Hyperthermia induces apoptosis by targeting Survivin in esophageal cancer

SIDA QIN[1], CHONGWEN XU[1], SHUO LI[1], XIFANG WANG[1,2], XIN SUN[1], PEILI WANG[1], BOXIANG ZHANG[1] and HONG REN[1]

[1]Department of Thoracic Surgery, The First Affiliated Hospital, School of Medicine, Xi'an Jiaotong University, Xi'an, Shaanxi 710061; [2]Department of Medical Oncology, Shaanxi Provincial People's Hospital, The Third Affiliated Hospital of School of Medicine, Xi'an Jiaotong University, Xi'an, Shaanxi 710068, P.R. China

Received June 5, 2015; Accepted July 7, 2015

DOI: 10.3892/or.2015.4252

Abstract. Hyperthermia is considered the fifth pillar of cancer treatment. It induces cancer cell apoptosis, however, its molecular mechanisms remain unclear. In the present study, the role of Survivin in hyperthermia-induced apoptosis in esophageal cancer was investigated. Different temperatures were used to treat EC109 esophageal cancer cells, and their viability was found to be significantly inhibited with a concomitant increase in apoptosis and necrosis. Necrosis increased in a temperature-dependent manner, whereas peak apoptosis was reached at 43˚C. The hyperthermia-induced apoptosis was due to the inhibition of Survivin and the activation of caspase-3. Subsequently, overexpression of Survivin inhibited the activation of caspase-3 and hyperthermia-induced apoptosis, however, this inhibition was reversed in the absence of XIAP. Immunoprecipitations showed that Survivin did not directly bind to caspase-3, whereas XIAP interacted with Survivin and caspase-3. Immunohistochemistry was performed to detect the expression of Survivin in esophageal cancer patient samples. A higher expression of Survivin in esophageal cancer tissues compared to normal tissues was observed, and a high expression correlated with poor prognosis. The results indicated that hyperthermia decreases the expression of Survivin, prevents its binding to XIAP, activates caspase-3 and induces apoptosis. Due to its correlation with poor prognosis, Survivin may be a target for hyperthermia in the treatment of esophageal cancer.

Introduction

Esophageal cancer is a common human gastrointestinal cancer, accounting for 400,000 mortalities and 480,000 new cases in 2008 worldwide (1). Despite advances in early detection and standardized treatment regimens, relatively low 5-year survival rates for esophageal cancer have been observed (2,3). In Eastern Asia and Africa, the incidence rates of esophageal cancer are extremely high (4). Countries in this region are developing countries and their patients may not be able to afford advanced treatments. Therefore, low-cost treatments, such as hyperthermia have been considered.

In the past decade, anticancer trials with multimodal therapies, including hyperthermia have been reported (5,6). These clinical trials demonstrated that hyperthermia increased overall survival by making normally inoperable tumors candidates for surgery and by improving the efficacy of chemo- or radiotherapy with reduced toxicity (7-9). Hyperthermia is considered the fifth pillar of cancer treatment following surgery, chemotherapy, radiotherapy and biotherapy. However, the mechanisms by which hyperthermia improves clinical outcomes remain unclear. Recent findings suggest that hyperthermia impairs protein synthesis, leading to apoptosis (10).

Apoptosis is a conserved and regulated cell suicide process, which is activated and executed by cysteine aspartate-specific proteases (caspases) (11). Survivin, a protein that is often overexpressed in cancer, suppresses apoptosis by inhibiting caspase activation (12). Due to its upregulation in almost all human tumors and its key role in apoptosis, proliferation and angiogenesis, Survivin is considered a crucial target for anticancer therapies (13). Downregulation of Survivin in human cancer cell lines and mouse models inhibits tumor growth and sensitizes tumor cells to chemo- or radiotherapy (14,15).

In the present study, we investigated the function of Survivin in hyperthermia-induced apoptosis in EC109 esophageal cancer cells and examined Survivin expression in patients with esophageal cancer, correlating its expression with clinicopathological characteristics. The results showed that, hyperthermia decreased the expression of Survivin, prevented its binding to XIAP, activated caspase-3 and induced apoptosis. In addition, Survivin expression was higher in esophageal cancer than in normal tissues, and this higher level of expression was associated with poor prognosis. Therefore, Survivin may be a crucial target for hyperthermia in the esophageal cancer treatment.

Correspondence to: Professor Hong Ren, Department of Thoracic Surgery, The First Affiliated Hospital, School of Medicine, Xi'an Jiaotong University, 277 West Yanta Road, Xi'an, Shaanxi 710061, P.R. China
E-mail: renhongs2000@gmail.com

Key words: hyperthermia, apoptosis, Survivin, esophageal cancer

Materials and methods

Construction of plasmids and transfection. Full-length Survivin and XIAP were generated by PCR from EC109 cell cDNA using the primers: Survivin sense, 5'-AGATCTGGA TCCGGTGCCCCGACGTTGCCCCC-3' and antisense, 5'-TCTAGAGCGGCCGCTCAATCCATGGCAGCCAGCT GCTCG-3'; XIAP sense, 5'-AGATCTGGATCCACTTTTAA CAGTTTTGAAGG-3' and antisense, 5'-TCTAGAGCGGCC GCTTAAGACATAAAAATTTTTTGCTTG-3'. The resulting fragments were digested by *Bam*HI and *Not*I (Thermo Scientific, Rockford, IL, USA) and cloned into pcDNA3.0-Flag. The resulting or control plasmids were transfected into the EC109 cells with Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA). Western blotting was used to identify protein overexpression in EC109 cells. The siRNAs for XIAP and the control siRNA were designed and produced by Shanghai GenePharma Co., Ltd. (Shanghai, China).

