μg ml/l; Sigma-Aldrich; Merck KGaA) for 10 min at 37˚C. The percentage of cells in the G1, G2 and S phase were analyzed using a cell cycle analysis kit (cat. no. C34568; Invitrogen; Thermo Fisher Scientific, Inc.), a BD FACSCalibur flow cytometer (BD Biosciences) and FCS Express IVD software (version 4; De Novo Software).

Animal experiments. A total of 80 specific pathogen-free (SPF) female BALB/c nude mice (age, 6-8 weeks) were purchased from Harbin Veterinary Research Institute (Harbin, China). All mice were feed under pathogen-free conditions. Mice were maintained at 22-24˚C in a 12 h light/dark cycle with *ad libitum* access to food and water. A total of $5x10^7$ HepG2 cells were injected into the right flank of female BALB/c nude mice at a total volume of 200 μl. Tumor-bearing mice then underwent intratumoral injection with TIPE-2 (6.0 mg/ml) or PBS (n=40/group), once tumor diameters reached 5-8 mm on day 6 after tumor inoculation. The treatment was continued 15 times at intervals of every two days for a total of 30 days. Tumor diameters were recorded once every 2 days and tumor volume was calculated using the following formula: 0.52 x smallest diameter^2 x largest diameter. Survival analysis was conducted over 120 days to analyze the therapeutic effects of TIPE-2 in tumor-bearing mice.

Immunohistochemistry. Immunohistochemical staining was performed according to the avidin-biotin-peroxidase technique. HCC tissues were isolated from experimental mice and paraffin-embedded tissue sections (4 μm) were prepared and epitope retrieval was performed by heating the tissue sections at 100˚C for 30 min in a citrate solution (10 mmol/l; pH 6.0) followed by dewaxing in xylene and rehydrating in a graded ethanol series for further analysis. Subsequently, paraffin-embedded sections were treated with hydrogen peroxide (3%) for 10-15 min and were blocked in 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 10-15 min at 37˚C. Finally, the sections were incubated with biotinylated goat anti-mouse caspase-3 (1:1,000; cat. no. ab13847), caspase-9 (1:1,000; cat. no. ab32539), PI3K (1:1,000; cat. no. ab191606), AKT (1:1,000; cat. no. ab8805), GRP78 (1:1,000; cat. no. ab21685) and CHOP (1:1,000; cat. no. ab179823) antibodies (Abcam) at 4˚C for 12 h. Samples were washed three times with PBS and then incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:2,000, cat. no. PV-6001; OriGene Technologies, Inc.) for 2 h at 37˚C. 3,3'-diaminobenzidene (0.05%) was used as the chromogen for 30 min at 37˚C and 1% hematoxylin as the nuclear counterstain for 30 min at 37˚C. The relative protein expression levels were analyzed using a chemiluminescence detection system (GE Healthcare). Tumor tissue images were captured with a ZEISS LSM 510 confocal microscope (magnification, x40;

Zeiss AG, Oberkochen, Germany). Relative protein expression levels were determined using Quantity-One software 3.0 (Bio-Rad Laboratories, Inc.) and are presented as the n-fold of β-actin expression levels.

Immunocytochemistry. HepG2 cells were treated with TIPE-2 (2 mg/ml) for 12 h at 37˚C. Following this, cells were washed with PBS at room temperature and fixed with 4% paraformaldehyde for 1 h at 37˚C. The cells were washed again with PBS three times, blocked with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 2 h at 37˚C and subsequently stained with the following antibodies for 12 h at 4˚C: Ki67 (1:1,000; cat. no. ab15580; Abcam), PCNA (1:1,000; cat. no. ab18197; Abcam), E-cadherin (1:1,000; cat. no. ab40772; Abcam) and fibronectin (1:1,000; cat. no. ab2413; Abcam). Cells were washed with PBS and subsequently incubated with an Oregon Green® 488-conjugated IgG (1:1,000; cat. no. O-6382; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h in a light-protected chamber at 37˚C. Immunofluorescence signals were detected using a laser scanning confocal microscope (magnification, x40; Zeiss AG).

Statistical analysis. All data are presented as the mean ± standard error of the mean of triplicate experiments. SPSS 19.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Unpaired data were analyzed by Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Analysis of the inhibitory effects of TIPE-2 on HCC cell growth and proliferation in vitro. As shown in Fig. 1A and B, TIPE-2 administration (0.5, 1, 1.5, 2.0 and 2.5 mg/ml) significantly inhibited HepG2 cell growth in a dose- and time-dependent manner. The results indicated that 2.0 mg/ml TIPE-2 markedly suppressed HCC cell proliferation and increased the number of cells in S phase of the cell cycle (Fig. 1C and D). Western blot analysis demonstrated that TIPE-2 administration (2.0 mg/ml) decreased the expression levels of cyclin D1, CDK1 and CDK2 in HepG2 cells (Fig. 1E). Immunofluorescence indicated that TIPE-2 administration (2.0 mg/ml) decreased the expression levels of Ki67 and proliferating cell nuclear antigen in HepG2 cells (Fig. 1F). These results suggested that TIPE-2 may significantly inhibit HCC cell growth and proliferation *in vitro*.

