Survivin regulates Bad gene expression by binding to its promoter and modulates cell cycle and apoptosis in esophageal carcinoma cell

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Abstract

Esophageal cancer (EC) is the eighth most prevalent cancer and the sixth leading cause of cancer-related mortality worldwide. As an anti-apoptotic and a pro-apoptotic protein respectively, survivin and Bad play important role in carcinogenesis of the most human cancers including EC. However the regulatory relationships between them remain unclear. We sought to investigate the effects of survivin knockdown and overexpression on the expression of Bad gene, cell cycle progression and apoptosis of esophageal carcinoma cell. The mRNA expression levels of survivin and Bad were determined in EC tissue samples. The knockdown and overexpression experiments were performed in ECA109 and KYSE450 cells via transfection with survivin overexpression and shRNA plasmids. RT-qPCR and Western blot analysis were used to detect mRNA and protein expression respectively. Cell cycle and apoptosis were analyzed by flow cytometry. The chromatin immunoprecipitation (ChIP) was conducted to determine the binding sites of survivin on the promoter of Bad gene. By analyzing the mRNA expression of survivin and Bad in 40 ESCC patient specimens, we found that the positive expression rate of survivin in tumor tissues (88%, 35/40) was remarkably high, compared with the distal non-tumor tissues (48%, 19/40, p<0.01). On the other hand, the positive expression rate of Bad in tumor tissues (70%, 28/40) was remarkably low, compared with the distal non-tumor tissues (95%, 38/40, p<0.01). Overexpression of survivin decreases Bad mRNA and protein expression, and promotes transformation of cell cycle to S phase. Conversely, knockdown of survivin increases Bad mRNA and protein expression, and induces cell cycle arrest and apoptosis. ChIP assays indicate that survivin directly binds to the Bad promoter region, diminishing the transcriptional activity of Bad. In conclusion, the result suggested that survivin regulates Bad gene expression by binding to its promoter and modulates cell cycle and apoptosis in esophageal carcinoma cell.
Introduction

Esophageal cancer (EC) is the eighth most prevalent cancer and the sixth leading cause of cancer-related mortality worldwide \[^{[1, 2]}\]. It has two major histologic types including esophageal squamous cell carcinoma (ESCC) and adenocarcinoma. It is estimated that more than 480,000 new cases are diagnosed yearly \[^{[2]}\] and more than 80% of esophageal cancers are ESCC \[^{[3]}\]. Lacking of sensitive method for early detection of ESCC, many patients with these tumors have adjacent invasion or distal metastases at the time of diagnosis. Despite recent advances in surgical techniques and chemoradiation, the prognosis of ESCC is relatively poor and the survival rate remains generally low \[^{[4]}\].

Recently, an increasing highlight has been focused on survivin as an important marker for diagnosis and prognosis, a molecular target for therapeutic interventions and a crucial mechanism for tumorigenesis \[^{[5-8]}\].

Survivin protein, a member of inhibitor of apoptosis (IAP) family, is encoded by the BIRC5 gene in humans \[^{[9]}\]. IAPs block apoptosis process by inhibiting the activation of caspases through direct binding to them. Survivin interact with effector caspases (caspase-3 and 7), functioning as a negative regulator of apoptosis process \[^{[10]}\]. Survivin has been extensively reported in various human cancers as a prognostic marker or a therapeutic target because of its important role in cell processes, such as apoptosis and cell division \[^{[5-10]}\]. However, there are rare reports on survivin as a transcriptional regulator of genes involving in tumorigenesis. Previously, we reported that survivin activates NF-κB (nuclear factor kappa-B) p65 by regulating the expression levels of IKKβ (inhibitor of nuclear factor κB kinase subunit β) in esophageal cancer cell lines \[^{[11]}\].

BH3-only protein Bad (Bcl-XI/Bcl-2-associated death promoter homologue), a member of
the Bcl-2 family, characters as a pro-apoptotic protein \(^{12}\). Dephosphorylated Bad translocates to mitochondrial membrane where it binds to and inactivates the anti-apoptotic protein including Bcl-2 and Bcl-xl \(^{13}\). Phosphorylation of Bad at Ser112, Ser136, and Ser155 inhibits its pro-apoptotic activity in response to growth and survival signal \(^{13}\). Thereby, Bad plays a crucial role in connecting the cell survival signaling pathway and apoptosis signaling pathway. Clinical significance of Bad has been identified in many types of cancer. Both high expression and low expression of Bad are associated with outcomes of patient with cancer \(^{14-16}\).