Cell culture and heat treatment. The human EC109 cells were obtained from the American Type Culture Collection; Manassas, VA, USA). The cells were maintained at 37˚C and 5% CO_2 in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS) (both from GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin and 100 g/ml streptomycin (both from Sigma, St. Louis, MO, USA). For heat treatment, EC109 cells were collected into 1.5 ml Eppendorf tubes and then incubated in a hot water bath at 37˚, 39˚, 41˚, 42˚, 43˚ or 45˚C for 40 min. Following heat treatment, the cells were recovered under standard culture conditions.

MTT assay. After heat treatment, $5x10^3$ EC109 cells were plated in each well of 96-well plates. The following day, 20 μl of MTT (Sigma) was added to each well, and the cells were incubated for an additional 4 h at 37˚C, prior to the addition of 150 μl dimethylsulfoxide (DMSO). After 20 min, absorbance at 490 nm (A490) was measured with a microplate reader (Bio-Rad Laboratories, Richmond, CA, USA). Cell viability (%) was calculated as: the average A490 in an experimental chemotherapy group/the average A490 in the blank control group x 100%.

Flow cytometry. The cells were incubated in a water bath at different temperatures for 40 min, and then plated into 24-well plates at a concentration of $1x10^5$ cells/well. After 24 h, the cells were trypsinized and incubated with 5 μl propidium iodide (PI) and 5 μl Annexin V-FITC (both from Invitrogen Life Technologies) for 15 min. The samples were then analyzed for apoptosis using a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis. Following heat treatment, EC109 cells were collected into a lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100 and protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA)], and incubated for 30 min on ice. Protein (30 μg) for each sample were separated on 12% SDS-PAGE gels (Invitrogen Life Technologies) and transferred to PVDF membranes (EMD Millipore, Billerica, MA, USA). After blocking with 5% BSA (Shanghai Bioleaf Biotech Co., Ltd., Shanghai, China) at room temperature for 1 h, the membranes were incubated with primary antibody overnight at 4˚C, incubated with horseradish peroxidase-labeled goat anti-rabbit IgG antibody (1:5,000; sc-45101; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at room temperature and developed using ECL detection (Thermo Scientific, Waltham, MA, USA). β-actin was used as a loading control. For immunodetection, the primary antibodies used were: anti-Survivin rabbit polyclonal IgG antibody (1:1,000; sc-10811; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) anti-Smac rabbit monoclonal antibody (1:1,000; 1012-1), anti-XIAP rabbit polyclonal antibody (1:2,000; S1022) (both from Epitomics, Burlingame, CA, USA), anti-bcl-2, anti-bax, anti-bcl-xl (rabbit polyclonal antibody, 1:1,000, sc-783, sc-493, sc-7195; Santa Cruz Biotechnology, Inc.), and anti-pro-caspase-3 rabbit monoclonal, anti-active caspase-3 rabbit polyclonal antibodies (1:1,000; ab32499; ab2302), and anti-β-actin rabbit polyclonal antibody (1:5,000; ab75186) (all from Abcam, Cambridge, UK).

Co-immunoprecipitation. The EC109 cells were transfected with Flag-Survivin, Flag-XIAP or Flag control vector for 48 h, collected in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100 and protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA)], and then incubated for 30 min on ice. Protein (500 μg) from the cell lysates was incubated with 20 μl Anti-FLAG M2 Affinity Gel (Sigma) for 2 h at 4˚C. The collected beads were washed three times with lysis buffer and then eluted with SDS-PAGE sample buffer. Immunoblotting was detected using western blot analysis.

Clinical samples. Primary tumor specimens were obtained from 86 patients diagnosed with esophageal squamous cell carcinomas, who underwent complete resection at the First Affiliated Hospital of Xi'an Jiaotong University from February, 2009 to February, 2011. Normal esophageal tissues were collected at >5 cm from the edge of the tumor. The tissues were fixed in 10% formalin immediately and embedded in paraffin within 24 h after surgical resection. Follow-up information was obtained from a review of the patient medical records. There were 64 males and 22 females and their mean age was 59.8 years (42-77 years). The patients had a single tumor, without distant metastasis and none of them had been previously treated with chemo- or radiotherapy. After surgical resection, the patients underwent standard therapeutic procedures according to the Clinical Oncology Information Network guidelines. Carcinomas were classified in accordance with the tumor-node-metastasis (TNM) classification system of the International Union Against Cancer (UICC) (16). The study design and procedure were approved by the Ethics Committee of the hospital and each participant signed an informed consent document prior to enrollment.

Immunohistochemistry. Resected specimens were fixed with 10% formaldehyde and embedded in paraffin blocks. Sections (5 μm) were deparaffinized with xylene and rehydrated in a series of ethanol concentrations. Endogenous peroxidase activity was blocked by immersion in 0.3% methanolic peroxide for 15 min. Immunoreactivity of the target antigens was enhanced by microwaving the sections for 10 min in

0.1 M citrate buffer, pH 6.0. Test sections were incubated with anti-Survivin rabbit polyclonal IgG antibody (sc-10811; Santa Cruz Biotechnology, Inc.) at a dilution of 1:100 in the blocking solution at 4˚C overnight and goat anti-rabbit IgG/HRP secondary antibody (bs-0295G-HRP; Beijing Biosynthesis Inc., Beijing, China) was used at a dilution of 1:250 for 1 h at room temperature. After washing, the signal was detected using a DAB kit (Beijing Biosynthesis, Inc.). The section was counterstained with hematoxylin and photographed under a light microscope (Nikon, Tokyo, Japan). For the negative controls, the anti-Survivin antibody was replaced with 1% bovine serum albumin (Shanghai Bioleaf Biotech Co., Ltd.) in phosphate-buffered saline (PBS). No staining was detected in any control section.

