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Original Article

Hyperthermia induced the apoptosis of esophageal squamous cell carcinoma cells and affected the nuclear translocation of Nrf2

Ya Yang[1,2*], Ying Wang[6*], Hongyao Yang[1*], Xiaoxiao Zuo[1,3], Yue Gu[1,4], Daoke Yang[1,2,5]

[1]Department III of Radiation Oncology, [2]Comprehensive Thermal Therapy Center, [3]Radiotherapy Center of China and The United States, [4]Radiotherapy and Tumor Intensive Medical Research Institution, [5]Key Laboratory of Molecular Radiotherapy, [6]Rhinology, The First Affiliated Hospital of Zhengzhou University, 1 Jianshe East Road, Zhengzhou 450000, Henan, China. [*]Equal contributors.

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Abstract: Hyperthermia has been accepted as an effective approach for multifarious tumors. It is not well understood that the mechanism of apoptosis induced by hyperthermia. The present study assessed that hyperthermia induced the apoptosis and subcellular localization of Nrf2 in esophageal squamous cell carcinoma. The cell apoptosis was monitored by flow cytometry, including control group; heat group; t-BHQ with heat group. The caspase activity assay was used to detect the Caspase-3 and Caspase-9 activity. The expression of Bcl-2 and XONA was determined by RT-PCR and western blotting. The subcellular localization of Nrf2 was evaluated by immunofluorescence staining. The apoptosis rate and caspase-3, caspase-9 activity of heat group were significantly higher than those of control group. The Bcl-2 expression was down-regulated in heat group compared to control group, on the contrary, the XONA expression was up-regulated in heat group compared to control group. The t-BHQ could inhibit the cell apoptosis and caspase-3 and caspase-9 activities, up-regulate the expression of Bcl-2 and suppress the expression of XONA. Nrf2 induced by hyperthermia shifted into cytoplasm, which was inhibited by t-BHQ. Hyperthermia could impact on subcellular localization of Nrf2 and then induce the apoptosis in esophageal squamous cell carcinoma cells.

Keywords: Hyperthermia, apoptosis, esophageal squamous cell carcinoma, nuclear translocation, Nrf2

Introduction

An estimated 455,800 new esophageal cancer cases and 400,200 deaths occurred in 2012 worldwide [1]. There are two main types of esophageal cancer, including esophageal squamous cell carcinoma (ESCC) and adenocarcinoma [2]. ESCC is one of the leading causes of cancer in China [3]. Substantial improvements in clinical management and outcomes accrued to advances in therapeutics such as endoscopic resection, surgery, radiotherapy and chemotherapy [4]. However, five-year survival was a dismal 5% in the mid 1970s compared with 20% now. While this represents significant progress, survival still remains poor [5].

For decades, hyperthermia has been accepted an effective approach for multifarious tumors, and has been uncovered to play a significant role in multi-mode concepts for cancer treatment [6-9]. Adding hyperthermia to standard treatment regimens had been proved effective, including cervical cancer, malignant melanoma, recurrent breast cancer, soft tissue sarcoma and bladder cancer [10-12]. As a physical treatment, there was fewer reports of complications than chemotherapy or radiotherapy [13]. As it has been corroborated that persistent heating treatment above certain temperature will result in cell necrosis and/or apoptosis [6, 9, 14]. However, it is not well understood the mechanism of the investigation in apoptosis induced by hyperthermia.

Nrf2 is a member of the NF-E2 family of the basic leucine zipper transcription factors. Under the resting state, the transcription factor Nrf2 was sequestered by the actin-anchored protein Keap1, largely localized in the cytoplasm. This

quiescent interaction suppressed basal expression of downstream genes of Nrf2. However, when Nrf2 was sustained accumulation or activated, it was released from Keap1, avoided proteasomal degradation, translocated to the nucleus and transactivated the expression of various cytoprotective genes [15, 16]. Nrf2 has been demonstrated to regulate the expression of detoxification enzymes, antioxidant proteins and xenobiotic transporters that enhance cell survival [17]. Sustained accumulation or activation of Nrf2 in cancer cells has been shown to tolerate chemotherapeutic agents and radiation and photodynamic therapy [18, 19]. However, there were fewer reports about hyperthermia related with Nrf2, Thus, the present study provides more useful information about hyperthermia combination with chemotherapy and/or radiotherapy.