Analysis of the inhibitory effects of TIPE-2 on HCC cell migration and invasion in vitro. Local migration and long-distance invasion are the most common characteristics of HCC. The present study investigated migration and invasion of HepG2 cells following treatment with TIPE-2 (2.0 mg/ml). Migration and invasion assays demonstrated that TIPE-2 significantly suppressed HepG2 cell migration and invasion *in vitro* (Fig. 2A and B). Western blot analysis demonstrated that TIPE-2 treatment decreased the expression levels of VIM, CT-I and Slug in HepG2 cells (Fig. 2C). In addition, TIPE-2 decreased JNK and NF-κB expression in HepG2 cells (Fig. 2D). Immunofluorescence demonstrated that E-cadherin/DNA and fibronectin/DNA expression levels were decreased in HepG2 cells following TIPE-2 treatment (Fig. 2E). HepG2 cell viability was also decreased by TIPE-2 treatment (Fig. 2F). These results indicated that TIPE-2 may significantly inhibit migration and invasion of HepG2 cells via inhibiting the expression levels of cancer cell migration-associated proteins.

Analysis of the effects of TIPE-2 on HCC cell apoptosis in vitro. As shown in Fig. 3A, TIPE-2 administration (2.0 mg/ml) promoted apoptosis of HepG2 cells after 48 h. Western blotting demonstrated that the expression levels of caspase-3 and caspase-8 were increased by TIPE-2 administration (Fig. 3B). In addition, TIPE-2 administration downregulated P53 and Bcl-2 expression levels in HepG2 cells (Fig. 3C). Results also indicated that the expression and levels of eIF2α and peIF2α were upregulated in HepG2 cells following TIPE-2 treatment (Fig. 3D). Furthermore, the expression levels of NRF2 and BIP were increased in HepG2 cells following TIPE-2 treatment (Fig. 3E). The expression levels of GRP78 and CHOP were also increased in HepG2 cells following TIPE-2 treatment (Fig. 3F). These results suggested that TIPE-2 may promote HCC apoptosis *in vitro*.

TIPE-2 regulates HCC cell apoptosis via regulation of the PI3K/AKT signaling pathway. As shown in Fig. 4A, the present study demonstrated that TIPE-2 significantly inhibited PI3K and AKT expression in HepG2 cells. In addition, N-ras and P27 expression were decreased by TIPE-2 treatment in HepG2 cells (Fig. 4B). pAKT levels were also decreased by TIPE-2 treatment in HepG2 cells when compared with pAKT levels in the control group (Fig. 4C). We observed that TIPE-2 decreased ratio of pAkt:Akt in HepG2 cells (Fig. 4D). As shown in Fig. 4E and F, pedue12.4-PI3K or pedue12.4-TIPE-2 upregulated PI3K and TIPE-2 mRNA expression, respectively, compared with pedue12.4-vector in HepG2 cells. Mechanistic analysis indicated that PI3K overexpression alone (PI3KOR) inhibited the apoptosis of HepG2 cells, whereas TIPE-2 treatment abolished this effect (Fig. 4G). In addition, N-ras and BIP expression was increased by PI3KOR in HepG2 cells (Fig. 4H). In addition, PI3KOR increased the expression levels of P53 and Bcl-2 in HepG2 cells when compared to the control group (Fig. 4I). These results suggested that TIPE-2 may regulate HCC cell apoptosis through regulation of the PI3K/AKT signaling pathway.

In vivo anticancer effects of TIPE-2 treatment on HepG2-bearing mice. The present study further investigated the antitumor effects of TIPE-2 treatment on HepG2-bearing nude mice. In order to avoid the interference of pathogenic microorganisms, SPF female BALB/c mice (body weight, 30-35 g) were implanted with HepG2 cells and treated with TIPE-2. As shown in Fig. 5A, TIPE-2 treatment (6 mg/kg) inhibited tumor growth during the 30-day observation period compared with in the control group. TUNEL assay demonstrated that TIPE-2 treatment increased the number of apoptotic cells in tumor tissues compared with in the PBS group (Fig. 5B). In addition, TIPE-2 treatment increased the expression levels of caspase-3 and caspase-8 (Fig. 5C), and upregulated PI3K and AKT in tumor tissues (Fig. 5D). Furthermore, TIPE-2 treatment increased GRP78 and CHOP expression in tumor tissues (Fig. 5E). Notably, survival of HepG2-bearing mice was prolonged by TIPE-2 treatment during the 120-day experiment (Fig. 5F). These results suggested that TIPE-2 may be a potential anticancer agent for the treatment of HCC.

