Survivin activates NF-κB p65 via the IKKβ promoter in esophageal squamous cell carcinoma

WEI ZENG[1,2*], HUI LI[3*], YAN CHEN[3*], HONGBO LV[4*], LING LIU[3], JIHUA RAN[5], XIAOHONG SUN[4], SHAYAHATI BIEERKEHAZHI[3], YINING LIU[3], XIAOMIAO LI[3], WENTING LAI[3], JIBIEKE WATIBIEKE[3], MEILIWUERTI DAWULIETIHAN[3], XIUMEI LI[6] and HUIWU LI[7]

[1]Department of Labour Hygiene and Sanitary Science, College of Public Health, Xinjiang Medical University; [2]First Department of Lung Cancer Chemotherapy, The Affiliated Cancer Hospital of Xinjiang Medical University; [3]Department of Biochemistry and Molecular Biology, School of Basic Medicine, Xinjiang Medical University; [4]Department of Thoracic Surgery, The Affiliated Cancer Hospital of Xinjiang Medical University, Ürümqi, Xinjiang Uyghur Autonomous Region 830011; [5]Clinical Laboratory Diagnosis Center of PLA, General Hospital of Lanzhou Command, Ürümqi, Xinjiang Uyghur Autonomous Region 830000; [6]Morphology Center, School of Basic Medicine, Xinjiang Medical University; [7]Cancer Institute, The Affiliated Cancer Hospital of Xinjiang Medical University, Ürümqi, Xinjiang Uyghur Autonomous Region 830011, P.R. China

Received March 23, 2015; Accepted December 2, 2015

DOI: 10.3892/mmr.2015.4737

Abstract. Survivin and transcription factor p65 (NF-κB p65) participate in the progression of esophageal squamous cell carcinoma (ESCC). However, the mechanism of NF-κB p65 activation in ESCC remains to be elucidated. The aim of the present study was to investigate the role of survivin in the activation of NF-κB p65 in ESCC. The expression levels of survivin, NF-κB p65, inhibitor of nuclear factor κB kinase subunit α (IKKα) and inhibitor of nuclear factor κB kinase subunit β (IKKβ) were detected in ESCC tissue samples. Eca109 and KYSE150 cells were cultured and survivin activity was modulated via transfection with an overexpression plasmid, a small hairpin RNA plasmid and a specific inhibitor. Quantitative reverse transcription-polymerase chain reaction and western blotting assays were conducted to assess the effects of survivin on the expression levels of IKKα, IKKβ and NF-κB p65. Cell cycle and apoptosis assays were conducted to detect survivin-dependent cellular behavior changes. In addition, the luciferase reporter gene assay and chromatin immunoprecipitation assay were conducted to determine the genomic sites responsible for survivin-induced activation of NF-κB p65. The present study demonstrated that the expression of survivin is positively correlated with IKKα and IKKβ in ESCC tissues. Survivin affected the mRNA and protein expression levels of IKKα, IKKβ, and NF-κB p65 in Eca109 and KYSE150 cells. Furthermore, survivin increased the transcriptional activity of the IKKβ promoter and bound to the IKKβ promoter region in the Eca109 cells. Downregulation of survivin arrested the cell cycle at the G_2/M phase and induced apoptosis. Results of the present study suggest that survivin activates NF-κB p65 in Eca109 cells via binding to the IKKβ promoter region and upregulating IKKβ promoter transcriptional activity. Survivin overexpression activates NF-κB p65, which is important in the acquisition and maintenance of the oncogenic characteristics of ESCC.

Correspondence to: Professor Xiumei Li, Morphology Center, School of Basic Medicine, Xinjiang Medical University, 393 Xinyi Road, Ürümqi, Xinjiang Uyghur Autonomous Region 830011, P.R. China
E-mail: dr.lxm@163.com

Professor Huiwu Li, Cancer Institute, The Affiliated Cancer Hospital of Xinjiang Medical University, 789 East Suzhou Street, Ürümqi, Xinjiang Uyghur Autonomous Region 830011, P.R. China
E-mail: professorhuiwuli@hotmail.com

*Contributed equally

Key words: esophageal squamous cell carcinoma, survivin, nuclear factor-κB p65, inhibitor of nuclear factor κB kinase subunit β

Introduction

Esophageal cancer is a prevalent type of cancer worldwide and is ranked sixth among cancer-associated mortalities (1). According to a recent study, in 2008 ~482,000 new esophageal cancer cases were diagnosed and 407,000 cancer-associated mortalities occurred globally (1). Almost half of newly diagnosed esophageal cancer cases occurred in China (2,3). Northern China is a high-incidence area for esophageal cancer and has been termed the Asian esophageal cancer belt (4). The highest-incidence areas of China include Linxian (Henan), Cixian (Hebei), Huai'an (Jiangsu) (5-7), a high incidence is also observed in Xinjiang, which has a population of various ethnic groups (3).

Table I. shRNA targeting sequences for survivin and the control.

Target gene	shRNA targeting sequence, 5'→3'
Survivin	GAAAGTGCGCCGTGCCATCTTCAAGAGAGATGGCACGGCGCACTTTCTT
Control	GCGCGCACAATCTACGCTAGTTTCAAGAGAACTAGCGTAGATTGTGCGCGCTT

shRNA, short hairpin RNA.

Esophageal squamous cell carcinoma (ESCC) is the predominant histological type of esophageal cancer in China, which contributes to >90% of all esophageal cancer incidences. Less common types include esophageal adenocarcinomas, melanoma, leiomyosarcoma and small-cell carcinoma (8-14).

Currently, esophageal cancer is treated using surgery, chemotherapy, radiotherapy, biotherapy, or a combination of these modalities (15). Despite improvements in surgical techniques and adjuvant chemoradiation, the overall 5-year survival rate of esophageal cancer has remained <10% in the USA (16). As the long-term survival rate is correlated with the clinical stage of esophageal cancer (17), early diagnosis and treatment would contribute to improving survival rates and the quality of life of esophageal cancer patients. However, early diagnosis is difficult as the majority of early-stage cases of esophageal cancer are asymptomatic (18).

Suppression of apoptosis is a notable biological behavior of cancer, and it is crucial in the oncogenesis and progression of ESCC (19-21). Elucidation of the regulatory mechanisms underlying gene expression in the process of apoptosis may contribute to early diagnosis and personalized therapy for ESCC patients.

Survivin is the smallest member of the inhibitor of apoptosis protein (IAP) family, and is important in the regulation of apoptosis (22). Survivin expression levels are correlated with the clinicopathological parameters and prognosis of esophageal cancer patients (23-27). Furthermore, overexpression of survivin has been associated with an increased likelihood of tumor relapse and poor overall survival (28,29). Thus, survivin detection has been used as a biomarker for monitoring tumor recurrence, and survivin has been targeted as a personalized therapeutic strategy in clinical trials (30-35).

The molecular mechanisms of high survivin expression in tumor tissues include amplification of the survivin locus (36), demethylation of the survivin promoter (37) and increased promoter activity (38). Furthermore, previous studies have observed that transcription factors, such as nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) (39), are important for increased survivin transcription activity. Activation of the NF-κB signaling pathway contributes to tumor progression by blocking apoptosis via upregulation of survivin (40,41).

NF-κB is a nuclear transcription factor that regulates immunoglobulin (Ig) κ light chain expression in B lymphocytes (42,43). The mammalian genome encodes five NF-κB subunits: RelA (p65), RelB, c-Rel and NF-κB1 (p50 and its precursor, p105), and NF-κB2 (p52 and its precursor, p100) (42).

Activation of the NF-κB signaling pathway is significantly associated with reduced overall survival in patients with ESCC (44,45). Inhibition of the NF-κB signaling pathway thus represents a promising approach for treatment of ESCC (46). However, the mechanism underlying activation of transcription factor p65 (NF-κB p65), which participates in the NF-κB canonical signaling pathway in ESCC, remains to be elucidated.

As NF-κB p65 and survivin contribute to regulating apoptosis, and are highly expressed or persistently activated in tumorigenesis and progression of ESCC (32,47), the present study hypothesized that survivin activates NF-κB p65 by regulating the expression levels of inhibitor of nuclear factor κB kinase subunit β (IKKβ) or inhibitor of nuclear factor κB kinase subunit α (IKKα) in ESCC. Thus, the aim of the current study was to investigate this hypothesis and establish the role of survivin in the activation of NF-κB p65 in ESCC.

Materials and methods

Tumor tissue specimens. Forty pairs of ESCC and healthy adjacent esophageal tissue samples were obtained from surgically excised specimens of ESCC from patients at the Affiliated Cancer Hospital of Xinjiang Medical University (Ürümqi, China) between July and December 2013. The tumor and adjacent healthy tissues were frozen in liquid nitrogen immediately following resection. The patients in the current study had not received chemotherapy or radiation therapy prior to surgery. The present study was approved by the Ethics Committee of the Affiliated Cancer Hospital of Xinjiang Medical University. Written informed consent was provided by the families of all of the patients.

Cell culture. The Eca109 cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Zhejiang Tianhang Biological Technology Co., Ltd., Zhejiang, China), penicillin (100 U/ml) and streptomycin (100 mg/ml) (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified 5% CO_2 atmosphere.