Immunohistochemical scoring. The stained slides were assessed by two independent pathologists, without knowledge of the patient clinical data. Ten randomly-selected fields for each slide were scored for the area and intensity of positively stained (brown) cytoplasm and/or cell membrane under light microscopy. The intensity of Survivin staining was scored as: 0, no signal; 1, weak; 2, moderate; and 3, marked. Percentage scores were assigned as: 1, 1-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%. The scores of each tumor sample were multiplied to give a final score of 0-12, and the tumors were designated as negative expression (-), score 0-4; weak expression (+), score 5-8; high expression (++), score 9-12. Tumor samples scored (+) to (++) were considered positive.

Statistical analysis. The χ^2 and Fisher's exact tests were used to compare frequencies. The Kaplan-Meier method was used to assess prognosis after surgery, and the log-rank test was used to compare survival curves. Univariate analysis was performed with the Cox regression model. Most pathological variables were used as dichotomized variables: Age (<60 vs. ≥60 years), location (upper+middle vs. lower thoracic), tumor differentiation (high+middle vs. low), tumor size (<5 vs. ≥5 cm), lymph node metastasis (N0 vs. N1+N2+N3) and stage (I+II vs. III). Statistical analyses were performed using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant result.

Results

Hyperthermia induces apoptosis and necrosis in EC109 cells. To analyze the effect of hyperthermia in esophageal cancer cells, we first treated EC109 cells at different temperatures (37˚C, 39˚C, 41˚C, 42˚C, 43˚C and 45˚C) for 40 min. After the cells were incubated at 37˚C in 5% CO_2 for another 24 h, cell viability was assessed using an MTT assay. With higher temperatures, EC109 cells showed significantly decreased viability (Fig. 1). To determine whether EC109 cells treated with hyperthermia underwent apoptosis, we treated cells as before, and after 24 h the cells were stained with Annexin V-FITC and PI to assess necrosis and apoptosis induction by flow cytometry. As shown in Fig. 2A, necrosis was calculated as Annexin V ($^-$) and PI ($^+$) while apoptosis was indicated by Annexin V ($^+$) and PI ($^\pm$). Necrotic EC109 cells increased with higher temperatures, whereas apoptotic cells reached their peak at 43˚C. The levels of apoptosis were similar between the last two groups, but at 45˚C the number of necrotic EC109 cells was significantly higher than at 43˚C (Fig. 2B).

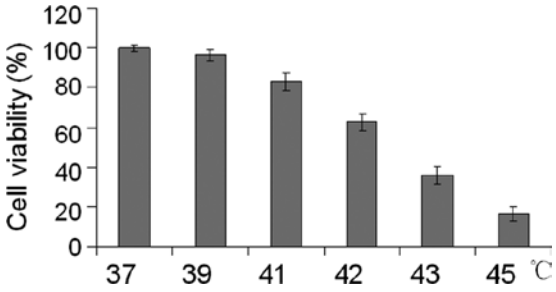

Figure 1. Hyperthermia reduces the viability of EC109 cells. Cells were treated with hyperthermia for 40 min and then incubated at 37˚C for 24 h. Cell viability was determined using MTT assays, and data are presented as the mean ± SD. With higher temperatures, EC109 cells showed significant decreases in viability.

Hyperthermia inhibits Survivin and activates caspase-3 in EC109 cells. To investigate the increased induction of apoptosis in EC109 cells following heat treatment, western blotting was performed for components of the apoptotic pathway. Following treatment with increased temperature, the expression of XIAP and Smac did not change, whereas active caspase-3 and Survivin were significantly altered. Hyperthermia reduced Bcl-2, while Bax and Bcl-xL were unaffected. Notably, the activation of caspase-3 coincided with the decrease of Survivin expression (Fig. 3). Survivin is a member of the IAP familiy, which inhibits caspase activation. It is possible that a reduced Survivin expression leads to caspase-3 activation during hyperthermia.

Overexpression of Survivin inhibits hyperthermia-induced apoptosis through caspase-3 inactivation. To investigate the function of Survivin in EC109 cells with heat treatment, we transfected pcDNA3.0-Flag-Survivin (FL-Sur) or empty vector (EV) into EC109 cells for 48 h and then incubated the cells at 43˚C for 40 min. The following day, western blotting confirmed that the expression of Survivin in the Survivin-transfected cells was higher than that in the control cells, and heat-induced active caspase-3 was inhibited by the overexpression of Survivin (Fig. 4A and B). Furthermore, Survivin-overexpressing and control cells were stained with PI and Annexin V-FITC to assess apoptosis induction by flow cytometry. At 43˚C cultures, the number of apoptotic EC109 cells with a higher Survivin expression was significantly lower than that in the control cells (P<0.05, Fig. 4C). These results indicated that the overexpression of Survivin inhibited the activation of caspase-3, resulting in decreased hyperthermia-induced apoptosis.

Survivin inactivates caspase-3 by binding to XIAP. We determined whether Survivin and caspase-3 physically interacted in EC109 cells. EC109 cells were transfected with Flag-Survivin, although immunoprecipitates of Flag from transfected cells rarely contained caspase-3 by western blotting. By contrast, XIAP was detected in Flag-Survivin immunoprecipitates from cells (Fig. 5A). When we overexpressed Flag-XIAP in EC109 cells and performed Flag immunoprecipitations,