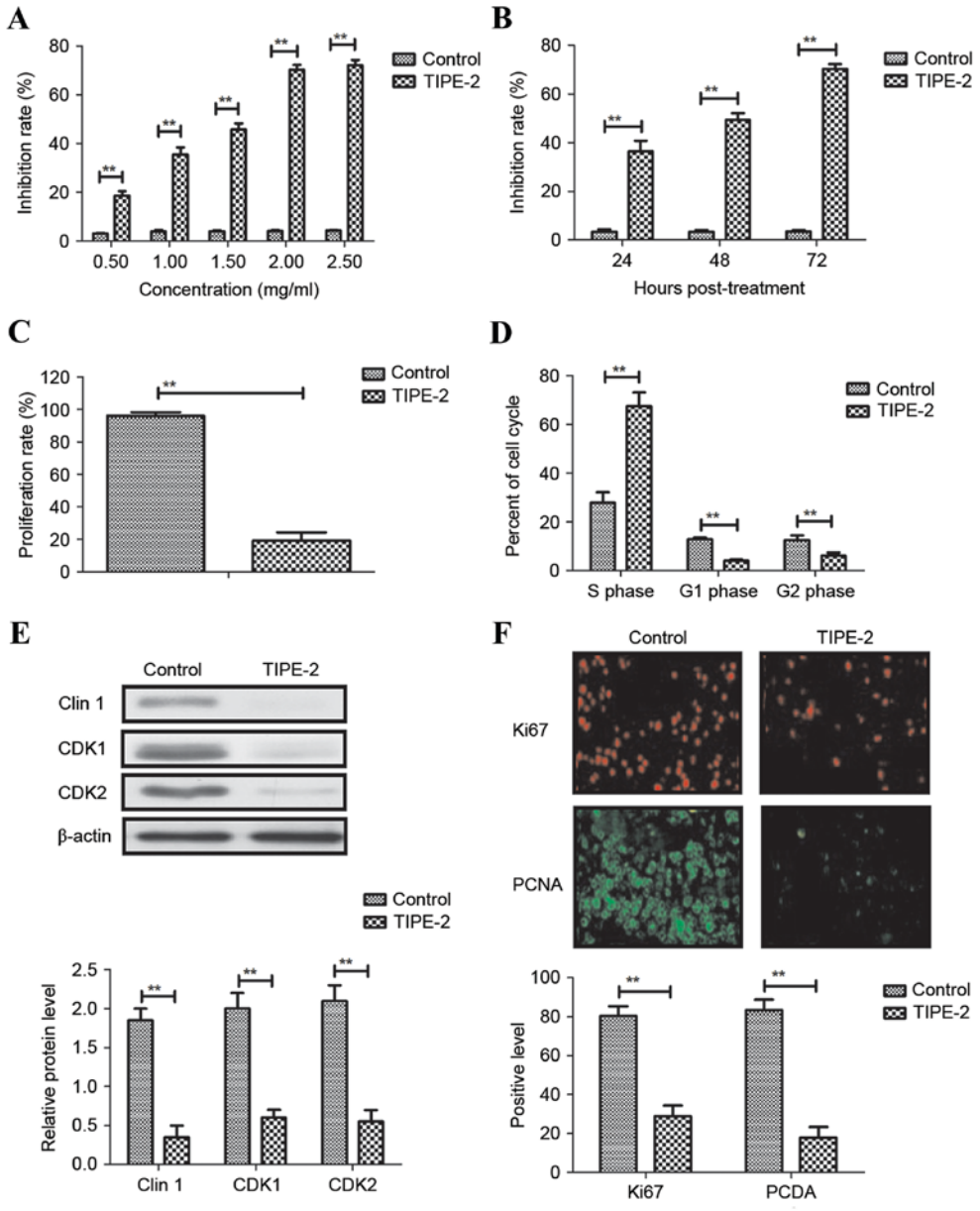

Figure 1. Inhibitory effects of TIPE-2 on HCC cell growth and proliferation *in vitro*. (A) TIPE-2 inhibited HepG2 cell growth in a dose- and (B) time-dependent manner. (C) TIPE-2 treatment suppressed HCC cell proliferation and (D) arrested HCC cells at S phase of the cell cycle. (E) Effects of TIPE-2 on the expression levels of cyclin D1 CDK1 and CDK2 in HepG2 cells. (F) Effects of TIPE-2 on the expression levels of Ki67 and PCNA in HepG2 cells, as determined by immunofluorescence. Magnification, x40. **P<0.01 vs. control group. CDK1, cyclin-dependent kinase; HCC, hepatocellular carcinoma; PCNA, proliferating cell nuclear antigen; TIPE-2, tumor necrosis factor-α-induced protein-8 like-2.