The KYSE150 cell line was purchased from the Beijing Institute of Cancer (Beijing, China). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37˚C in a humidified 5% CO_2 atmosphere.

LV3-survivin shRNA interference plasmid construction. The LV3 vector was purchased from Shanghai GenePharma Co.,

Table II. Primers for GV142-survivin overexpression plasmid and the GV142-control plasmid construction.

Target gene	Primers, 5'→3'	
	Forward	Reverse
Survivin	TGCCAAGCTTATGGGTGCCCCGACGTTGC	TCCGCTCGAGTATCCATGGCAGCCAGCTGCTC
Control	TTATGGGTGCCCCGACGTTGC	TCCGCTCGAGTATCCTGCCAAGCATGGCAGCCAGCTGCTC

Table III. Primers for survivin, NF-κB p65, IKKα and IKKβ for quantitative reverse transcription-polymerase chain reaction.

Target gene	Primers, 5'→3'		Product size, bp
	Forward	Reverse	
Survivin	ACCGAGACCTAAAGTCCAAC	AGACAGATAGCCACGACCC	305
NF-κB	GTCTTCGTGCTCGGTGATG	AGGACCTCTGACCCAAATG	147
IKKβ	TTTACAGTATGCCTCCACC	GTTTACTCCCGTCTGCTTC	365
IKKα	GCCCTTGTCCCTGTCCCTA	GCAGAGTATTTCCCTTTGGTTTGA	374
GAPDH	TCTTAGGGAAGGTCAGCATT	GCCTCTAGTTCCYYGGCATCA	325

NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; IKKβ, inhibitor of nuclear factor κB kinase subunit β; IKKα, inhibitor of nuclear factor κB kinase subunit α.

Ltd., Shanghai, China). The small hairpin RNA (shRNA) targeting sequences for survivin and the control are presented in Table I. The sequences were inserted between the *Hin*dIII and *Xho*I sites of the LV3 vector, which yielded LV3-survivin shRNA and LV3-control shRNA plasmids (synthesized by Shanghai GenePharma Co. Ltd.). The constructs were verified by DNA sequence analysis.

GV142-survivin overexpression plasmid construction. The GV142 plasmid was purchased from GeneChem Co., Ltd. (Shanghai, China). For the GV142-survivin overexpression and GV142-control plasmid construction, GV227 (GeneChem Co., Ltd.) was used as the template, and the survivin and control polymerase chain reaction (PCR) primers used are presented in Table II. The resulting PCR products were inserted into the GV142 vector between *Hin*dIII and *Xho*I sites, yielding GV142-survivin overexpression and GV142-control plasmids.

Plasmid transient transfection. Prior to transfection, $2x10^5$ cells/well were placed into 6-well plates cultivated in serum-free culture medium and antibiotics, and grown overnight until they reached 70-80% confluence. Plasmid transfection was performed using Lipofectamine™ 2000 transfection reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The plasmids and Lipofectamine™ 2000 were diluted in Opti-MEM I Reduced Serum Medium (Thermo Fisher Scientific, Inc.) separately and incubated for 10 min at room temperature. The diluted solutions were mixed and incubated for 20 min at room temperature. Subsequently, the mixtures were added to each well containing cells and medium. The cells with only the transfection reagent served as a blank control. Cell culture plates were incubated for 6 h at 37˚C in a CO_2 incubator. Culture medium containing 10% FBS was added and cells were incubated under the above-mentioned conditions.

YM155 treatment and cell viability assay. Cells were seeded in 96-well plates at a density of $5x10^3$ cells/well in a volume of 100 μl culture medium per well. After 24 h, cells were exposed to survivin inhibitor, YM155 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at various concentrations (0, 0.005, 0.05, 0.5, 5 and 50 μM) for 48 h. Following incubation at 37˚C, 0.4% Trypan Blue solution (Sigma-Aldrich, St. Louis, MO, USA) was added to each well. Viability was assessed by counting the relative number of live, unstained cells to total cells, and the half-inhibitory concentration (IC_{50}) for YM155 was derived from a logarithmic plot.

Cells treated with YM155 (concentrations at, above and below the IC_{50}) were harvested at 48 h, and the total RNA and protein was extracted to determine the expression levels of survivin, IKKα, IKKβ and NF-κB p65 by reverse transcription quantitative (RT-q) PCR and western blotting.

RT-qPCR. At 48 h post-transfection, total cellular RNA was extracted using TRIzol (Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 1 μg total RNA using Maloney Murine Leukemia Virus Reverse Transcriptase (Promega Corporation, Madison, WI, USA) and an oligo-deoxy-thymine nucleotide primer (Promega Corporation) according to the manufacturer's protocols. DNA content was measured using a UV/visible spectrophotometer (Ultrospec 2000; GE Healthcare Life Sciences, Chalfont, UK). Primer sequences are presented in Table III. Primers used in the current study were synthesized by Shanghai Shenggong Biology Engineering Technology Service, Ltd. (Shanghai, China). RT-qPCR assays

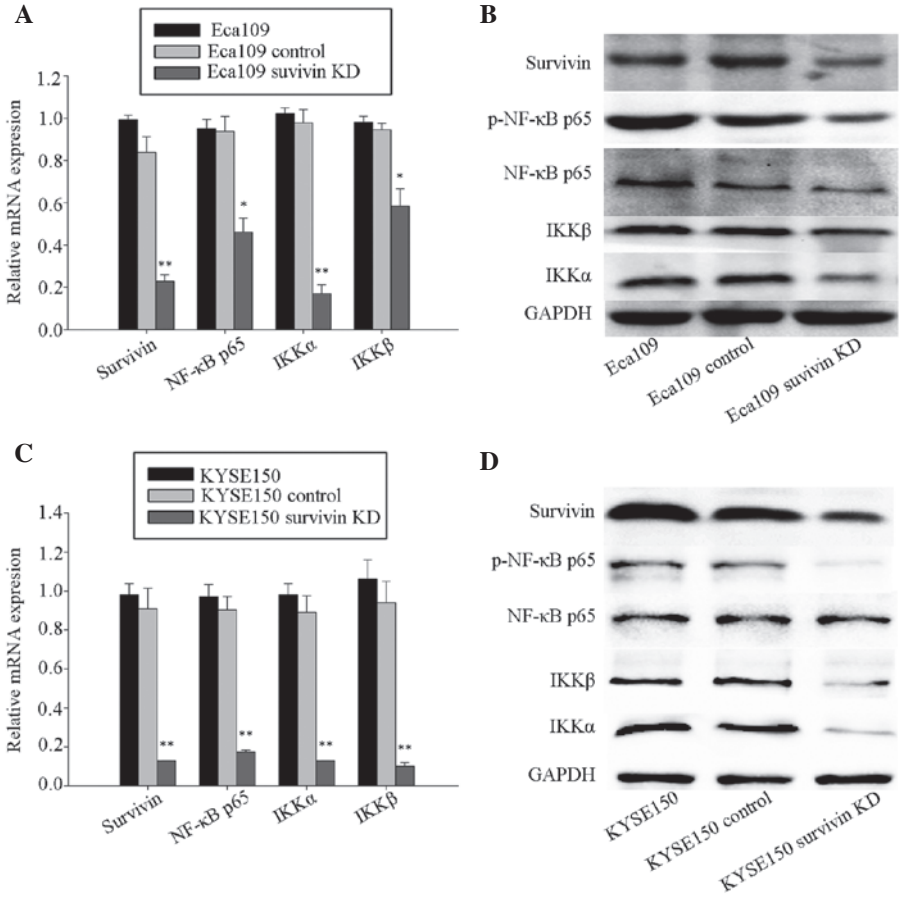

Figure 1. Survivin knockdown results in downregulation of IKKα, IKKβ and NF-κB p65 in Eca109 and KYSE150 cells. Eca109 and KYSE150 cells were transfected with LV3-survivin shRNA plasmid (Eca109 survivin KD and KYSE150 survivin KD) and LV3-survivin control plasmids (Eca109 control and KYSE150 control), respectively. The expression levels of survivin, NF-κB, IKKα and IKKβ were measured by quantitative reverse transcription-polymerase chain reaction and western blotting in (A and B) Eca109 cells and (C and D) KYSE150 cells. GADPH served as an internal and loading control. Columns present the mean values from triplicate experiments and the error bars indicate the standard deviation. *P<0.05; **P<0.01 vs. control. KD, knockdown; NF-κB p65, transcription factor p65; IKKα, inhibitor of nuclear factor κB kinase subunit α; IKKβ, inhibitor of nuclear factor κB kinase subunit β; p, phosphorylated.

used the TaqMan® Fast Virus 1-Step Master mix kit (Thermo Fisher Scientific, Inc.). The reaction system was: 12 μl SYBR® green reagent (Thermo Fisher Scientific, Inc.), 0.2 μM each primer, 1 μl cDNA template, and 6 μl nuclease-free distilled water (Thermo Fisher Scientific, Inc.). GAPDH served as an internal standard to evaluate the relative expression levels of the target genes. qPCR analysis was performed on an Applied Biosystems® 7500 Fast Real Time PCR instrument (Thermo Fisher Scientific, Inc.). The PCR conditions, performed for 40 cycles, were as follows: 2 min at 50˚C, 2 min at 95˚C, 15 sec at 95˚C, 15 sec at 55-60˚C, 1 min at 72˚C. The relative quantification transcript levels were determined using the $2^{-\Delta\Delta Cq}$ method. Specificities of all PCR amplifications were confirmed by melting curve analysis. All experiments were performed in triplicate and results are presented as the mean ± standard deviation.