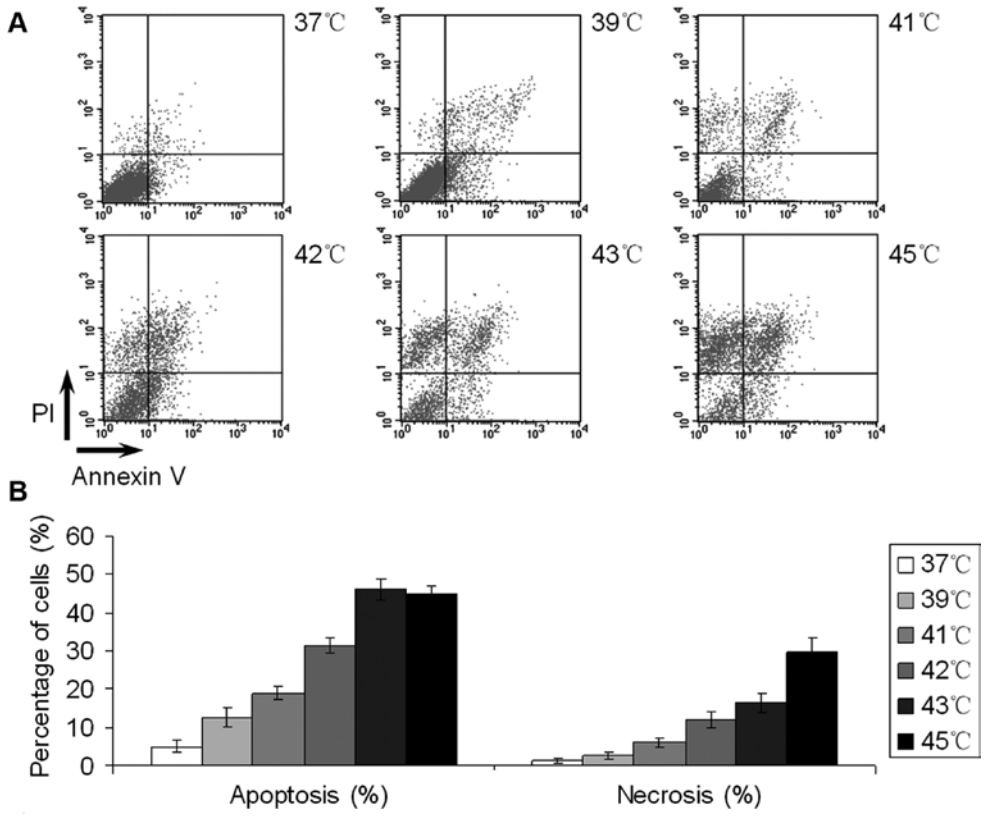

Figure 2. Hyperthermia induces apoptosis and necrosis of EC109 cells. (A) Cells were treated with hyperthermia for 40 min and were then incubated at 37˚C for 24 h. Annexin V/PI staining was used to detect apoptosis. (B) Statistical analysis of FACS in (A). The necrotic EC109 cells increased with higher temperatures, whereas the apoptotic cells reached their peak at 43˚C.

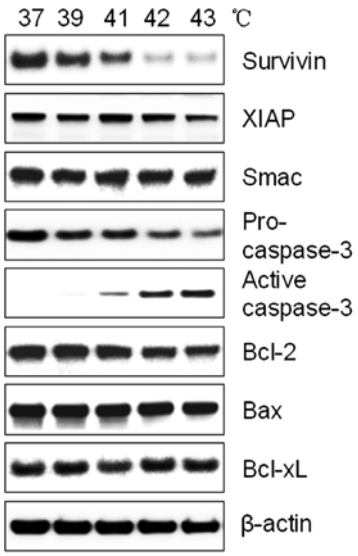

Figure 3. Hyperthermia promotes apoptosis by inhibiting Survivin and activating caspase-3. EC109 cells were treated with hyperthermia for 40 min and then incubated at 37˚C for 24 h. Western blotting was used to analyze the changes in protein expression of members of the apoptotic pathway. The expression of XIAP and Smac did not change, whereas activated caspase-3 and Survivin changed significantly. Hyperthermia reduced Bcl-2, while Bax and Bcl-xL were not affected.

there was an interaction between XIAP and Survivin or caspase-3 (Fig. 5B). To demonstrate the interaction of XIAP with Survivin and caspase-3, we co-transfected Flag-Survivin and SiXIAP into EC109 cells, and then treated cells with hyperthermia (43˚C for 40 min). The inhibitions of active caspase-3 and apoptosis by Survivin were reversed in the absence of XIAP (Fig. 5C-E). These results supported the model that Survivin inactivated caspase-3 by binding XIAP.

Table I. Comparison of Survivin expression in esophgeal cancer and para-tumor tissues.

		Survivin		
Tissues	Total	+	-	Positive rate (%)
Esophageal cancer	86	49	37	57.0
Para-tumor	62	16	46	25.8

χ^2=14.212, P<0.001.

Expression of Survivin in esophageal cancer and para-tumor tissues. Using immunohistochemistry and patient samples, we found that the expression rate of Survivin in the esophageal cancer tissues was 57.0% (49 of 86 patients), and positive staining was identified in the cytoplasm. Of the 62 para-tumor tissues, 16 samples exhibited positive staining, and the remaining stained negative. The positive staining rate was 25.8% (16 of 62 patients) (Fig. 6). The expression of Survivin in esophageal cancer was significantly higher than that in the para-tumor tissues (P<0.01) (Table I).