In present study, mRNA expression of survivin and Bad in 40 paired tumor tissues of ESCC patients was examined. Subsequently, the experiments of up-regulation and down-regulation of survivin were performed by infecting EC cell lines (KYSE450, ECA109) with overexpression and shRNA plasmids. After transfection 48 hours, mRNA and proteins expression levels of survivin and Bad were examined. Meanwhile apoptosis rate and cell cycle distribution were evaluated. Finally, in order to confirm the transcriptional regulation between survivin and Bad, ChIP assay were conducted to determine whether survivin binds to the promoter region of Bad gene. Our findings demonstrated that Survivin regulates Bad gene expression by binding to its promoter and modulates cell cycle and apoptosis in EC cell lines.

**Method And Materials**

**Tumor tissue specimens**

Forty pairs of tumor and distal normal tissue samples were prospectively collected from surgically excised specimens of patients with ESCC at the Affiliated Cancer Hospital of Xinjiang Medical University (Urumqi, China) between July and December 2016. The tumor and distal tissues were frozen in liquid nitrogen immediately following resection. All
patients in the current study did not received chemotherapy or radiation therapy prior to surgery. The 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

ECA109 cell line was obtained from the Cell Bank of Xinjiang Medical University (Urumqi, China). KYSE450 cell line was purchased from the Cell Bank of the Chinese Academy of Sciences. ECA109 and KYSE450 cells were cultured in RPMI1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heatinactivated fetal bovine serum (FBS; Zhejiang Tianhang Biological Technology Co., Ltd., Zhejiang, China), 100 units/ml penicillin, and 100 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified 5% CO2 atmosphere.

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 2. The resulting PCR products were inserted into the GV142 vector between HindIII and XhoI sites, yielding GV142-survivin overexpression and GV142-control plasmids.

| Target gene | Primers 5’→3’ |
|-------------|---------------|
|             | Forward | Reverse |
| Survivin    | TGCCAAGCTTTGTTGCCCCGAC | GCCAATTCATGGGCAGCCA |
| Control     | TTATGGGTGCCCCGACGTTGC | GCGGTAGCTCAGCTGCTGTC |

LV3-survivin shRNA plasmid constructs

The LV3 vector was purchased from Shanghai GeneChem Co., Ltd. (Shanghai, China). The
sequences of small shRNA targeting survivin were designed as follows:
GAAAGTGCGCCGTGCCATCTTTCAAGAGAGATGGCACGGCGCACTTTCTT. The sequences of negative control were designed as follows:
GCGCGCACAATCTACGCTAGTTTCAAGAGAACTAGCGTAGATTGTGCACGCTT. The sequences were inserted between the HindIII and XhoI sites of the LV3 vector chemically synthesized by Shanghai GeneChem Co., Ltd. The constructs were verified by DNA sequence analysis.

Plasmid transfection

Prior to electroporation, KYSE450 and Eca109 cells were ensured in exponential growth phase. Culture medium was removed and replaced with fresh serum-free Opti-MEMI medium. Cells were clicked and re-suspended before centrifugation. After centrifugation the supermatant was discarded. This step was repeated twice. 400 ul cell suspension was transferred into electroporation cuvette. Then plasmid was added to cuvette. The cuvette was subjected to the electroporation (500 V, 15 ms and square wave). After electroporation, the cells were transferred into 6-well plate containing complete medium to culture. Transfection efficiency was checked 24 hours after transfection by watching the glowing cell under fluorescence microscope because the plasmid contained the fluorescent protein gene.

Groups of cell infected with plasmids

Cancer cells including KYSE450 and ECA109 were divided into three groups both in survivin overexpression and knock-down experiment. Groups in survivin overexpression are UP group, NC group and BC group. Cells in the UP group were transfected with GV142-survivin. Cells in the NC group were transfected with GV142-negative control. BC group meaning blank control, Cells in the BC group were not treated with any plasmid during the electroporation. Groups in survivin knockdown are KD group, NC group and BC group. Cells
in the KD group were transfected with LV3-survivin shRNA. Cells in the NC group were transfected with LV3-negative control. Cells in the BC group were not treated with any plasmid during the electroporation.