Materials and methods

Experimental reagents

Tert-butyl hydroquinone (t-BHQ) and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich.

Cell Lines and cell culture

ESCC cell line (Eca-109) was obtained from the Cell Bank of Shanghai (Shanghai, China) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies)/high glucose medium supplemented with 10% heat-inactivated newborn calf serum at 37°C in a humidified incubator under 5% carbon dioxide atmosphere.

Cell apoptosis analyses

Apoptosis was assessed using annexin V, a protein that binds to phosphatidylserine (PS) residues, which are exposed on the cell surface of apoptotic cells. Eca-109 cells were seeded into culture flask and incubated at 37°C for 24 h prior to treatment. After 24 h, fresh growth medium was added into each culture flask, there were three groups, including control group; heat group (43°C for 1 h); t-BHQ with heat group (43°C for 1 h, then t-BHQ 50 μM). Following incubation at 37°C for 24 h, cells were collected, washed twice with precold PBS and then were stained with Annexin V-FITC and PI in the dark, according to the manufacturer's protocol. Double-labeling was performed at room temperature for 10 min in the dark before the flow cytometric analysis. Finally, number of apoptotic cells was quantified by Cell Quest software. Each assay was done at least three times.

Caspase activity assay

Eca-109 cells were treated as above, the activities were investigated using the caspase activitiy assay kits (Caspase-3 and Caspase-9), according to the manufacturer's instructions (Nanjing Jiancheng Corp). Measurements were made using a fluorescence microplate reader at 405 nm.

RNA extraction and quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted using TRIzolreagent (Invitrogen), according to the manufacturer's instructions. RNA was analyzed and reverse-transcribed into cDNA using the Superscript II Reverse transcriptase kit (Takara). Quantitative, real-time RT-PCR was performed (Roche Diagnostic Systems). The primer sequences of the target genes were provided as follows: Bcl-2 forward, 5'-GGAGGATTGTGGCCTTCTTT-3', reverse, 5'-GCCGTACAGTTCCACAAAGG-3'; NOXA forward, 5'-TGGAAGTCGAGTGTGCTACTCAA-3', reverse, 5'-CAGAAGAGTTTGGATATCAGATTCAGA-3'; Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) forward, 5'-AGA AGG CTG GGG CTC ATT TG-3' reverse, 5'-AGG GGC CAT CCA CAG TCT TC-3'. The PCR condition consisted of 95°C (30 seconds), 95°C (5 seconds), 60°C (30 seconds), and 72°C (60 seconds) for 30 cycles. The quantity of each transcript was standardized against that of GAPDH. The relative expression was calculated using the equation relative quantification (RQ) = $2^{-\Delta\Delta CT}$.

Western blot analysis

Whole-cell lysates were extracted with RIPA lysis buffer kit (Santa Cruz), the protein concentrations were determined by BCA Protein Assay Kit (Bio-Rad). An equal amount of protein was electrophoresed using 15% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (Merck Millipore). Membranes were blocked in tris buffered saline with 5% nonfat dry milk and then treated overnight with primary antibody, Bcl-2, NOXA and β-Actin (Santa Cruz), followed by secondary antibodies for 1 h at room temperature. Blots were developed using a peroxidase reaction and visualized with