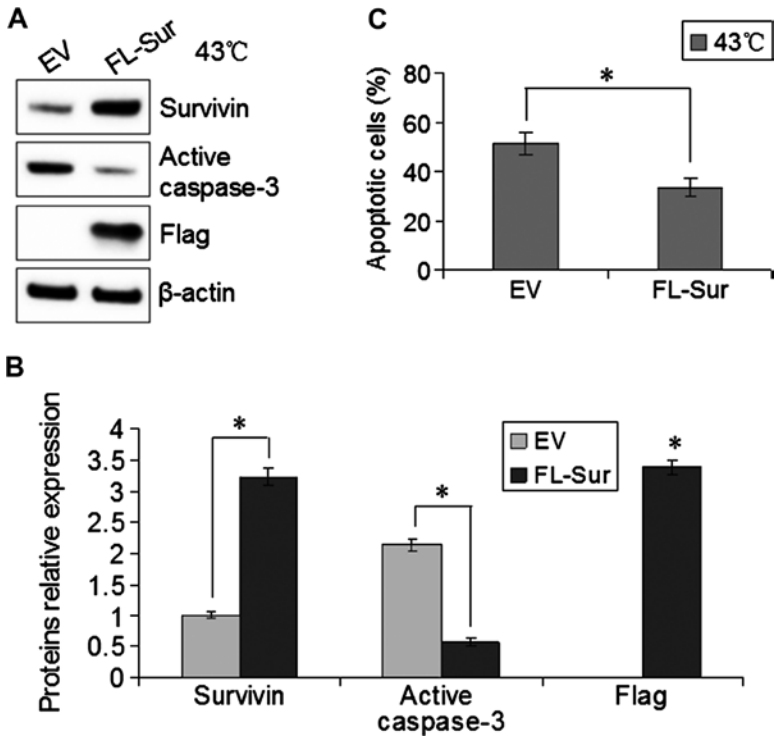

Figure 4. Overexpression of Survivin inhibits hyperthermia-induced apoptosis. (A) EC109 cells were transfected with pcDNA3.0-Flag-Survivin (FL-Sur) or empty vector (EV) for 48 h and then incubated at 43˚C for 40 min. The protein expression was evaluated by western blotting. (B) Values are presented as the mean ± SD, *P<0.05. (C) Annexin V/PI staining was used to detect apoptosis and the apoptotic rates were provided as mean ± SD, *P<0.05.

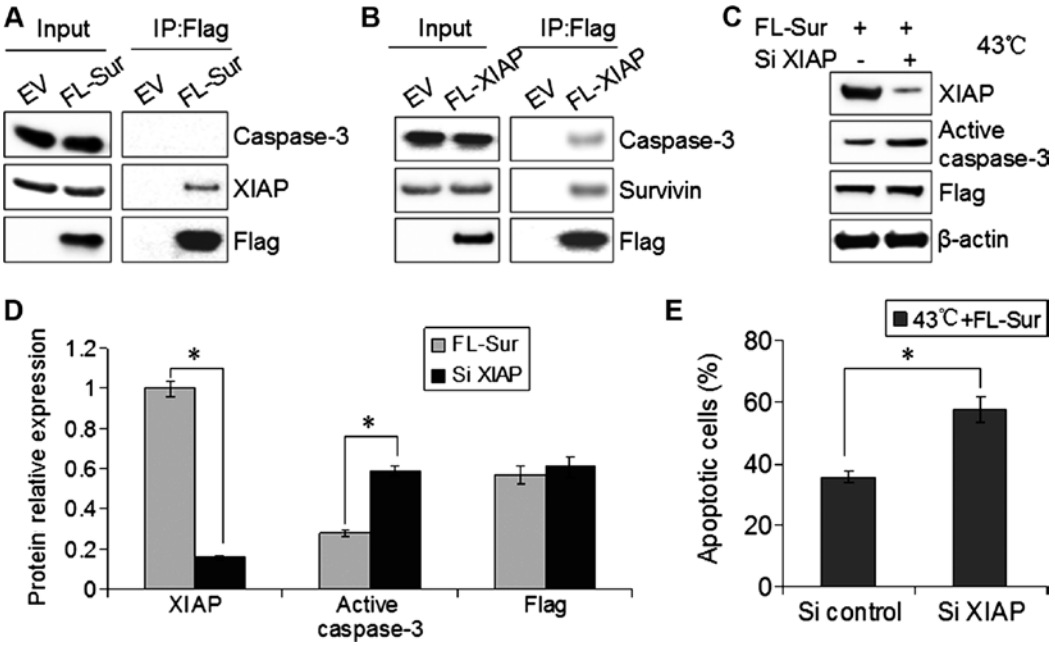

Figure 5. Survivin affects caspase-3 by binding XIAP. (A) EC109 cells were transfected with pcDNA3.0-Flag-Survivin (FL-Sur) or empty vector (EV) for 48 h. The cell supernatant was collected and immunoprecipitated with Anti-FLAG M2 Affinity Gel. Western blotting was performed for XIAP and caspase-3. (B) EC109 cells transfected with pcDNA3.0-Flag-XIAP (FL-XIAP) or EV were immunoprecipitated with Anti-FLAG M2 Affinity Gel and western blotting with Survivin and caspase-3. (C) EC109 cells were transfected with Flag-Survivin and SiXIAP or Si control. The cells were incubated at 43˚C for 40 min. The following day, protein expression was evaluated by western blotting. (D) Values are presented as the mean ± SD, *P<0.05. (E) Annexin V/PI staining was used to detect apoptosis and the apoptotic rates were provided as mean ± SD, *P<0.05.

Correlation between Survivin expression and clinicopathological characteristics. To examine the association between Survivin expression and the clinicopathological characteristics of patients, we compared Survivin expression with common parameters, such as age, gender, tumor size, tumor location, pathological type, tumor differentiation, tumor size,