**RT-PCR for analysis of mRAN from patient’s tissue**

Total RNA was isolated with TriZOL (Thermo Fisher Scientific, Inc.) following instructions provided by manufacturer. Specific PCR products were generated using the following primers: Survivin: forward primer 5’-CCCTGCCTGGCAGCCCTTTC-3’ and reverse primer 5’-CTGGCTCCAGCCTTCCA-3’; Bad: forward primer 5’-CAGAGTTTGAGCCGAGTGAGC-3’ and reverse primer 5’-CCCATCCCCTCGTCGTCC-3’; GAPDH: forward primer 5’-GGGAAACTGTGGCGTGAT-3’ and reverse primer 5’-AAAGGTGGAGGAGTGTTGGT-3’. RNA samples were quantified with ultraviolet spectrophotometer and severed as templates to generate cDNA.

**Real-Time quantitative PCR for analysis of cellular mRNA**

48 hours after transfection, total cellular RNA extraction from cultured cell lines was performed using TRlzol (Thermo Fisher Scientific, Inc.) following instructions provided by manufacturer. One µg of total RNA extracted from the cells was subjected to reverse transcription. Maloney Murine Leukemia Virus Reverse Transcriptase (Promega Corporation, Madison, WI, USA) was used for cDNA synthesis. Specific primers were as following. Survivin: forward primer 5’-CCCTGCCTGGCAGCCCTTTC-3’ and reverse primer 5’-CTGGCTCCAGCCTTCCA-3’; Bad: forward primer 5’-CAGAGTTTGAGCCGAGTGAGC-3’ and reverse primer 5’-CCCATCCCCTCGTCGTCC-3’; Caspase-3: forward primer 5’-GCTATTGTGAGGCGGTGT-3’ and reverse primer 5’-AGCAGGGCTCGTAACTC-3’; Caspase-9: forward primer 5’-CGAACTAACAGGCAAGCA-3’ and reverse primer 5’-GCACCGACATCACCAAT-3’; GAPDH: forward primer 5’-GGGAAACTGTGGCGTGAT-3’ and
reverse primer 5′-AAAGGTGGAGGAGTGGGT-3′. Real-time PCR (RT-qPCR) was used to quantify the expression level of the Survivin, Bad, Caspase-3 and Caspase-7 gene in the ESCC cell lines ECA109 and KYSE450 using the TaqMan® Fast Virus 1-Step Master mix kit (Thermo Fisher Scientific, Inc.), according to the protocol supplied by manufacturer. Amplification conditions 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 calculated using the 2-ΔΔCq method. The experiments were performed in triplicate for each cell line and results are presented as the mean ± standard deviation.

**Western blotting assay**

Forty-eight hours after transfection, attached and floating cells were harvested on ice. The cells were washed with cold PBS and subsequently lysed in cold RIPA 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 H2O] for 30 min on ice. Clear protein extracts were obtained by centrifugation at 18,407 x g for 15 min at 4°C. Protein concentrations were determined by Pierce BCA protein assay (Thermo Fisher Scientific, Inc.) and 20 mg of protein mixed with loading buffer was loaded per lane, and separated by 10-15% polyacrylamide gels (Wuhan Boster Biological Technology, Ltd.). Proteins were transferred to PVDF membranes. Nonspecific binding was blocked by 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. Membranes were incubated with the primary antibody overnight at 4°C. The primary antibodies include rabbit anti-survivin, anti-Bad, anti-Bad, anti-Caspase 3, anti-Caspase 7 and anti-GAPDH served as a loading control. Then the membranes were washed three times with TBST at room temperature and incubated with
appropriate horseradish peroxidase-linked goat anti-rabbit secondary antibodies at a dilution of 1:1,000 (cat. no. BA1054; WuhanBoster Biological Technology, Ltd.) diluted in TBST for 1 h at room temperature. The immunoreactive bands were visualized using an Enhanced Chemiluminescence Detection kit (Thermo Fisher Scientific, Inc.).