Western blotting assays. At 48 h post-transfection, total protein was extracted, by washing twice with ice-cold phosphate-buffered saline (PBS; Thermo Fisher Scientific, Inc.), and resuspended in lysis buffer [1 M Tris HCl (pH 7.4), 5M NaCl, 0.5 M ethylene glycol tetraacetic acid, 0.5 M EDTA, NP-40, 10% SDS (Wuhan Boster Biological Technology, Ltd., Wuhan, China), glycerine, 10 μg/μl aprotinin, 10 μg/μl leupeptin, 10 μg/μl Pepstatin A, 10 mM phenylmethylsulfonyl fluoride, double-distilled H_2O] for 30 min on ice. Suspensions were centrifuged at 18,407 x g for 15 min at 4˚C. The supernatant containing the protein was collected and the protein concentration of each lysate was determined by Pierce BCA protein assay (Thermo Fisher Scientific, Inc.). Protein (20 μg) was loaded for each sample. Proteins were denatured, subjected to SDS-PAGE using 10-15% polyacrylamide gels (Wuhan Boster Biological Technology, Ltd.), electrophoresed (stacking gel, 60 V for 45 min; separating gel, 100 V for 90 min) and electrophoretically transferred onto nitrocellulose membranes (Whatman; GE Healthcare Life Sciences). The membranes were blocked with 5% non-fat dried milk (Sigma-Aldrich) in Tris-buffered saline and Tween-20 (TBST; Beijing Solarbio Science & Technology Co., Ltd.,Beijing,China) at room temperature for 2 h. The membranes were incubated overnight at 4˚C with the following primary antibodies: Rabbit polyclonal anti-GAPDH (1:400; cat. no. BA2913; Wuhan Boster Biological Technology, Ltd.), which served as a loading control; rabbit polyclonal anti-survivin (1:1,000; cat. no. sc-10811; Santa Cruz Biotechnology Inc.); rabbit polyclonal anti-phosphorylated (p)-NF-κB p65 (pSer^{536}) (1:1,000; cat. no. AF2006; Affinity

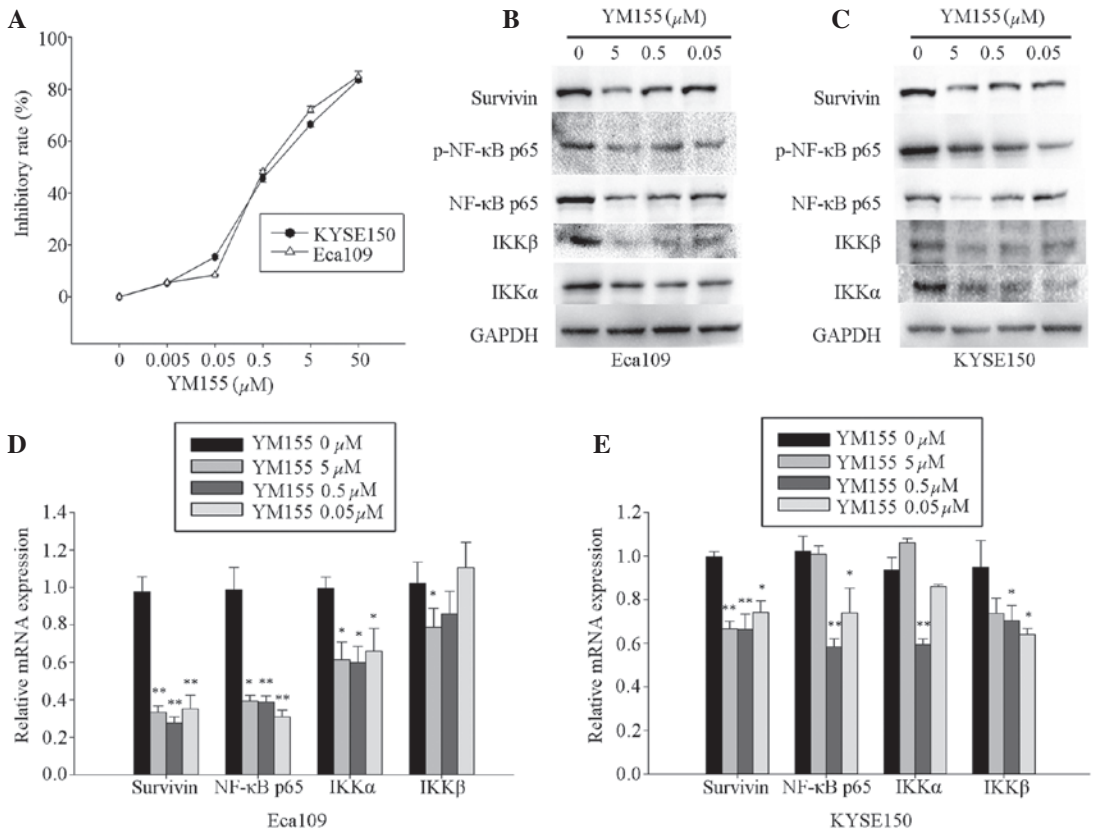

Figure 2. YM155 inhibited the expression of survivin, NF-κB p65, IKKα and IKKβ in Eca109 and KYSE150 cells. (A) Eca109 and KYSE150 cells were incubated with 0.005, 0.05, 0.5, 5 and 50 μM YM155 for 48 h. Cells incubated with the culture medium served as the control group. Experiments were performed in triplicate. Eca109 and KYSE150 cells were treated with 0 (blank), 0.05, 0.5, and 5.0 μM YM155. The expression of survivin, NF-κB p65, IKKβ and IKKα were analyzed by (B and C) western blotting and (D and E) RT-qPCR. Western blotting of survivin, p-NF-κB p65, NF-κB p65, IKKβ and IKKα proteins was conducted using total protein isolated from cells, with GADPH serving as a loading control. Transcript levels were measured by RT-qPCR of total isolated RNA, with GADPH serving as an internal control. YM155 inhibited expression of survivin and NF-κB p65, IKKα and IKKβ in (B and D) Eca109 and (C and E) KYSE150 cells. Columns demonstrate the mean values from triplicate experiments and the error bars indicate standard deviation. *P<0.05; **P<0.01 vs. control. RT-qPCR, quantitative reverse transcription-polymerase chain reaction; NF-κB p65, transcription factor p65; IKKα, inhibitor of nuclear factor κB kinase subunit α; IKKβ, inhibitor of nuclear factor κB kinase subunit β; p, phosphorylated; mRNA, messenger RNA.

Biosciences, Cell Signal Transduction, Cincinnati, OH, USA); rabbit polyclonal anti-NF-κB p65 (1:400; cat. no. BA0610; Wuhan Boster Biological Technology, Ltd.); rabbit polyclonal anti-IKKα (1:400; cat. no. BA1594-2; Wuhan Boster Biological Technology, Ltd.); and rabbit polyclonal anti-IKKβ (1:400; cat. no. BA4458-2; Wuhan Boster Biological Technology, Ltd.) were blocked in TBST. Membranes were washed three times (10 min per wash) with TBST at room temperature. Subsequently, the membranes were incubated with appropriate horseradish peroxidase-linked goat anti-rabbit secondary antibodies at a dilution of 1:1,000 (cat. no. BA1054; Wuhan Boster Biological Technology, Ltd.) diluted in TBST for 1 h at room temperature. Protein bands were visualized using an Enhanced Chemiluminescence Detection kit (Thermo Fisher Scientific, Inc.).

Luciferase reporter gene assay. The human IKKβ promoter sequences (3,000 bp upstream) and random control sequence (size, 3,000 bp) were obtained by PCR amplification and inserted between the *Hin*dIII and *Xho*I sites of the pGL3 vector (Promega Corporation) yielding pGL3-IKKβ and pGL3-random control plasmids (synthesized by GeneChem Co., Ltd.). Eca109 and KYSE150 cells were seeded at $5x10^5$ cells per well in 6-well dishes one day prior to transfection. The cells were co-transfected with 0.1 μg pGL3-random control, pGL3-IKKβ or pGL3-basic firefly luciferase reporter construct, 0.01 μg pRL-TK *Renilla* luciferase reporter plasmid and the GV142-survivin overexpression plasmid, using Lipofectamine™ 2000. pRL-TK *Renilla* luciferase reporter plasmid was co-transfected to assess the transfection efficiency. Post-transfection (48 h), cells were harvested and lysed with 1X lysis buffer (Promega Corporation). Cell extracts (20 μl) were assayed for luciferase activity using the Dual-luciferase Reporter assay system kit (Promega Corporation) according to the manufacturer's protocols. Relative levels of reporter gene expression were expressed as ratios of firefly luciferase activity to *Renilla* luciferase (LU/RL). All experiments were performed in triplicate.