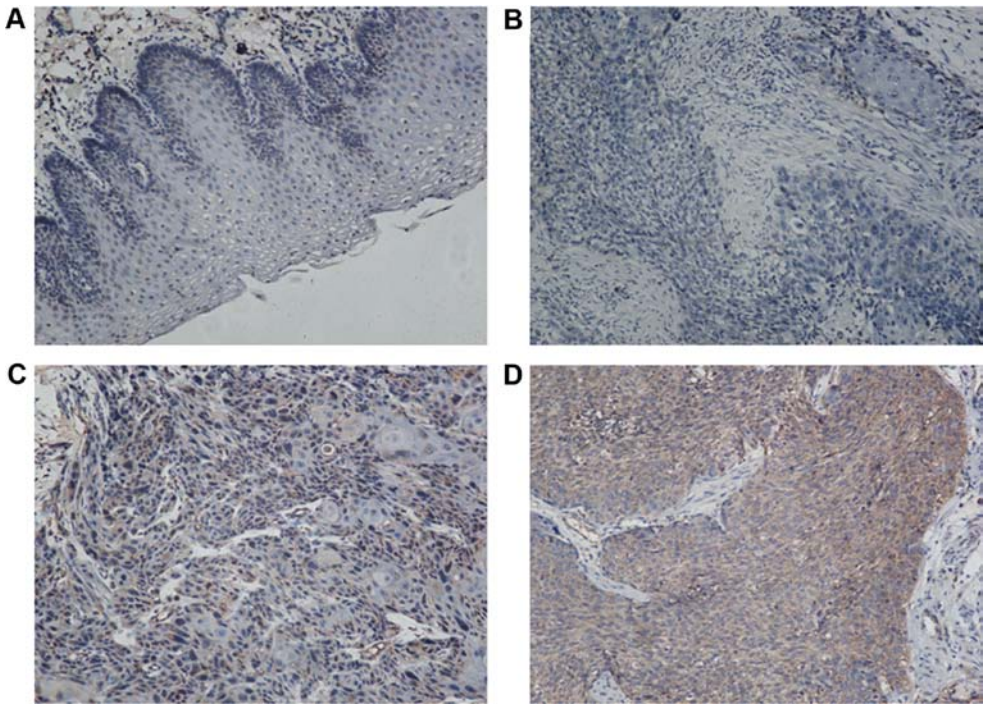

Figure 6. Survivin expression in esophageal cancer and para-tumor tissues by immunohistochemistry. (A) Negative expression of Survivin in normal esophageal tissue. (B) Negative expression (score 0-4) of Survivin in esophageal cancer with stage I. (C) Weak positive expression (score 5-8) of Survivin in esophageal cancer with stage II. (D) Strong positive expression (score 9-12) of Survivin in esophageal cancer with stage III. Original magnification, x100.

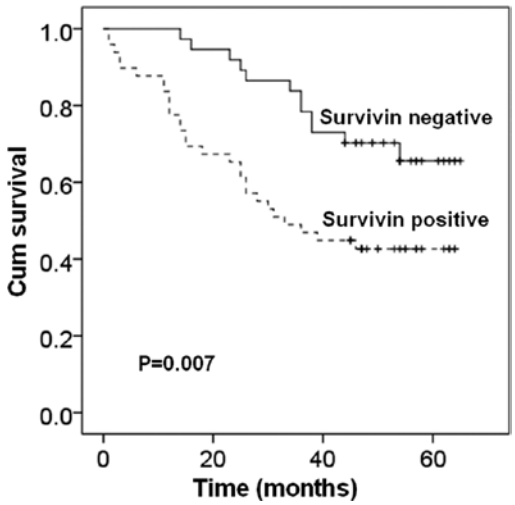

Figure 7. Association between the expression of Survivin and survival of patients with esophageal cancer. Patients with a negative expression of Survivin had a significantly longer overall survival than those with a positive expression of Survivin (P=0.007). Analyses were constructed using the Kaplan-Meier method. The log-rank test was used to compare survival curves between Survivin positive and negative. P<0.05, significant difference.

depth of tumor invasion, lymph node metastasis and clinical stage (Table II). In the 86 esophageal squamous carcinoma samples, the expression of Survivin was directly correlated with clinical stage and lymph node metastasis (P<0.05), but not age, gender, tumor location, pathological type, tumor differentiation, tumor size or depth of tumor invasion (P>0.05).

Prognostic value of Survivin and other clinical characteristics. We used the Kaplan-Meier survival curves and the log-rank test to examine the prognostic value of Survivin and other clinical characteristics (Fig. 7). We found that the patients with a negative expression of Survivin had a significantly longer overall survival than those with a positive expression of Survivin (P=0.007). Lymph node metastasis and clinical stage also showed a significant relationship with overall survival (P<0.001). Other clinical characteristics, such as age, gender, tumor location, had no correlation with overall survival of patients (P>0.05). Univariate Cox regression analysis results revealed that, Survivin expression (P=0.009), tumor differentiation (P=0.028), lymph node metastasis (P<0.001) and clinical stage (P<0.001) were the best predictors of survival of esophageal cancer patients.

Discussion

Survivin is an essential regulator of cell death, apoptosis, cell division and proliferation, which is rarely expressed in normal tissues. However, it is upregulated in the majority of cancers (17,18). As a tumor-specific molecule, Survivin promoted angiogenesis, antagonized apoptosis and facilitated resistance to chemotherapy/radiotherapy (19). Survivin is a good diagnostic biomarker for estimating the prognosis of cancer patients (20). Therefore, it is a potentially valuable target for drug identification. In our experiments, we found that Survivin is expressed at higher levels in esophageal cancer tissues than in normal tissues, and its high expression correlated with poor prognosis, suggesting that Survivin is also a target for esophageal cancer treatment.

Hyperthermia, the heating of tumors to 42-43˚C, is considered the fifth pillar of cancer treatment and is strongly supported by evidence-based medicine (21,22). However,

Table II. Relationship between the expression of Survivin and the characteristics of patients.

Characteristics	Total	The expression of Survivin			χ^2	P-value
		Positive	Negative	Positive rate (%)		
Age (years)						
<60	45	27	18	60.0	0.352	0.553
≥60	41	22	19	53.7		
Gender						
Male	64	36	28	56.3	0.054	0.861
Female	22	13	9	59.1		
Location						
Upper+middle thoracic	43	25	18	58.1	0.047	0.828
Lower thoracic	43	24	19	55.8		
Pathological type						
Polypoid	9	5	4	55.6	0.051[a]	0.829[a]
Medullary	41	24	17	58.5		
Ulceration	29	18	11	62.1		
Erosive	7	2	5	28.6		
Tumor differentiation						
High+middle	60	33	27	55.0	0.316	0.574
Low	26	16	10	61.5		
Tumor size (cm)						
<5	59	35	24	59.3	0.422	0.516
≥5	27	14	13	51.9		
Depth of invasion						
T1 (submucosa)	12	5	7	41.7	1.251	0.263
T2 (muscle)	15	8	7	53.3		
T3 (serosa)	41	26	15	63.4		
T4 (adjacent organs)	18	10	8	55.6		
Lymph node metastasis						
Yes	37	28	9	75.7	9.263	0.002
No	49	21	28	42.9		
Stage						
I+II	46	20	26	43.5	7.351	0.007
III	40	29	11	72.5		
Total	86	49	37	57.0		

[a]Fisher's exact test.