**Cycle and cell apoptosis analysis by flow cytometry**

ECA109 and KYSE450 cells were directly incubated, at 37°C for 48 h, in 6well plates and collected 48 h after transfection. Then, the cells were treated with the indicative reagent propidium iodide (PI) and Annexin V staining kit (BestBio Co. Ltd.). For the cell cycle analysis, the cells were washed with phosphate buffered saline (PBS) for 5 min and subsequently centrifugation at 900 g. The cells were collected and fixed in icecold 70% ethanol for 2 hours 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 prior to cell cycle analysis. Analysis of the samples was performed within 24 h. To determine the apoptosis rate, the transfected cells were washed twice with icecold PBS, and resuspended in 195 µl 1X Binding Buffer (EMD Millipore) to a concentration of 1 × 104 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 fluorescenceactivated cell sorting with a BD FACSMicroCount™ system (BD Biosciences, Franklin Lakes, NJ, USA). The experiments were performed independently in triplicate for each cell line.

**Chromatin immunoprecipitation assay (ChIP)**
To determine whether survivin binds to the Bad promoter region, ChIP assays were performed using the ChIP kit (EPIGENTEK, USA) according to the manufacturer's protocols. DNA and proteins in ECA109 and KYSE450 cells were cross-linked by treatment with 1% formaldehyde (Sigma-Aldrich) for 10 min. The cells were washed twice with 1X PBS, lysed, and sonicated to reduce DNA lengths to within the range of 200-1,000 bp. Immunoprecipitation was then performed using 4 µg rabbit antibody against survivin to incubate. The group which incubated with normal mouse IgG served as the negative control. The group which incubated with 1 µl Anti-RNA Polymerase II (dilution, 1:1,000) served as the positive control. The immune complexes were precipitated, eluted, reverse-crosslinked and treated with proteinase K [Tiangen Biotech (Beijing) Co., Ltd., Beijing, China]. The primers designed to amplify various regions of the human Bad gene promoters were as follows: region 1(196 bp): 5’-GAGGTTCAATAAGCGTCAAGGT-3’(forward) and 5’-GTATGGGACAAAGCTC-3’(reverse); region 2 (252 bp): 5’-CCTTCGCCCGCAGTAATC-3’ (forward) and 5’-CCTCGTCCGCATCCTTTT-3’ (reverse); region 3(431 bp): 5’-CTGGGCAAAGTAGAGGTTCAT-3’(forward) and 5’-TCCGTATTTATTTCCCTGGTC-3’ (reverse); region 4(490): 5’-CTGGGCAAAGTAGAGGTTCAT - 3’(forward) and 5’-TCCGTATTTATTTCCCTGGTC-3’ (reverse). The group which did not add any primers served as the primer blank control. PCR fragments were separated and visualized on 1.8% agarose gels stained with ethidium bromide (Shanghai Bioleaf Biotech Co., Ltd., Shanghai, China). All ChIP assays were performed independently in triplicate and the most representative results are illustrated in the figures.

Statistical analysis

All statistical analyses were performed with SPSS version 17.0 for Windows (SPSS, Inc., Chicago, IL, USA). Difference and correlation were analyzed by χ² test. P < 0.05 was considered statistically significant. Data (mRNA/protein levels, cell cycle, and cell
apoptosis) were expressed as the mean ± standard deviation from three independent experiments. Data were analyzed by one-way analysis of variance followed by the LSD post hoc test used to compare mean differences in two groups. P < 0.05 was considered to indicate a statistically significant difference.

Result

Expression of survivin and Bad in esophageal cancer studied by RT-PCR

We studied 40 patients with ESCC. Tumor samples and paired distal normal tissues for mRNA expression of survivin and Bad were examined by RT-PCR. Survivin was expressed in 35 of 40 tumor tissue, expression rate was 88%; and expressed in the 19 of 40 normal tissues, expression rate was 48%. The positive expression rate of survivin in tumor tissues was remarkably high, compared with the distal non-tumor tissues (p < 0.01, Table 1). Bad was expressed in 28 of 40 tumor tissue, expression rate was 70%; and expressed in the 38 of 40 normal tissues, expression rate was 95% (Table 1). The positive expression rate of Bad in tumor tissues was remarkably low, compared with the distal non-tumor tissues (p < 0.01, Table 1).