Chromatin immunoprecipitation assay (ChIP). ChIP assays were performed using the EZ ChIP™ kit (EMD Millipore, Billerica, MA, USA) according to the manufacturer's protocols. Eca109 and KYSE150 cells were transfected with the GV142-survivin overexpression plasmid and fixed with 1% formaldehyde (Sigma-Aldrich) for 10 min. The Eca109 and KYSE150 cells were washed twice with 1X PBS, lysed, and sonicated to reduce DNA lengths to within the range of 200-1,000 bp. The survivin/DNA complexes were incubated

Table IV. Promoter-specific primers for IKKα and IKKβ used in the chromatin immunoprecipitation assay.

Target gene	Primer, 5'→3'	Product site, bp
IKKα promoter-1	F: TGTGGATGGAGGCGTAGAG R: AGCCAGAAGGGAAGAATGAG	Upstream, 2596-2576
IKKα promoter-2	F: GAATCCTCCAGGGAGACCAAAGTAA R: TTACTTTGGTCTCCCTGGAGGATTC	Upstream, 1758-1737
IKKα promoter-3	F: CCCTGACATAACCCCAGCCACA R: ACAGCCCCACCATCCCCATT	Upstream, 267-247
IKKα promoter-4	F: GCCCTTCAGGAGCAACTAA R: TGACGCCTACCATAGCACTC	Upstream, 1695-1675
IKKα promoter-5	F: TCAAGGCGATAATGCTCACT R: TCTCCACTTTCAGCCGTTT	Upstream, 801-781
IKKα promoter-6	F: CAAGGTGGACTAGGGTTGTAAA R: TGTGAGAATCTAATGCCTGATG	Upstream, 1402-1424
IKKα promoter-7	F: AAATAACTTGCTCCATACCCTG R: GGGGAATGGCAGTTGTGA	Upstream, 788-808
IKKα promoter-8	F: TCTTGGGTAGGGAAGTATGGG R: GGTCTGGGAAGTCTTGCTTTA	Upstream, 1197-1006
IKKα promoter-9	F: AGTTTATAGGGCAAGAATCGAG R: GGTAAAGGTAGTATTGGGCAAC	Upstream, 176-156
IKKβ promoter-1	F: AAAGAAAGAAACCAAGTAGCCG R: TGAGGTATTGATAGCAGCAGTG	Upstream, 270-248
IKKβ promoter-2	F: TCTTCAGGTTCCTTTGGTAGTT R: TGAGTTTCTCCGTTTTATGGG	Upstream, 860-849
IKKβ promoter-3	F: GGTAGGCAAGGGCAGTTCT R: GACCGTGCTCACCGATTT	Upstream, 1520-1502

F, forward; R, reverse; IKKα, inhibitor of nuclear factor κB kinase subunit α; IKKβ, inhibitor of nuclear factor κB kinase subunit β.

with 4 μg rabbit antibody against survivin, 1 μl Normal Mouse IgG (dilution, 1:1,000), which served as the negative control, and 1 μl Anti-RNA Polymerase II (dilution, 1:1,000; both included in the EZ-ChIP™ kit) served as the positive control. The mixes were incubated at room temperature for 60-90 min. The immune complexes were precipitated, eluted, reverse-crosslinked and treated with proteinase K [Tiangen Biotech (Beijing) Co.,Ltd., Beijing, China]. The resulting DNA samples were amplified using primers for the putative survivin site in the human IKKα and IKKβ promoter region. The primer sequences are presented in Table IV. PCR fragments were separated and visualized on 1.8% agarose gels stained with ethidium bromide (Shanghai Bioleaf Biotech Co., Ltd., Shanghai, China). The ratios of the PCR products of survivin (IKKα, IKKβ and NF-κB p65) to GAPDH were used to determine the expression levels of target genes.

Flow cytometric analysis. For cell cycle analysis, DNA labeling was conducted using the Cycle Test Plus DNA reagent kit (BestBio Co., Ltd., Shanghai, China). Labeling with propidium iodide (PI) and Annexin V was performed using an Annexin V staining kit (BestBio Co. Ltd.) for the detection of apoptotic cells and the assays were performed according to the manufacturer's protocols. Eca109 and KYSE150 cells were directly incubated, at 37˚C for 48 h, in 6-well plates and collected 48 h following transfection. For the cell cycle analysis, the cells were washed with PBS for 5 min and subsequently centrifugation at 900 x g. The cells were collected and fixed in ice-cold 70% ethanol (Saihongrui Biotechnology Co., Ltd., Nanjing, China) for a minimum of 2 h at 4˚C, followed by treatment with 0.2 mg/ml RNase A (EMD Millipore) in PBS for 30 min at 37˚C. PI was added (final concentration, 25 μg/ml) and the cells were incubated for 30 min at 4˚C in the dark. Analysis of the samples was conducted within 24 h. For the apoptosis assay, the transfected cells were washed twice with ice-cold PBS, and resuspended in 195 μl 1X Binding Buffer (EMD Millipore) to a concentration of $1x10^4$ cells/ml. Annexin V (5 μl) and PI were gently mixed with the cells and incubated for 15 min at room temperature in the dark. The dyes were washed out by centrifugation for 5 min at 94 x g and the cells were resuspended in 190 μl 1X Binding Buffer. PI staining solution (10 μl) was gently mixed in and incubated on ice and in the dark. The samples were analyzed within 1 h. All samples for the two assays consisted of 10,000 cells and were analyzed by fluorescence-activated cell sorting with a BD FACSMicroCount™ system (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis. SPSS version 17.0 for Windows (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. All data were expressed as the mean ± standard deviation

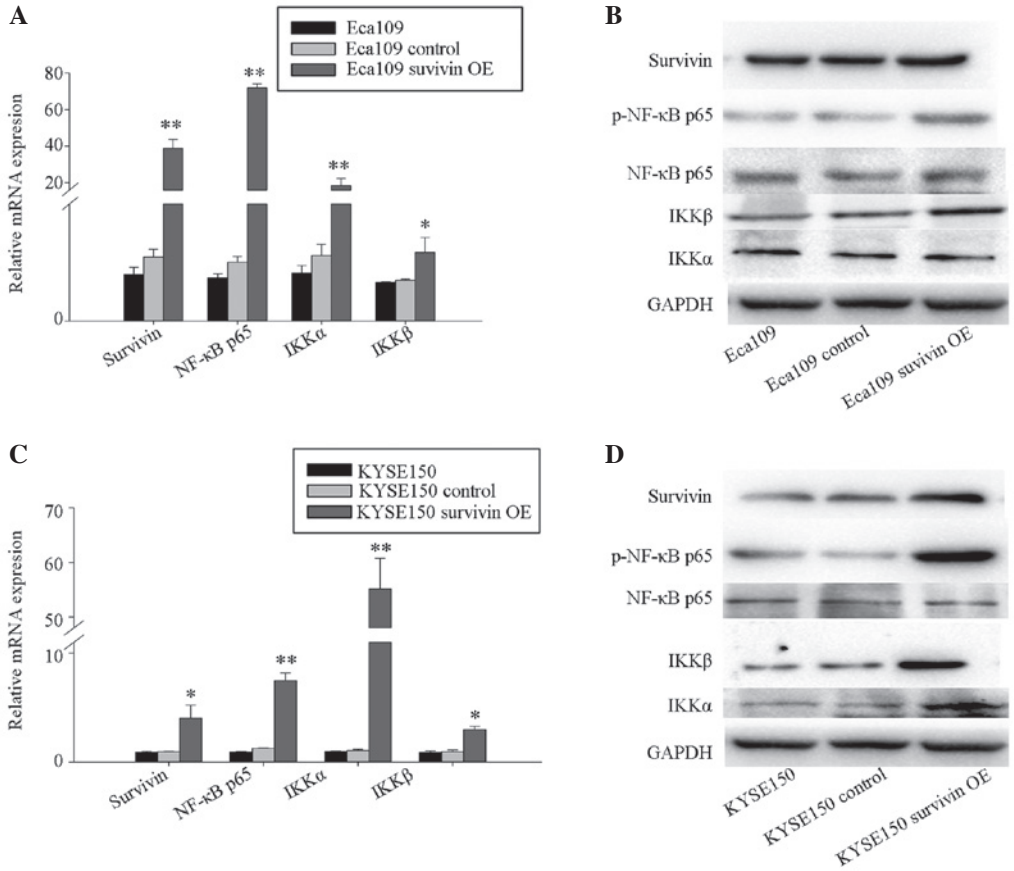

Figure 3. Survivin overexpression results in upregulation of NF-κBp65, IKKα, IKKβ in Eca109 and KYSE150cells. (A and B) Eca109 and (C and D) KYSE150 cells were transfected with GV142-survivin overexpression plasmid (Eca109 survivin OE and KYSE150 survivin OE), GV142-control plasmid (Eca109 control and KYSE150 control), and mock transfection. Expression levels of survivin, NF-κB p65, IKKβ, and IKKα were analyzed by (A and C) RT-qPCR and (B and D) western blotting of total protein. Transcript levels were measured by RT-qPCR of total isolated RNA, with GADPH serving as an internal control. Columns indicate the mean values from triplicate experiments and the error bars indicate standard deviation. *P<0.05; **P<0.01 vs. control. OE, overexpression; RT-qPCR, quantitative reverse transcription-polymerase chain reaction; NF-κB p65, transcription factor p65; IKKα, inhibitor of nuclear factor κB kinase subunit α; IKKβ, inhibitor of nuclear factor κB kinase subunit β; p, phosphorylated; mRNA, messenger RNA.