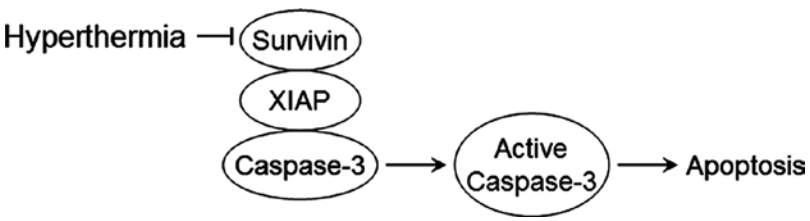

Figure 8. A model for hyperthermia induction of apoptosis by targeting Survivin. Hyperthermia decreases the expression of Survivin, prevents its binding to XIAP, activates caspase-3 and induces apoptosis.

the mechanisms by which hyperthermia improves clinical outcomes remain unclear. There are possible mechanisms that are to be considered. First, hyperthermia induces apoptosis via the cell death pathway. Second, hyperthermia alters the cell

cycle by disrupting S phase and blocking of mitosis. Third, hyperthermia causes chronic ischemia inside the tumor and reduces vessel regulation (23,24). In the present study, hyperthermia was found to induce esophageal cancer apoptosis by inhibiting Survivin. Due to the high expression of Survivin in esophageal cancer tissues, hyperthermia is a valuable therapy for targeting Survivin in esophageal cancer.

Survivin plays a crucial role in inhibiting caspase-dependent apoptosis in different tumors, such as lung and thyroid tumors. As an IAP family member, Survivin contains a BIR domain that may bind to caspases and interfere with their functions (12). There is a debate concerning the Survivin ability to directly bind to caspases (such as other IAP members) with its single BIR domain. Previous findings have demonstrated that Survivin binds to caspase-3 and -7 under specific conditions (25). By contrast, other studies have shown that Survivin inhibited the activation of caspases by binding and stabilizing XIAP rather than directly binding to caspases (26,27). In the present study, we found that hyperthermia inhibits Survivin, activates caspase-3 and induces apoptosis. Overexpression of Survivin reduced hyperthermia-induced apoptosis by inhibiting the activation of caspase-3. However, Survivin did not interact with caspase-3 directly. Caspase-3 and Survivin bind to XIAP and the inhibition of activated caspase-3 by Survivin was reversed in the absence of XIAP. Therefore, Survivin assisted XIAP in inactivating caspase-3 and inhibiting apoptosis.

It has been shown that inhibiting Survivin increases the sensitivity of cancer cells to chemo- or radiotherapy, *in vitro* and *in vivo*. For example, YM155, a small molecule inhibitor of Survivin, is potent in targeting various renal cancer and lymphoma cell lines. It inhibits the transcription of Survivin and participates in numerous antitumor activities (28-30). LY2181308, a Survivin antisense oligonucleotide, has been proven to be effective by itself or in combination with chemotherapy in pre-clinical and clinical studies. However, these small molecules have many adverse effects in patients, including fatigue, nausea, pyrexia or even renal failure (31,32). In the present study, we reported that hyperthermia is a new method to treat cancer by inhibiting Survivin. Due to its low adverse effects, hyperthermia behaved as a better Survivin inhibitor and improved the function of chemotherapy or radiotherapy for esophageal cancer patients. However, more studies are required to precisely identify the effects of hyperthermia in esophageal cancer models and to determine whether there is synergy in combination with chemotherapy or radiotherapy.

In conclusion, hyperthermia decreases the expression of Survivin, prevents its binding to XIAP, activates caspase-3 and induces apoptosis (Fig. 8). Additionally, the expression of Survivin is higher in esophageal cancer than in the normal tissues, and its higher level in cancer is associated with poor prognosis. Therefore, Survivin may be an essential target for hyperthermia in the treatment of esophageal cancer.