Discussion

Advanced stage HCC possesses aggressive potential for migration to adjacent and distant cells, and/or organs (26,27). Clinical therapies are required that inhibit the migration and invasion of HCC cells, in order to prolong the survival of patients with HCC (28,29). It has previously been suggested that TIPE-2 serves an important role in tumorigenesis, and tumor growth, proliferation, aggressiveness and apoptosis. Although a previous study indicated the relevance of targeting the tumor suppressor gene TIPE-2, the molecular mechanism underlying TIPE-2-mediated apoptosis remains to be fully elucidated (30). The present study evaluated the inhibitory effects of TIPE-2 on growth, aggressiveness and apoptosis of HCC cells *in vitro*, as well as the underlying mechanism of TIPE-2-mediated apoptosis of HepG2 cells. The results demonstrated that TIPE-2 administration significantly inhibited HCC cell growth, proliferation and aggressiveness. In addition, TIPE-2 administration promoted HCC cell apoptosis through regulation of the PI3K/AKT signaling pathway. Notably, the present findings suggested that TIPE-2 may be a potential anticancer agent for the treatment of HCC, as it was able to inhibit tumor growth and prolong survival. However, it must be noted that the HepG2 cell line is potentially misidentified, and may be derived from hepatoblastoma, rather than HCC (31). Therefore, the present study may identify the inhibitory effects of TIPE-2 on hepatic cancer cells in general, rather than on HCC cells specifically.

Induction of apoptosis and tumor cell death, thus resulting in the inhibition of growth and aggressiveness in patients, is the

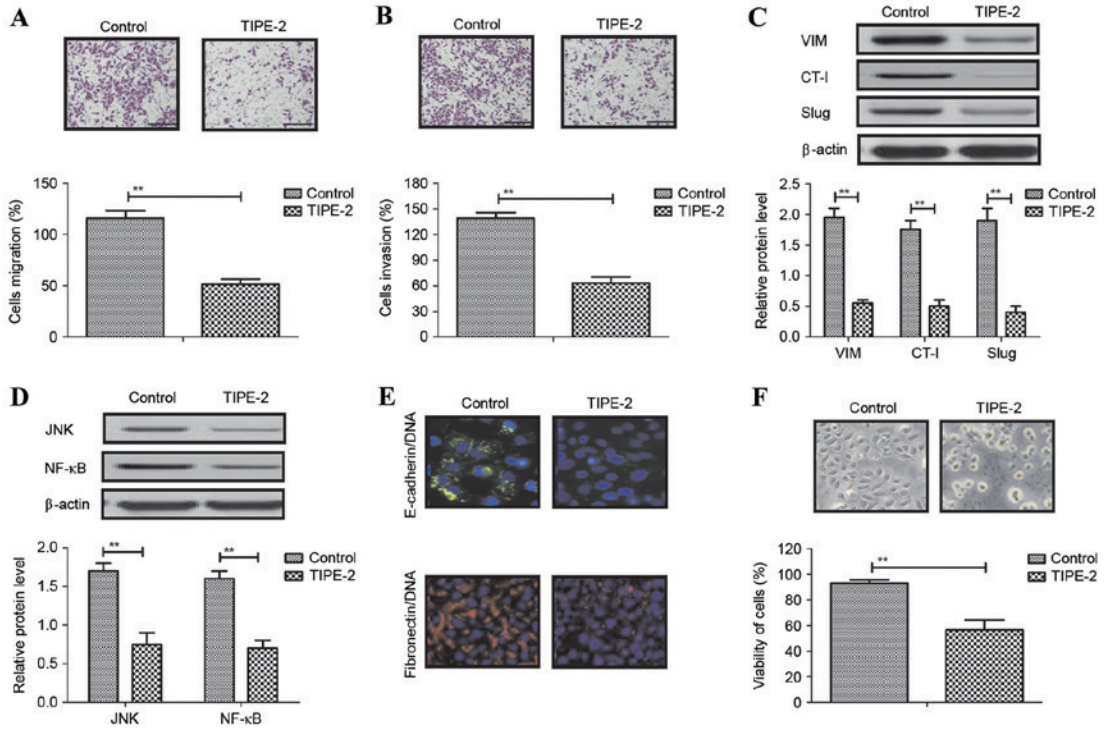

Figure 2. Inhibitory effects of TIPE-2 (2.0 mg/ml) on hepatocellular carcinoma cell migration and invasion *in vitro*. Effects of TIPE-2 on (A) migration and (B) invasion of HepG2 cells. Effects of TIPE-2 on the expression levels of (C) VIM, CT-I and Slug, and (D) JNK and NF-κB in HepG2 cells. (E) Immunofluorescence analysis of E-cadherin/DNA and fibronectin/DNA expression levels following TIPE-2 treatment in HepG2 cells. (F) Effects of TIPE-2 on viability of HepG2 cells. Magnification, x40. **P<0.01 vs. control group. CT-I, collagen type I; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-κB; TIPE-2, tumor necrosis factor-α-induced protein-8 like-2; VIM, vimentin.