Table V. Association between survivin and NF-κB p65, IKKα, and IKKβ mRNA expression levels in esophageal squamous cell carcinoma samples.

	IKKα		IKKβ		NF-κB p65	
Parameter	+	-	+	-	+	-
Survivin						
+	22	4	20	6	16	10
-	7	7	6	8	10	4
Statistical value						
r	0.370		0.341		0.154	
P-value	0.019		0.031		0.350	

IKKα, inhibitor of nuclear factor κB kinase subunit α; IKKβ, inhibitor of nuclear factor κB kinase subunit β; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; r, correlation coefficient.

from three experiments. The two-tailed Student's t-test was used to analyze the difference between groups and Fisher's exact test was used to analyze correlation between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Survivin expression is positively correlated with IKKα and IKKβ expression in ESCC tissue samples. The expression levels of survivin, IKKα, IKKβ and NF-κB p65 were evaluated by RT-qPCR in 40 paired ESCC and healthy tissue samples, as presented in Table V. In the present study, the expression of survivin was observed to be positively correlated with IKKα (r =0.370; P<0.05) and IKKβ mRNA expression levels (r =0.341; P<0.05) in ESCC samples.

Survivin knockdown deactivates NF-κB signaling in ESCC cells. Cells transfected with LV3-survivin shRNA and LV3-control shRNA plasmids were designated the survivin knockdown group and control group, respectively. The cells cultured with the transfection reagent only were considered as a blank group. At 48 h after transfection, cells were harvested for RT-qPCR and western blotting assays. The RT-qPCR analysis (Fig. 1) demonstrated that expression levels of survivin, NF-κB p65, IKKα and IKKβ were significantly reduced in the survivin knockdown group, compared with the control and

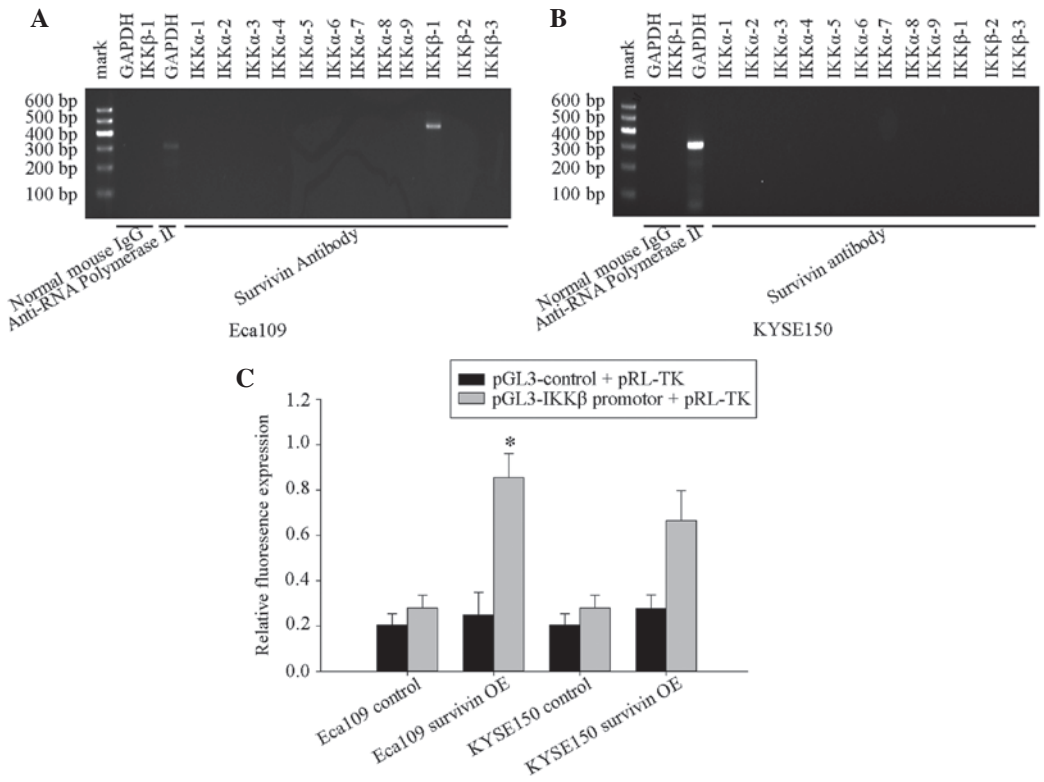

Figure 4. Survivin binds to IKKβ promoter and increases IKKβ promoter luciferase activity in Eca109 cells, but not in KYSE150 cells. (A) Eca109 and (B) KYSE150 cells were transfected with GV142-survivin overexpression plasmid for 48 h. ChIP assay was performed using the EZ ChIP kit. The DNA/protein crosslinks were immunoprecipitated using survivin antibodies and subjected to polymerase chain reaction with primers spanning the IKKα and IKKβ promoters. Anti-RNA polymerase II antibody and GAPDH primers served as positive controls. Nonspecific mouse IgG pulldown with GAPDH and IKKβ-1 primers served as a negative control. (C) Eca109 and KYSE150 cells were transfected with pGL3-control plasmid or pGL3-IKKβ promoter plasmid, in addition to the luciferase reporter system and GV142-control plasmid (Eca109 control and KYSE150 control) and GV142-survivin overexpression plasmid (Eca109 survivin OE and KYSE150 survivin OE). Luciferase activity was detected using a Dual-Luciferase Reporter gene kit. Columns demonstrate the mean values from triplicate experiments and the error bars indicate standard deviation. *P<0.05 vs. control. IKKα, inhibitor of nuclear factor κB kinase subunit α; IKKβ, inhibitor of nuclear factor κB kinase subunit β; p, phosphorylated; ChIP, chromatin immunoprecipitation; OE, overexpression; pRL-TK, internal control reporter.

blank group in Eca109 (Fig. 1A) and KYSE150 cells (Fig. 1C). Western blotting analysis also demonstrated that protein expression levels of survivin, the p-NF-κB p65, IKKα and IKKβ expression levels were similarly reduced in the survivin knockdown group of the two cell lines (Fig. 1B and D).

YM155 reduces cell viability and downregulates survivin, NF-κB p65, IKKα and IKKβ. YM155, an inhibitor of survivin, was used to investigate the effect of survivin on cell viability, as well as the interaction between survivin and NF-κB p65, IKKα, and IKKβ expression levels in Eca109 and KYSE150 cells. YM155 was administered at concentrations from 0.005 to 50 μM, which significantly reduced cell viability in a dose-dependent manner in the Eca109 and KYSE150 cells at 48 h following administration. According to the logarithmic curve, the IC_{50} of YM155 for Eca109 and KYSE150 cells was ~0.5 μM (Fig. 2A). Concentrations at, above, and below the IC_{50} (0.05, 0.5 and 5 μM) were selected for further experiments.

Eca109 and KYSE150 cells were treated with YM155 at the selected concentrations for 48 h, and the expression levels of survivin and NF-κB p65, IKKα and IKKβ were measured by RT-qPCR and western blotting assays. The results demonstrated that YM155 effectively inhibited mRNA expression levels of survivin and NF-κB p65, IKKα and IKKβ, and protein expression levels of survivin and the p-NF-κB p65, IKKα and IKKβ in Eca109 (Fig. 2B and D) and KYSE150 (Fig. 2C and E) cells, which was comparable with the survivin shRNA knockdown.

Survivin overexpression activates NF-κB signaling in ESCC cells. Cells transfected with GV142-survivin overexpression plasmid and GV142-control plasmid were designated the survivin overexpression group and the control group, respectively. The cells with the transfection reagent only served as the blank group. At 48 h after transfection, cells were harvested for RT-qPCR and western blotting assays. RT-qPCR analysis demonstrated increased mRNA expression levels of survivin, NF-κB p65, IKKα and IKKβ in the survivin overexpression group, when compared with the control group and the blank control group in Eca109 (Fig. 3A and B) and KYSE150 (Fig. 3C and D) cells. In addition, western blotting analysis indicated that the protein levels of survivin, and phosphorylation of NF-κB p65 and IKKβ were also increased in the survivin overexpression group in the Eca109 (Fig. 3B) and KYSE150 (Fig. 3D) cells. Furthermore, KYSE150 cells demonstrated increased expression levels of IKKα protein (Fig. 3D).