Acknowledgements

We would like to thank J.R. Skaar for critically reading the manuscript. The present study was supported by the National Natural Science Foundation of China, approved ID nos.: 81402506 and 81272418.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
2. Kato H and Nakajima M: Treatments for esophageal cancer: A review. Gen Thorac Cardiovasc Surg 61: 330-335, 2013.
3. Kim T, Grobmyer SR, Smith R, Ben-David K, Ang D, Vogel SB and Hochwald SN: Esophageal cancer - the five year survivors. J Surg Oncol 103: 179-183, 2011.
4. Zhang Y: Epidemiology of esophageal cancer. World J Gastroenterol 19: 5598-5606, 2013.
5. Lordick F, Hölscher AH, Haustermans K and Wittekind C: Multimodal treatment of esophageal cancer. Langenbecks Arch Surg 398: 177-187, 2013.
6. van Hagen P, Hulshof MC, van Lanschot JJB, Steyerberg EW, van Berge Henegouwen MI, Wijnhoven BP, Richel DJ, Nieuwenhuijzen GA, Hospers GA, Bonenkamp JJ, *et al*; CROSS Group: Preoperative chemoradiotherapy for esophageal or junctional cancer. N Engl J Med 366: 2074-2084, 2012.
7. Heijkoop ST, van Doorn HC, Stalpers LJA, Boere IA, van der Velden J, Franckena M and Westermann AM: Results of concurrent chemotherapy and hyperthermia in patients with recurrent cervical cancer after previous chemoradiation. Int J Hyperthermia 30: 6-10, 2014.
8. Gadaleta-Caldarola G, Infusino S, Galise I, Ranieri G, Vinciarelli G, Fazio V, Divella R, Daniele A, Filippelli G and Gadaleta CD: Sorafenib and locoregional deep electro-hyperthermia in advanced hepatocellular carcinoma: A phase II study. Oncol Lett 8: 1783-1787, 2014.
9. Petryk AA, Giustini AJ, Gottesman RE, Kaufman PA and Hoopes PJ: Magnetic nanoparticle hyperthermia enhancement of cisplatin chemotherapy cancer treatment. Int J Hyperthermia 29: 845-851, 2013.
10. Januszewski A and Stebbing J: Hyperthermia in cancer: Is it coming of age? Lancet Oncol 15: 565-566, 2014.
11. Qin S, Yang C, Li S, Xu C, Zhao Y and Ren H: Smac: Its role in apoptosis induction and use in lung cancer diagnosis and treatment. Cancer Lett 318: 9-13, 2012.
12. Altieri DC: Survivin, cancer networks and pathway-directed drug discovery. Nat Rev Cancer 8: 61-70, 2008.
13. Pennati M, Folini M and Zaffaroni N: Targeting survivin in cancer therapy. Expert Opin Ther Targets 12: 463-476, 2008.
14. Ryan BM, O'Donovan N and Duffy MJ: Survivin: A new target for anti-cancer therapy. Cancer Treat Rev 35: 553-562, 2009.
15. Or YY, Chow AK, Ng L, Fan ST, Yau TC, Poon RT and Pang RW: Survivin depletion inhibits tumor growth and enhances chemosensitivity in hepatocellular carcinoma. Mol Med Rep 10: 2025-2030, 2014.
16. Rice TW: Staging of esophageal cancer: TNM and beyond. Esophagus 7: 189-195, 2010.
17. Kanwar JR, Kamalapuram SK and Kanwar RK: Survivin signaling in clinical oncology: A multifaceted dragon. Med Res Rev 33: 765-789, 2013.
18. Coumar MS, Tsai FY, Kanwar JR, Sarvagalla S and Cheung CH: Treat cancers by targeting Survivin: Just a dream or future reality? Cancer Treat Rev 39: 802-811, 2013.
19. Mobahat M, Narendran A and Riabowol K: Survivin as a preferential target for cancer therapy. Int J Mol Sci 15: 2494-2516, 2014.
20. Waligórska-Stachura J, Jankowska A, Waśko R, Liebert W, Biczysko M, Czarnywojtek A, Baszko-Błaszyk D, Shimek V and Ruchała M: Survivin - prognostic tumor biomarker in human neoplasms - review. Ginekol Pol 83: 537-540, 2012.
21. Zhao P, Jiang H, Su D, Feng J, Ma S and Zhu X: Inhibition of cell proliferation by mild hyperthermia at 43˚C with Paris Saponin I in the lung adenocarcinoma cell line PC-9. Mol Med Rep 11: 327-332, 2015.
22. Soares PI, Ferreira IM, Igreja RA, Novo CM and Borges JP: Application of hyperthermia for cancer treatment: Recent patents review. Recent Patents Anticancer Drug Discov 7: 64-73, 2012.
23. Palazzi M, Maluta S, Dall'Oglio S and Romano M: The role of hyperthermia in the battle against cancer. Tumori 96: 902-910, 2010.
24. Ahmed K and Zaidi SF: Treating cancer with heat: Hyperthermia as promising strategy to enhance apoptosis. J Pak Med Assoc 63: 504-508, 2013.
25. Shin S, Sung BJ, Cho YS, Kim HJ, Ha NC, Hwang JI, Chung CW, Jung YK and Oh BH: An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and -7. Biochemistry 40: 1117-1123, 2001.

26. Dohi T, Okada K, Xia F, Wilford CE, Samuel T, Welsh K, Marusawa H, Zou H, Armstrong R, Matsuzawa S, *et al*: An IAP-IAP complex inhibits apoptosis. J Biol Chem 279: 34087-34090, 2004.
27. Hu D, Liu S, Shi L, Li C, Wu L and Fan Z: Cleavage of survivin by granzyme M triggers degradation of the survivin-X-linked inhibitor of apoptosis protein (XIAP) complex to free caspase activity leading to cytolysis of target tumor cells. J Biol Chem 285: 18326-18335, 2010.
28. Koike H, Nitta T, Sekine Y, Arai S, Furuya Y, Nomura M, Matsui H, Shibata Y, Ito K, Oyama T, *et al*: YM155 reverses rapamycin resistance in renal cancer by decreasing Survivin. J Cancer Res Clin Oncol 140: 1705-1713, 2014.
29. Winter GE, Radic B, Mayor-Ruiz C, Blomen VA, Trefzer C, Kandasamy RK, Huber KV, Gridling M, Chen D, Klampfl T, *et al*: The solute carrier SLC35F2 enables YM155-mediated DNA damage toxicity. Nat Chem Biol 10: 768-773, 2014.
30. Kaneko N, Mitsuoka K, Amino N, Yamanaka K, Kita A, Mori M, Miyoshi S and Kuromitsu S: Combination of YM155, a survivin suppressant, with bendamustine and rituximab: A new combination therapy to treat relapsed/refractory diffuse large B-cell lymphoma. Clin Cancer Res 20: 1814-1822, 2014.
31. Talbot DC, Ranson M, Davies J, Lahn M, Callies S, André V, Kadam S, Burgess M, Slapak C, Olsen AL, *et al*: Tumor survivin is downregulated by the antisense oligonucleotide LY2181308: A proof-of-concept, first-in-human dose study. Clin Cancer Res 16: 6150-6158, 2010.
32. Tanioka M, Nokihara H, Yamamoto N, Yamada Y, Yamada K, Goto Y, Fujimoto T, Sekiguchi R, Uenaka K, Callies S, *et al*: Phase I study of LY2181308, an antisense oligonucleotide against survivin, in patients with advanced solid tumors. Cancer Chemother Pharmacol 68: 505-511, 2011.