| Tumor tissue | Normal tissue | $\chi^2$ | P     |
|--------------|---------------|---------|-------|
| Survivin     |               |         |       |
| (+)          | (+)           | 14.587  | 0.000 |
| (-)          | (-)           |         |       |
| Bad          |               |         |       |
| (+)          | (+)           | 8.658   | 0.003 |
| (-)          | (-)           |         |       |

Overexpression of survivin decreases Bad mRNA and protein expression

To further explore the regulation relationship between survivin and pro-apoptotic factor Bad, we examined whether overexpression of survivin was able to modulate the expression of Bad and other apoptosis-associated proteins including Casepase-3 and 7.

Following the transfection of KYSE450 and ECA109 cells with GV142-survivin overexpression plasmid and controls, the mRNA levels of survivin, Bad, Casepase-3 and 7
were examined by RT-qPCR. The results indicated that when survivin was overexpression in KYSE450 cells, Bad (P < 0.05, Fig. 1A) and Casepase-3 (P < 0.05) were downregulated but Casepase-7 (P > 0.05) (Fig. 1A). When survivin was overexpression in ECA109 cells, only Bad (P < 0.05, Fig. 1B) was downregulated, compared to blank and negative control (Fig. 1). Immunoblotting confirmed that overexpression of survivin can downregulate expression of Bad protein both in KYSE450 and ECA109 cells (Fig. 1).

Knockdown of survivin increases Bad mRNA and protein expression

Following the transfection of KYSE450 and ECA109 cells with LV3-survivin shRNA plasmid and controls, the mRNA levels of survivin, Bad, Casepase-3 and 7 were examined by RT-qPCR. The results indicated that when survivin downregulation in KYSE450 cells, Bad (P < 0.01) was up-regulated but there were no significant differences in Casepase-3 (P > 0.05) and Casepase-7 (P > 0.05) (Fig. 1). When survivin was downregulation in ECA109 cells, Bad (P < 0.01) was up-regulated but Casepase-3 (P > 0.05) and Casepase-7 (P > 0.05) (Fig. 1), compared to BC and NC group (Fig. 1). Immunoblotting confirmed that downexpression of survivin can upregulate expression of Bad protein both in KYSE450 and ECA109 cells (Fig. 1).

Survivin overexpression promotes transformation of cell cycle to S phase

To explore the effects of survivin overexpression on the viability of esophageal cancer cell, flow cytometry was adopted to detect alterations in cell cycle progression and apoptosis following survivin overexpression. In KES450 cell lines, cytometry showed that the proportions of cells in the G1 phase among the BC, NC and UP group were (50.52 ± 0.67)%, (50.71 ± 1.01)% and (52.09 ± 1.27)%, respectively. For these groups, the proportions of cells in the G2/M phase were (23.11 ± 1.23)%, (21.20 ± 0.63)% and (9.96 ± 1.38)% respectively, and the proportions of cells in the S phase were (26.37 ± 0.80)%,
(28.09 ± 1.47)% and (37.85 ± 1.26)%, respectively. Compared with the BC and NC group, the proportion of cells in S phase in the UP group was significantly increased (P < 0.01, Fig. 2A), whereas the ratio of cells in the G2/M phase was significantly decreased (P < 0.01, Fig. 2A). However, there were no significant differences between the BC and NC groups. The significantly alteration of apoptotic rate was not found among the UP group (24.01 ± 1.75%), the BC (6.42 ± 0.95%) and NC (10.23 ± 0.56%) groups (P > 0.05, Fig. 3A).

In ECA109 cell lines, approximately similar results of cell cycle progression and apoptosis were observed (Fig. 2B and Fig. 3A). These results suggested that survivin overexpression promotes transformation of cell cycle to S phase.