Survivin binds to IKKβ promoter and increases the transcriptional activity of the IKKβ promoter in Eca109 cells. To further determine whether survivin recognized and bound to the IKKβ promoter *in vivo,* and increased its transcriptional

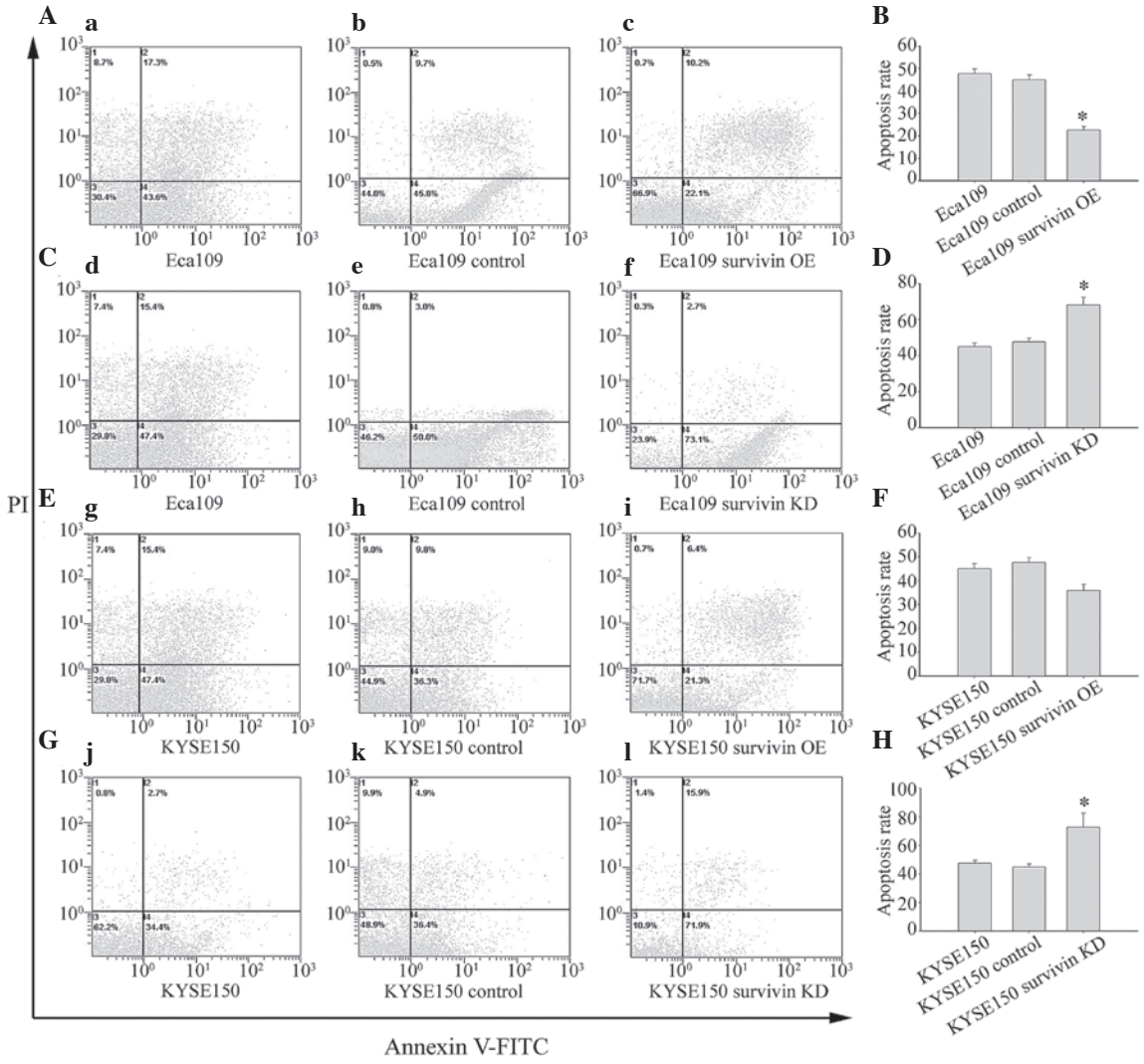

Figure 5. Survivin overexpression significantly inhibited, whereas survivin knockdown significantly induced, apoptosis in Eca109 cells. (A-D) Eca109 and (E-H) KYSE150 cells were transfected with GV142-survivin overexpression plasmid (c, Eca109 survivin OE; i, KYSE150 survivin OE), GV142-control plasmid (b, Eca109 control; h, KYSE150 control), and transfection reagent alone (a, Eca109; g, KYSE150); or LV3-survivin shRNA plasmid (f, Eca109 survivin KD; l, KYSE150 survivin KD), LV3-control plasmid (e, Eca109 control; k, KYSE150 control), and transfection reagent alone (d, Eca109; j, KYSE150). Cells were stained with Annexin V and analyzed by FACScan flow cytometry. Columns demonstrate the mean values from triplicate experiments and the error bars indicate standard deviation. *P<0.05; **P<0.01 vs. control. OE, overexpression; FITC, fluorescein isothiocyanate; PI, propidium iodide; KD, knockdown.

activity, the ChIP assay was performed in Eca109 cells. DNA was immunoprecipitated using anti-survivin polyclonal antibodies and subjected to PCR with promoter-specific primers for IKKβ. Positive amplification in Eca109 cell lines demonstrated that survivin may bind the upstream 700 bp IKKβ promoters (Fig. 4A and B).

To investigate whether overexpression of survivin affects the promoter transcriptional activities of IKKβ in Eca109 and KYSE150 cells, a Luciferase reporter gene assay was performed and overexpressed survivin was observed to significantly increase the transcriptional activity of the IKKβ promoter in Eca109 cells, but not in the KYSE150 cells (Fig. 4C).

Survivin knockdown induces apoptosis and G_2/M phase arrest in vitro. To analyze the effect of survivin on apoptosis, flow cytometric analysis with PI and Annexin V staining was performed (Fig. 5). Results demonstrated that survivin knockdown and subsequent reduction in activation of NF-κB p65 increased apoptosis in Eca109 (Fig. 5C and D) and KYSE150 (Fig. 5G and H) cells. Conversely, survivin overexpression and concomitant activation of NF-κB p65 decreased apoptosis in Eca109 (Fig. 5A and B) and KYSE150 (Fig. 5E and F) cells.

To analyze the effect of survivin on the cell cycle, flow cytometry was performed. The results indicated that survivin knockdown increased the fraction of cells arrested in the G_2/M phase in Eca109 and KYSE150 cells (Fig. 6).

Discussion

Survivin, the smallest member of the IAP family, is overexpressed in ESCC. Survivin detection is correlated with the clinical stage, metastasis, relapse rate and the overall survival of ESCC patients, and provides valuable information to predict