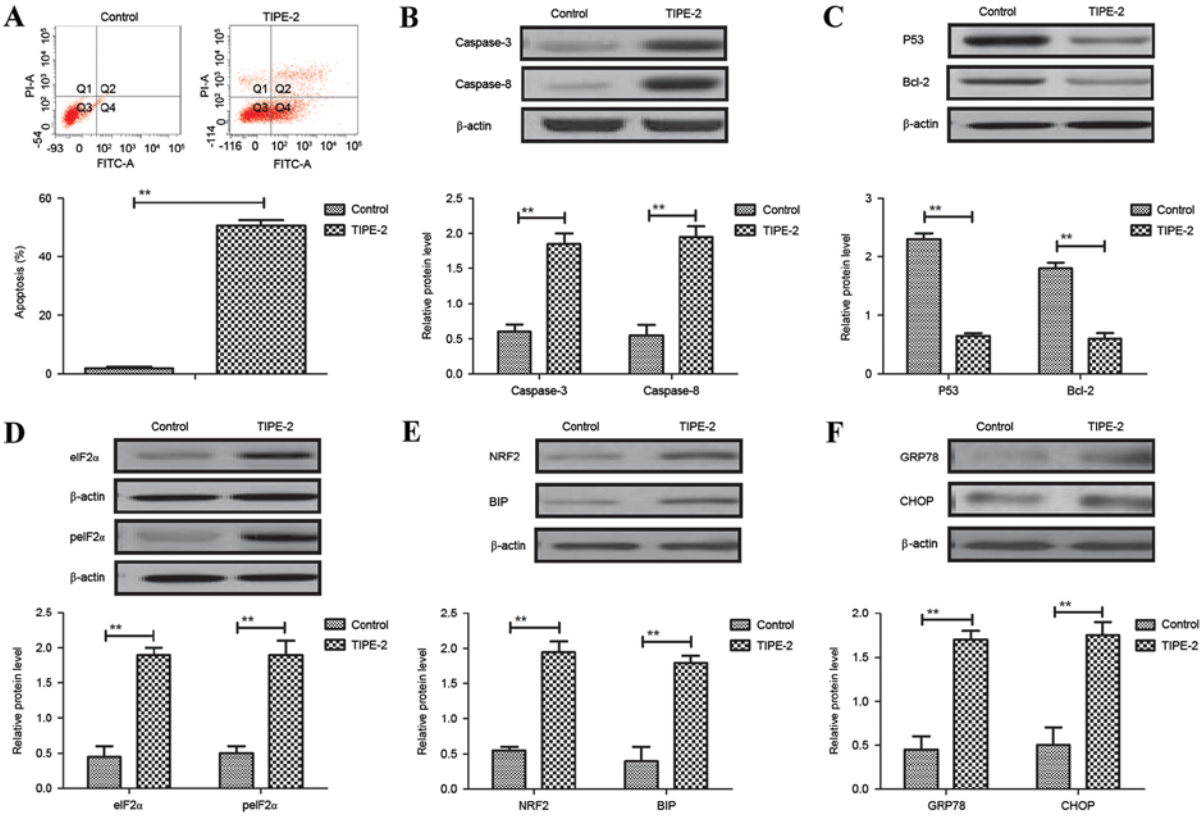

Figure 3. Effects of TIPE-2 (2.0 mg/ml) on hepatocellular carcinoma cell apoptosis *in vitro*. (A) Effects of TIPE-2 on apoptosis of HepG2 cells after 48 h, as determined by flow cytometry. Effects of TIPE-2 treatment on the expression levels of (B) caspase-3 and caspase-8, (C) P53 and Bcl-2, (D) peIF2α and eIF2α, (E) NRF2 and BIP, and (F) GRP78 and CHOP in HepG2 cells, as determined by western blotting. **P<0.01 vs. control group. Bcl-2, B-cell lymphoma 2; BIP, binding immunoglobulin protein; CHOP, CCAAT-enhancer-binding protein homologous protein; eIF2α, eukaryotic initiation factor; FITC, fluorescein isothiocyanate; GRP78, glucose-regulated protein 78; peIF2α, phosphorylated-eIF2α; PI, propidium iodide; TIPE-2, tumor necrosis factor-α-induced protein-8 like-2.