Survivin knockdown induces cell cycle arrest and apoptosis

In KES450 cell lines, cytometry showed that the proportions of cells in the G1 phase among the BC, NC and KD group were (50.52 ± 0.67)%, (50.71 ± 1.01)% and (56.83 ± 1.96)%, respectively. For these groups, the proportions of cells in the G2/M phase were (23.11 ± 1.23)%, (21.20 ± 0.63)% and (27.13 ± 1.09)% respectively, and the proportions of cells in the S phase were (26.37 ± 0.80)%, (28.09 ± 1.47)% and (16.04 ± 1.63)%, respectively. Compared with the BC and NC group, the proportion of cells in S phase in the KD group was significantly decreased (Fig. 2C, P < 0.01), whereas the ratio of cells in the G2/M phase was significantly increased (Fig. 2C, P < 0.01). Apoptotic rate of the KD group (24.01 ± 1.75%) was significantly higher compared with the BC (6.42 ± 0.95%) and NC (10.23 ± 0.56%) groups (P < 0.01) (Fig. 3B). However, there were no significant differences between the BC and NC groups. In ECA109 cell lines, approximately similar results of cell cycle progression and apoptosis were observed (Fig. 2D and Fig. 3B). These results suggested that survivin knockdown induces cell cycle arrest and apoptosis in esophageal carcinoma cell.
Survivin binds to Bad promoter and regulates Bad mRAN expression in KESE450 and Eca109 cell lines

A ChIP assay was performed to further confirm the direct interaction between survivin and the Bad promoter regions. Chromatin samples were incubated with anti-survivin antibody, Anti-RNA Polymerase II (positive control) and normal mouse IgG (negative control). The recovery DNA fragments from ChIP experiment in KESE450 and ECA109 cell lines were subjected to PCR with promoter-specific primers for Bad. Four primers were designed to amplify various regions of the human Bad gene promoter. The group which did not add any primers served as primer blank control. The positive results of amplified fragment in the 4th and 5th lane of agarose gel indicated that there were bind sites of survivin protein in the promoter region of Bad gene (Fig. 4). These findings indicate that survivin directly binds to the Bad promoter region, diminishing the transcriptional activity of Bad.

Discussion

As an anti-apoptotic and a pro-apoptotic protein respectively, survivin and Bad play important role in carcinogenesis of most human cancers. A number of studies have indicated survivin are highly expressed in different cancer cells and primary tumor biopsy samples, but it is present at low levels or is completely absent in healthy cells and tissues [6]. However, expression of Bad is down regulated in many tumor tissues [16]. Our data showed that mRNA level of survivin was increased, while mRNA level of Bad was decreased in ESCC tissues compared to normal tissues.

Based on their expression characteristics in ESCC samples and the relevant literature reports, we speculate that there may be regulatory relationship between survivin and Bad. In order to test this hypothesis, we conducted a series of experiments to manipulate expression of survivin by transfecting KESE450 and ECA109 cells with survivin expression
vector and siSurvivin. We measured the mRNA and protein expression of Bad, Casepase-3 and Casepase-7 in KESE450 and ECA109 cells after transfection of the survivin expression vector and siSurvivin. Western blot and qPCR analyses demonstrated that downregulation of survivin induced increases in Bad mRNA and protein levels in both cell lines, whereas overexpression of survivin resulted in decreased Bad mRNA and protein. However, there were not same effects in Casepase-3 and 7. It indicted that survivin might not regulate the expression of Casepase-3 and 7, but binds directly to them, inhibiting the caspase-mediated cascade leading to apoptosis. Meanwhile, the biology behaviors of two cell lines including apoptosis rate and cell cycle distribution were also evaluated. Cytometry showed that survivin overexpression promotes transformation of cell cycle to S phase and knockdown induces cell cycle arrest and apoptosis. This result was consistent with our previous study and literature reports [11].

These findings made us to suspect the putative roles for survivin as a transcription regulator for Bad. A ChIP assay was conducted to further confirm the direct interaction between survivin and the Bad promoter. The ChIP assay indicated that survivin directly binds to the Bad promoter region, diminishing the transcriptional activity of Bad. According to our best knowledge, this is the first study to find that survivin as a transcriptional regulator regulates the expression of Bad. The result suggested that survivin is a novel transcriptional regulator of Bad. Survivin regulates Bad gene expression by binding to its promoter and modulates cell cycle and apoptosis in esophageal carcinoma cell.