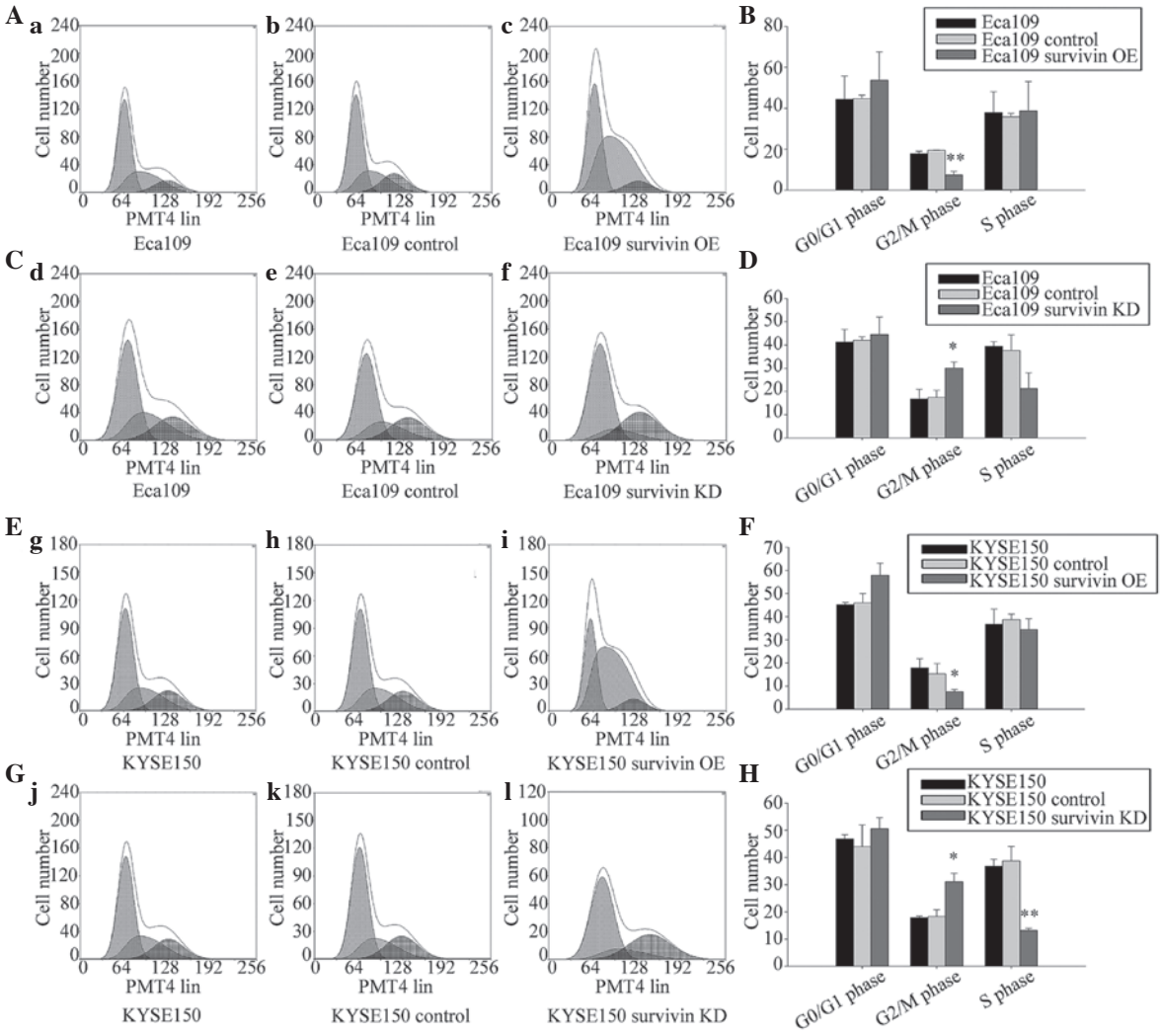

Figure 6. Survivin overexpression significantly increased the cell number in the G_2/M phase, whereas survivin knockdown significantly delayed the cell cycle in the G_2/M phase in Eca109 and KYSE150 cells. (A-D) Eca109 and (E-H) KYSE150 cells were transfected with GV142-survivin overexpression plasmid (c, Eca109 survivin OE; i, KYSE150 survivin OE), GV142-control plasmid (b, Eca109 control; h, KYSE150 control), and transfection reagent alone (a, Eca109; g, KYSE150); or LV3-survivin shRNA plasmid (f, Eca109 survivin KD; l, KYSE150 survivin KD), LV3-control plasmid (e, Eca109 control; k, KYSE150 control), and transfection reagent alone (d, Eca109; j, KYSE150). The cell cycle distribution was assessed by FACScan flow cytometry. Columns demonstrate mean values from triplicate experiments and the bars indicate standard deviation. *P<0.05, **P<0.01 vs. control group. KD, knockdown; OE, overexpression.

the prognosis of ESCC (48,49). The present study demonstrated that survivin overexpression inhibited cell apoptosis and induced cell proliferation. Conversely, survivin knockdown increased cell apoptosis; thus, as survivin inhibits apoptosis it is involved in the progression of ESCC.

Activated NF-κB has been associated with acid-induced esophageal epithelial cell transformation (50). NF-κB nuclear expression was significantly increased in ESCC tissue samples compared with healthy esophageal tissues (51-53). NF-κB activation indicates a poorly differentiated cancer and is associated with a low survival rate in ESCC patients (54). Interference with the NF-κB signaling pathway increases the chemotherapeutic sensitivity in ESCC, and suppresses metastasis (55), as activated NF-κB p65 is involved in the progression of ESCC via activating multiple apoptosis-associated genes, including survivin (56,57). However, the underlying mechanism of NF-κB p65 activation in ESCC remains unclear. In the present study, it was observed that survivin regulated the expression of IKKα, IKKβ, and NF-κB p65 in Eca109 and KYSE150 cells. Knockdown of survivin was demonstrated to deactivate NF-κB p65, which induced cell apoptosis and arrested cells in the G_2/M phase. Notably, survivin overexpression activated NF-κB p65, inhibiting cell apoptosis and indicating that activation of NF-κB p65 by survivin is potentially important in cell apoptosis, cell proliferation and the progression of ESCC.

NF-κB dimers are observed in the majority of resting cells and retained in the cytosol via interaction with one of the prototypical IκB proteins (58). Extracellular stimulating factor induces degradation of IκB kinase (IKK) proteins upon their phosphorylation by the IKK complex, and inducing NF-κB dimer translocation into the nucleus and resulting in target gene transcription (59). IKKα and IKKβ are important IKKs, which phosphorylate nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α (IκBα) proteins and determine NF-κB cytosolic localization (60). The present study demonstrated that overexpression of survivin increased the expression

and transcriptional activity of IKKβ in Eca109 cells by binding to the IKKβ promoter. Overexpression of survivin may result in binding of survivin to the IKKβ promoter and increase the transcriptional activity of IKKβ, which phosphorylates IκBα and releases NF-κB p65 to translocate into the nucleus.

In conclusion, survivin performs its biological functions by affecting cell apoptosis and proliferation, and increases the activity of the inducible transcription factor, NF-κB p65 via maintaining a high expression level of IKKβ and upregulating the phosphorylation level of IκBα via IKKβ, and finally releasing NF-κB p65 from the cytoplasm to the nucleus in ESCC cells.

The present study provides valuable data toward an increased understanding of constant high expression and activation of NF-κB p65 in ESCC. In addition, investigation into the underlying mechanisms of survivin/NF-κB p65 regulation in the tumorigenesis and progression of ESCC may result in the development of a novel biomarker for the early diagnosis and personalized therapeutic strategies for the treatment of ESCC.

Acknowledgements

The present study was supported by a grant from the National Natural Science Foundation of China (grant no. 81460359).

References

1. Jemal A, Bray F, Center MM, Ferlay vvJ, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
2. Holmes RS and Vaughan TL: Epidemiology and pathogenesis of esophageal cancer. Semin Radiat Oncol 17: 2-9, 2007.
3. Lin Y, Totsuka Y, He Y, Kikuchi S, Qiao Y, Ueda J, Wei W, Inoue M and Tanaka H: Epidemiology of esophageal cancer in Japan and China. J Epidemiol 23: 233-242, 2013.
4. Wu KS, Huo X and Zhu GH: Relationships between esophageal cancer and spatial environment factors by using Geographic Information System. Sci Total Environ 393: 219-225, 2008.
5. Tran GD, Sun XD, Abnet CC, Fan JH, Dawsey SM, Dong ZW, Mark SD, Qiao YL and Taylor PR: Prospective study of risk factors for esophageal and gastric cancers in the Linxian general population trial cohort in China. Int J Cancer 113: 456-463, 2005.
6. Muñoz N, Crespi M, Grassi A, Qing WC, Qiong S and Cai LZ: Precursor lesions of oesophageal cancer in high-risk populations in Iran and China. Lancet 1: 876-879, 1982.
7. Blot WJ, Li JY, Taylor PR, Guo W, Dawsey SM and Li B: The Linxian trials: Mortality rates by vitamin-mineral intervention group. Am J Clin Nutr 62 (Suppl): 1424S-1426S, 1995.
8. Jemal A, Center MM, DeSantis C and Ward EM: Global patterns of cancer incidence and mortality rates and trends. Cancer Epidemiol Biomarkers Prev 19: 1893-1907, 2010.
9. Chen CP, Braunstein S, Mourad M, Hsu IC, Haas-Kogen D, Roach M III and Fogh SE: Quality improvement of International Classification of Diseases, 9th revision, diagnosis coding in radiation oncology: Single-institution prospective study at University of California, San Francisco. Pract Radiat Oncol 5: e45-e51, 2015.
10. Macefield RC, Avery KN and Blazeby JM: Integration of clinical and patient-reported outcomes in surgical oncology. Br J Surg 100: 28-37, 2013.
11. Daly JM, Fry WA, Little AG, Winchester DP, McKee RF, Stewart AK and Fremgen AM: Esophageal cancer: Results of an American College of Surgeons Patient Care Evaluation Study. J Am Coll Surg 190: 562-573, 2000.
12. Enzinger PC and Mayer RJ: Esophageal cancer. N Engl J Med 349: 2241-2252, 2003.
13. Lepage C, Rachet B, Jooste V, Faivre J and Coleman MP: Continuing rapid increase in esophageal adenocarcinoma in England and Wales. Am J Gastroenterol 103: 2694-2699, 2008.
14. Pohl H and Welch HG: The role of overdiagnosis and reclassification in the marked increase of esophageal adenocarcinoma incidence. J Natl Cancer Inst 97: 142-146, 2005.
15. Kranzfelder M, Büchler P and Friess H: Surgery within multimodal therapy concepts for esophageal squamous cell carcinoma (ESCC): the MRI approach and review of the literature. Adv Med Sci 54: 158-169, 2009.
16. Lightdale CJ; American College of Gastroenterology: Esophageal cancer. Am J Gastroenterol 94: 20-29, 1999.
17. Iizuka T, Isono K, Kakegawa T and Watanabe H; Japanese Committee for Registration of Esophageal Carcinoma Cases: Parameters linked to ten-year survival in Japan of resected esophageal carcinoma. Chest 96: 1005-1011, 1989.
18. Toh Y, Egashira A and Yamamoto M: Epigenetic alterations and their clinical implications in esophageal squamous cell carcinoma. Gen Thorac Cardiovasc Surg 61: 262-269, 2013.
19. Shang L and Wang M: Molecular alterations and clinical relevance in esophageal squamous cell carcinoma. Front Med 7: 401-410, 2013.
20. Takeno S, Yamashita S, Takahashi Y, Ono K, Kamei M, Moroga T and Kawahara K: Survivin expression in oesophageal squamous cell carcinoma: Its prognostic impact and splice variant expression. Eur J Cardiothorac Surg 37: 440-445, 2010.
21. Akyürek N, Memiş L, Ekinci O, Köktürk N and Oztürk C: Survivin expression in pre-invasive lesions and non-small cell lung carcinoma. Virchows Arch 449: 164-170, 2006.
22. Altieri DC: Validating survivin as a cancer therapeutic target. Nat Rev Cancer 3: 46-54, 2003.
23. Yang X, Xiong G, Chen X, Xu X, Wang K, Fu Y, Yang K and Bai Y: Polymorphisms of survivin promoter are associated with risk of esophageal squamous cell carcinoma. J Cancer Res Clin Oncol 135: 1341-1349, 2009.
24. Kato J, Kuwabara Y, Mitani M, Shinoda N, Sato A, Toyama T, Mitsui A, Nishiwaki T, Moriyama S, Kudo J and Fujii Y: Expression of survivin in esophageal cancer: Correlation with the prognosis and response to chemotherapy. Int J Cancer 95: 92-95, 2001.
25. Ikeguchi M and Kaibara N: Survivin messenger RNA expression is a good prognostic biomarker for oesophageal carcinoma. Br J Cancer 87: 883-887, 2002.
26. Grabowski P, Kühnel T, Mühr-Wilkenshoff F, Heine B, Stein H, Höpfner M, Germer CT and Scherübl H: Prognostic value of nuclear survivin expression in oesophageal squamous cell carcinoma. Br J Cancer 88: 115-119, 2003.
27. Dabrowski A, Filip A, Zgodziński W, Dabrowska M, Polańska D, Wójcik M, Zinkiewicz K and Wallner G: Assessment of prognostic significance of cytoplasmic survivin expression in advanced oesophageal cancer. Folia Histochem Cytobiol 42: 169-172, 2004.
28. Mita AC, Mita MM, Nawrocki ST and Giles FJ: Survivin: Key regulator of mitosis and apoptosis and novel target for cancer therapeutics. Clin Cancer Res 14: 5000-5005, 2008.
29. Li C, Li Z, Zhu M, Zhao T, Chen L, Ji W, Chen H and Su C: Clinicopathological and prognostic significance of survivin over-expression in patients with esophageal squamous cell carcinoma: A meta-analysis. PLoS One 7: e44764, 2012.
30. Altieri DC: Survivin, cancer networks and pathway-directed drug discovery. Nat Rev Cancer 8: 61-70, 2008.
31. Altieri DC: Survivin and IAP proteins in cell-death mechanisms. Biochem J 430: 199-205, 2010.
32. Kanwar JR, Kamalapuram SK and Kanwar RK: Targeting survivin in cancer: The cell-signalling perspective. Drug Discov Today 16: 485-494, 2011.
33. Ryan BM, O'Donovan N and Duffy MJ: Survivin: A new target for anti-cancer therapy. Cancer Treat Rev 35: 553-562, 2009.
34. Saito T, Hama S, Izumi H, Yamasaki F, Kajiwara Y, Matsuura S, Morishima K, Hidaka T, Shrestha P, Sugiyama K and Kurisu K: Centrosome amplification induced by survivin suppression enhances both chromosome instability and radiosensitivity in glioma cells. Br J Cancer 98: 345-355, 2008.
35. Sharma H, Sen S, Lo Muzio L, Mariggiò A and Singh N: Antisense-mediated downregulation of anti-apoptotic proteins induces apoptosis and sensitizes head and neck squamous cell carcinoma cells to chemotherapy. Cancer Biol Ther 4: 720-727, 2005.
36. Islam A, Kageyama H, Takada N, Kawamoto T, Takayasu H, Isogai E, Ohira M, Hashizume K, Kobayashi H, Kaneko Y and Nakagawara A: High expression of Survivin, mapped to 17q25, is significantly associated with poor prognostic factors and promotes cell survival in human neuroblastoma. Oncogene 19: 617-623, 2000.