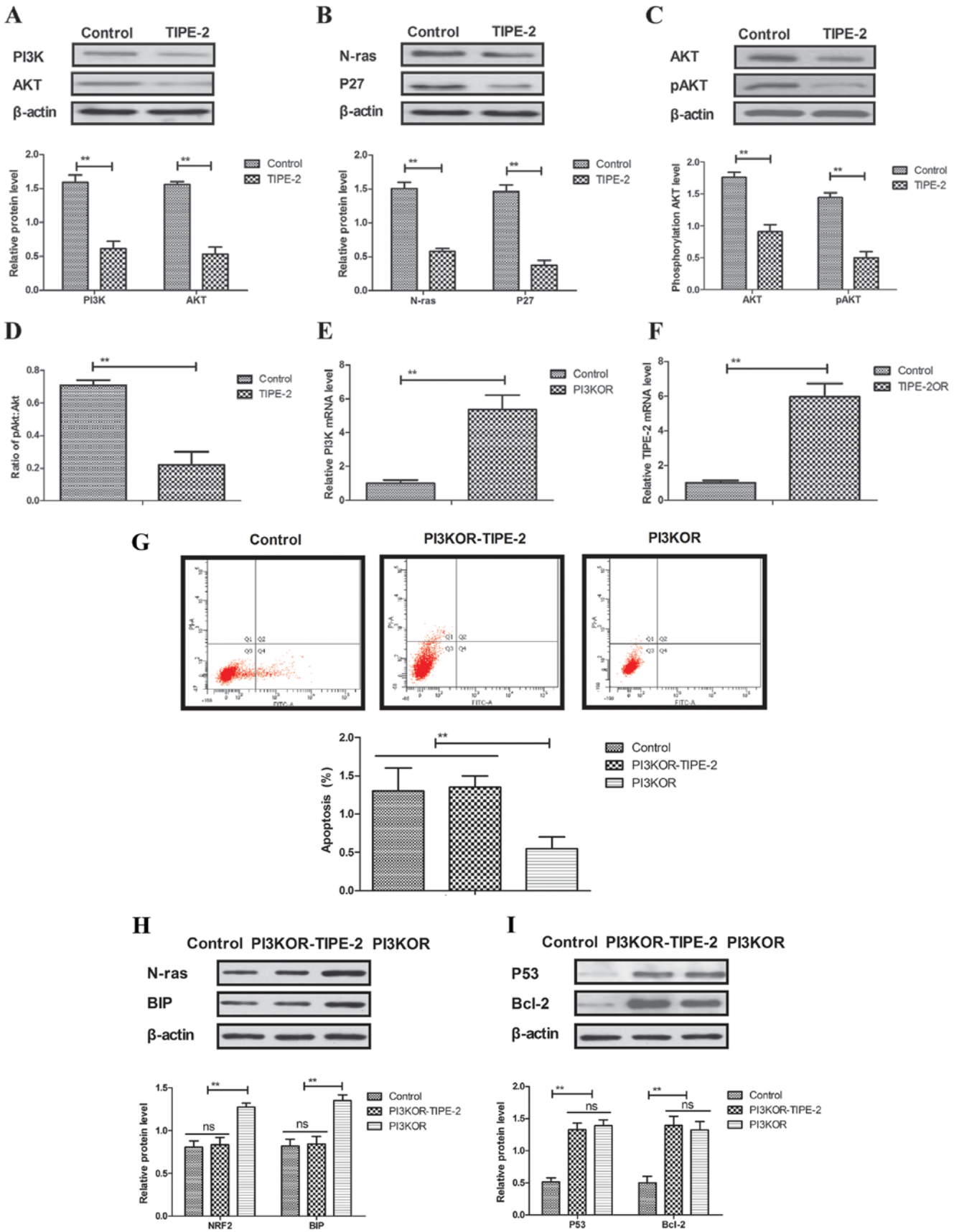

Figure 4. TIPE-2 regulates hepatocellular carcinoma cell apoptosis through regulation of the PI3K/AKT signaling pathway. Effects of TIPE-2 treatment on the expression levels of (A) PI3K and AKT, (B) N-ras and P27, and (C) pAKT in HepG2 cells. (D) TIPE-2 decreased the ratio of pAkt:Akt in HepG2 cells. Transfection with (E) pedue12.4-PI3K or (F) TIPE-2OR upregulates PI3K or TIPE-2 mRNA expression in HepG2 cells, respectively. **P<0.01 vs. control group. (G) PI3KOR prevented the apoptosis promoted by TIPE-2 treatment in HepG2 cells **P<0.01, PI3KOR-TIPE-2 vs. PI3KOR or control group. Effects of PI3KOR on (H) N-ras and BIP, and (I) P53 and Bcl-2 expression in HepG2 cells. **P<0.01, PI3KOR-TIPE-2 or PI3KOR vs. control group. AKT, protein kinase B; Bcl-2, B-cell lymphoma 2; FITC, fluorescein isothiocyanate; N-ras, neuroblastoma Ras viral oncogene; OR, overexpression; pAKT, phosphorylated-AKT; PI, propidium iodide; PI3K, phosphoinositide 3-kinase; TIPE-2, tumor necrosis factor-α-induced protein-8 like-2.