Conclusions

In conclusion, our study demonstrated that mRNA level of survivin was increased, while mRNA level of Bad was decreased in ESCC tissues compared to normal tissues.
Downregulation of survivin induced increases in Bad mRNA and protein levels in KESE450 and ECA109 cell lines, whereas overexpression of survivin resulted in decreased Bad mRNA and protein. Survivin overexpression promotes transformation of cell cycle to S phase and knockdown induces cell cycle arrest and apoptosis. A ChIP assay confirmed that the direct interaction between survivin and the Bad promoter. The result suggested that survivin is a novel transcriptional regulator of Bad. Survivin regulates Bad gene expression by binding to its promoter and modulates cell cycle and apoptosis in esophageal carcinoma cell.

Declarations

Author's contribution

YC made substantial contributions to conception and design of project. SB designed and conducted most of the experiments. XL and LM cultured the cells and performed plasmids transfection. WY collected patient sample and clinical data. JR contributed to design of project and writing manuscript. All authors contributed toward data analysis, drafting and critically revising the paper. All authors approved the final submitted version.

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Competing interests

The authors declared that they have no conflict of interest in relation to the article.

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Figures
The effects of survivin overexpression and knockdown on the expression of Bad, casepase-3 and casepase-7 gene. The expressions of survivin, Bad, casepase-3 and 7 were determined by qRT-PCR and confirmed by western blot. GADPH served as an internal and loading control. A: When survivin was overexpression in KYSE450 cells, Bad(*P<0.05) and casepase-3(*P<0.05) were downregulated. B: When survivin was overexpression in ECA109 cells, Bad(**P<0.01) was downregulated. C: When survivin was downregulation in KYSE450 cells, Bad(P<0.01) was up-regulated but there were no significant differences in Casepase-3(P>0.05) and Casepase-7 (P>0.05). D: When survivin was downregulation in ECA109 cells, Bad(P<0.01) was up-regulated but Casepase-3(P>0.05) and Casepase-7 (P>0.05) (Fig 1), compared to BC and NC group. Data are presented as the means ± standard deviation from triplicate experiments. BC: Blank control. NC, UP, KD: Cells in these groups were transfected with negative control, overexpression and shRNA plasmid respectively. Statistically significant differences compared to BC and NC group are indicated (*P<0.05, **P<0.01).
Figure 2

The effects of survivin overexpression and knockdown on the cell cycle progression of esophageal carcinoma cell. A: The UP groups exhibited a decreased number of cells in the G2 phase, but an increased number of cells in the S phases in KYSE450 cells. B: The UP groups exhibited a decreased number of cells in the G2 phase, but an increased number of cells in the S phases in ECA109 cells. C: The KD groups exhibited an increased number of cells in the G2 phase, but a decreased number of cells in the S phases in KYSE450 cells. D: The KD groups exhibited an increased number of cells in the G2 phase, but a decreased
number of cells in the S phases in ECA109 cells. Data are presented as the means ± standard deviation from triplicate experiments. BC: Blank control. NC, UP, KD: Cells in these groups were transfected with negative control, overexpression and shRNA plasmid respectively. Statistically significant differences compared to BC and NC group are indicated (*P<0.05, **P<0.01).

Figure 3

The effects of survivin overexpression and knockdown on the apoptosis of esophageal carcinoma cell. A: There was not significantly alteration of apoptotic rate among the UP group, the BC and NC groups both in KYSE450 and ECA109 cells. B: Apoptotic rate of the KD group was significantly higher compared with the BC and NC groups. Data are presented as the means ± standard deviation from triplicate experiments. BC: Blank control. NC, UP, KD: Cells in these groups were transfected with negative control, overexpression and shRNA plasmid respectively. Statistically significant differences compared to BC and NC group are indicated (*P<0.05, **P<0.01).
The representative PCR results of recovery DNA fragments from ChIP experiment in KESE450 and ECA109 cell lines. The positive results of amplified fragment in the 4th and 5th lane of agarose gel indicated that there were bind sites of survivin protein in the promoter region of Bad gene. 1st lane of agarose gel: primer blank control, the group which did not add any primers. 2nd: negative control, the group which incubated with normal mouse IgG. 3rd: positive control, the group which incubated with 1 µl Anti-RNA Polymerase II (dilution, 1:1,000). 4-7th: 4 primers designed to amplify various regions of the human Bad gene promoter.