37. Hattori M, Sakamoto H, Satoh K and Yamamoto T: DNA demethylase is expressed in ovarian cancers and the expression correlates with demethylation of CpG sites in the promoter region of c-erbB-2 and survivin genes. Cancer Lett 169: 155-164, 2001.
38. Li F and Altieri DC: Transcriptional analysis of human survivin gene expression. Biochem J 344: 305-311, 1999.
39. Kawakami H, Tomita M, Matsuda T, Ohta T, Tanaka Y, Fujii M, Hatano M, Tokuhisa T and Mori N: Transcriptional activation of survivin through the NF-kappaB pathway by human T-cell leukemia virus type I tax. Int J Cancer 115: 967-974, 2005.
40. Schreck R, Albermann K and Baeuerle PA: Nuclear factor kappa B: An oxidative stress-responsive transcription factor of eukaryotic cells (a review). Free Radic Res Commun 17: 221-237, 1992.
41. Takizawa BT, Uchio EM, Cohen JJ, Wheeler MA and Weiss RM: Downregulation of survivin is associated with reductions in TNF receptors' mRNA and protein and alterations in nuclear factor kappa B signaling in urothelial cancer cells. Cancer Invest 25: 678-684, 2007.
42. Hayden MS and Ghosh S: Signaling to NF-kappaB. Genes Dev 18: 2195-2224, 2004.
43. Sen R and Baltimore D: Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. Cell 47: 921-928, 1986.
44. Scheidereit C: IkappaB kinase complexes: Gateways to NF-kappaB activation and transcription. Oncogene 25: 6685-6705, 2006.
45. Kim HJ, Hawke N and Baldwin AS: NF-kappaB and IKK as therapeutic targets in cancer. Cell Death Differ 13: 738-747, 2006.
46. Karin M: NF-kappaB and cancer: Mechanisms and targets. Mol Carcinog 45: 355-361, 2006.
47. Lin C, Song L, Gong H, Liu A, Lin X, Wu J, Li M and Li J: Nkx2-8 downregulation promotes angiogenesis and activates NF-κB in esophageal cancer. Cancer Res 73: 3638-3648, 2013.
48. Zhu H, Wang Q, Hu C, Zhang W, Quan L, Liu M, Xu N and Xiao Z: High expression of survivin predicts poor prognosis in esophageal squamous cell carcinoma following radiotherapy. Tumour Biol 32: 1147-1153, 2011.
49. Cao M, Yie SM, Wu SM, Chen S, Lou B, He X, Ye SR, Xie K, Rao L, Gao E and Ye NY: Detection of survivin-expressing circulating cancer cells in the peripheral blood of patients with esophageal squamous cell carcinoma and its clinical significance. Clin Exp Metastasis 26: 751-758, 2009.
50. Debruyne PR, Witek M, Gong L, Birbe R, Chervoneva I, Jin T, Domon-Cell C, Palazzo JP, Freund JN, Li P, *et al*: Bile acids induce ectopic expression of intestinal guanylyl cyclase C through nuclear factor-kappaB and Cdx2 in human esophageal cells. Gastroenterology 130: 1191-1206, 2006.
51. Konturek PC, Nikiforuk A, Kania J, Raithel M, Hahn EG and Mühldorfer S: Activation of NFkappaB represents the central event in the neoplastic progression associated with Barrett's esophagus: A possible link to the inflammation and overexpression of COX-2, PPARgamma and growth factors. Dig Dis Sci 49: 1075-1083, 2004.
52. Kang MR, Kim MS, Kim SS, Ahn CH, Yoo NJ and Lee SH: NF-kappaB signalling proteins p50/p105, p52/p100, RelA, and IKKepsilon are over-expressed in oesophageal squamous cell carcinomas. Pathology 41: 622-625, 2009.
53. Kausar T, Sharma R, Hasan MR, Tripathi SC, Saraya A, Chattopadhyay TK, Gupta SD and Ralhan R: Clinical significance of GPR56, transglutaminase 2, and NF-κB in esophageal squamous cell carcinoma. Cancer Invest 29: 42-48, 2011.
54. Izzo JG, Malhotra U, Wu TT, Ensor J, Luthra R, Lee JH, Swisher SG, Liao Z, Chao KS, Hittelman WN, *et al*: Association of activated transcription factor nuclear factor kappab with chemo-radiation resistance and poor outcome in esophageal carcinoma. J Clin Oncol 24: 748-754, 2006.
55. Li B, Li YY, Tsao SW and Cheung AL: Targeting NF-kappaB signaling pathway suppresses tumor growth, angiogenesis, and metastasis of human esophageal cancer. Mol Cancer Ther 8: 2635-2644, 2009.
56. Song L, Gong H, Lin C, Wang C, Liu L, Wu J, Li M and Li J: Flotillin-1 promotes tumor necrosis factor-α receptor signaling and activation of NF-κB in esophageal squamous cell carcinoma cells. Gastroenterology 143: 995-1005, 2012.
57. Tian F, Zhang C, Tian W, Jiang Y and Zhang X: Comparison of the effect of p65 siRNA and curcumin in promoting apoptosis in esophageal squamous cell carcinoma cells and in nude mice. Oncol Rep 28: 232-240, 2012.
58. Oeckinghaus A and Ghosh S: The NF-kappaB family of transcription factors and its regulation. Cold Spring Harb Perspect Biol 1: a000034, 2009.
59. Sakurai H, Chiba H, Miyoshi H, Sugita T and Toriumi W: IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain. J Biol Chem 274: 30353-30356, 1999.
60. Hayden MS and Ghosh S:S hared principles in NF-kappaB signaling. Cell 132: 344-362, 2008.