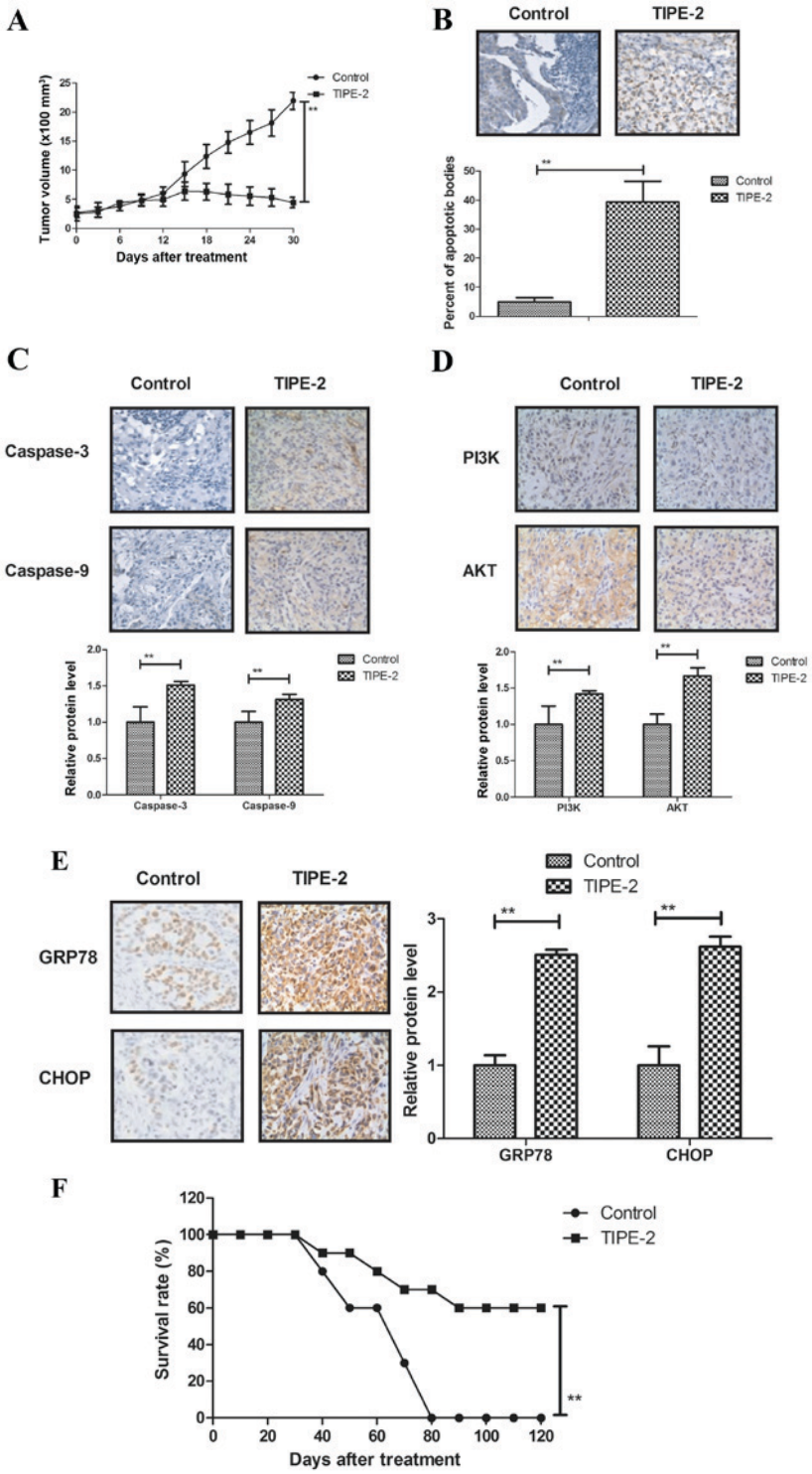

Figure 5. *In vivo* anticancer effects of TIPE-2 (6.0 mg/kg) treatment on hepatocellular carcinoma-bearing mice. (A) Antitumor effects of TIPE-2 treatment on HepG2-bearing nude mice. (B) TIPE-2 treatment promoted apoptosis of cells within tumor tissues. (C) TIPE-2 treatment increased the expression levels of caspase-3 and caspase-8 in tumor tissues. Effects of TIPE-2 treatment on the expression levels of (D) PI3K and AKT, and (E) GRP78 and CHOP in tumor tissues. Magnification, x40. (F) Survival rate of HepG2-bearing mice following treatment with TIPE-2 or PBS. **P<0.01 vs. control group. AKT, protein kinase B; CHOP, CCAAT-enhancer-binding protein homologous protein; GRP78, glucose-regulated protein 78; PI3K, phosphoinositide 3-kinase; TIPE-2, tumor necrosis factor-α-induced protein-8 like-2.

ultimate goal in neoplastic therapy (32). TIPE-2 is able to inhibit TLR4-mediated development of colon cancer via the down-regulation of caspase-8 activity. In the present study, TIPE-2 increased caspase-8 expression in HCC cells, thus promoting apoptosis of HepG2 cells. A previous study demonstrated that TIPE-2 may inhibit TNF-α-mediated HCC cell metastasis through the downregulation of extracellular signal-regulated kinase and the NF-κB signaling pathway (33). In addition, it has previously been indicated that regulation of P53-, Bcl-2- and caspase-dependent signaling pathways in drug-induced apoptosis of HepG2 cells contributes to anti-proliferative effects (34). Notably, enhancement of P53 protein nuclear

export may inhibit cisplatin-induced HepG2 cell apoptosis, which may hinder anticancer drug-induced HepG2 cell apoptosis (35). The present study demonstrated that TIPE-2 not only downregulated the expression of anti-apoptotic proteins (P53 and Bcl-2), but also indicated that TIPE-2 regulated apoptosis of HCC cells via the PI3K/AKT signaling pathway. In addition, another study revealed that bortezomib may arrest the proliferation of HepG2 HCC cells by increasing P27 (kip1) (36). Furthermore, Lin and Chiang (37) suggested that increasing P27 levels is conducive to anticancer drug-induced inhibition of human HCC HepG2 cells proliferation. The present data indicated that TIPE-2 treatment downregulated p27 expression, which may lead to inhibition of HepG2 cell proliferation.

Apoptosis induction and G_2/M cell cycle arrest in human cancer cells via the Ras/Raf/mitogen-activated protein kinase and PI3K/AKT signaling pathways has been reported in previous studies (38,39). The present results indicated that TIPE-2 decreased PI3K and AKT expression in HCC cells, which may have promoted apoptosis, and inhibited HepG2 cell growth and aggressiveness. Yue *et al* (40) reported that PI3K/AKT activation underlies human prostate cancer aggressiveness, and may be a target signaling pathway for human cancer therapy. The present findings indicated that TIPE-2 inhibited growth and aggressiveness of HCC cells through downregulation of the expression levels of VIM, CT-I and Slug. Notably, the present findings suggested that TIPE-2 promoted apoptosis in HCC *in vitro* and *in vivo*.

Endoplasmic reticulum stress is implicated in the progression of human carcinoma via the regulation of apoptotic signaling pathways in tumor cells (41). Edagawa *et al* (42) indicated that endoplasmic reticulum stress induced sensitization of P53-deficient human colon cancer cells to TNF-related apoptosis-inducing ligand-mediated apoptosis via the upregulation of death receptor 5, which may further lead to tumor growth inhibition. In the present study, the results demonstrated that TIPE-2 could promote HCC cell apoptosis through endoplasmic reticulum stress *in vitro*. Although research has indicated that TIPE-2 overexpression suppresses the proliferation, migration and invasion of prostate cancer cells by downregulation of the PI3K/AKT signaling pathway, to the best of our knowledge, TIPE-2-mediated apoptosis has been not reported in a previous study in HCC cells (43). In addition, TIPE-2 has been reported to suppress angiogenesis and invasiveness of non-small cell lung cancer via inhibiting Rac1 activation and vascular endothelial growth factor expression (44). The present study indicated that TIPE-2 treatment may regulate HCC cell apoptosis via regulation of the PI3K/AKT signaling pathway, which may result in activation of endoplasmic reticulum stress in HCC cells through promoting NRF2 and BIP expression (45).

In conclusion, the present study identified that TIPE-2 administration may efficiently inhibit HCC cell growth and aggressiveness via downregulation of the expression levels of VIM, CT-I and Slug. In addition, the results indicated that TIPE-2 administration promoted apoptosis of HCC cells through the PI3K/AKT signaling pathway, which may further contribute to the inhibition of tumor growth and may prolong survival of HepG2-bearing mice. These findings suggested that TIPE-2 may be a promising anticancer agent for the treatment of HCC.

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