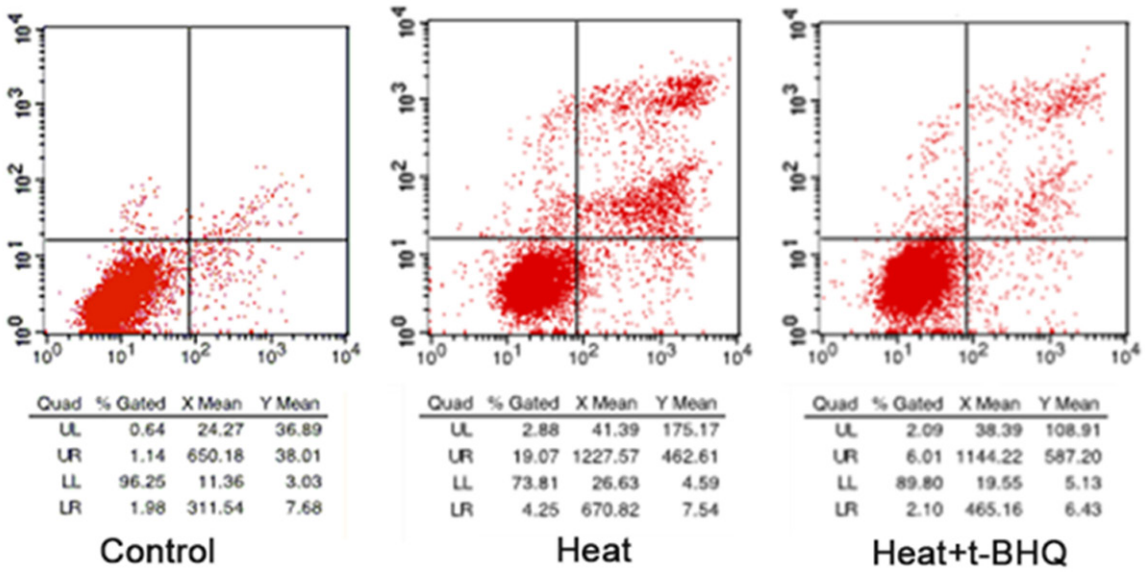

Figure 1. Apoptotic rates of Eca109 cells after treatment with heat or heat and t-BHQ as detected by flow cytometry. One representative experiment was shown.

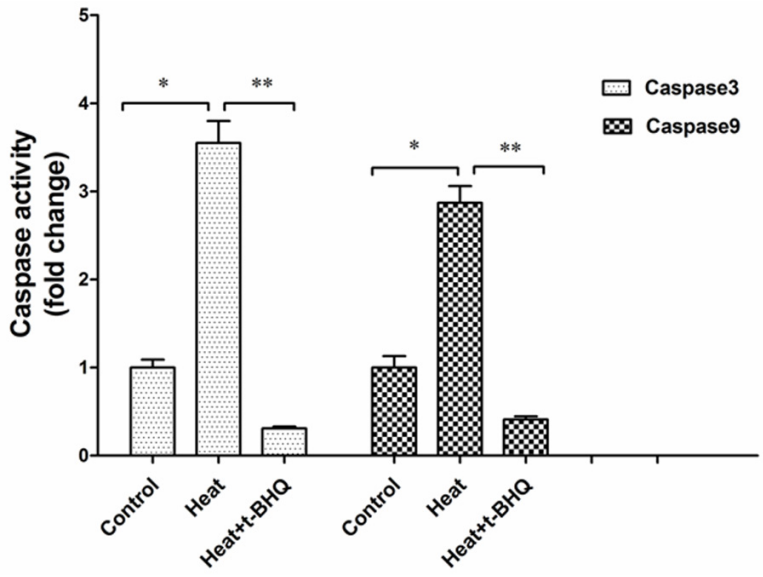

Figure 2. Hyperthermia induced the caspase-3 and caspase-9 activity of Eca109 cell that was inhibited by the t-BHQ. (*P < 0.05).

the ECL detection system (Merck Millipore). β-Actin was used as an internal positive control. The densitometry was analyzed by the use of the program Quantity One.

Immunofluorescence staining

Eca109 cells were seeded on glass coverslips and were divided into three groups, including control group; heat group (43°C for 1 h); t-BHQ with heat group (43°C for 1 h, then t-BHQ 50 μM). The cells were fixed in 4% paraformaldehyde for 15 min at room temperature and washed with cold PBS three times, and then permeabilized with 0.5% Triton X-100 for 10 min. To investigate the cellular localization of Nrf2, cells were incubated with primary antibody against Nrf2 (Santa Cruz) overnight at 4°C temperature. After washing with PBS, the cells were incubated with Cy3-conjugated goat antirabbit IgG as secondary antibody for 1 h at room temperature. Nuclei were stained with 1 μg/mL of DAPI for 5 min in the dark, and then analyzed by a fluorescence microscope (Olympus IX71).

Statistical analysis

Statistical analyses were performed using GraphPadPrism. For each treatment and control, data from the independent replicate trials were pooled, and the results were expressed as the means ± standard error. Student's t test or one-way ANOVA was used to compare normally distributed variables. Results were considered statistically significant if $P < 0.05$ (*$P < 0.05$, **$P < 0.01$).

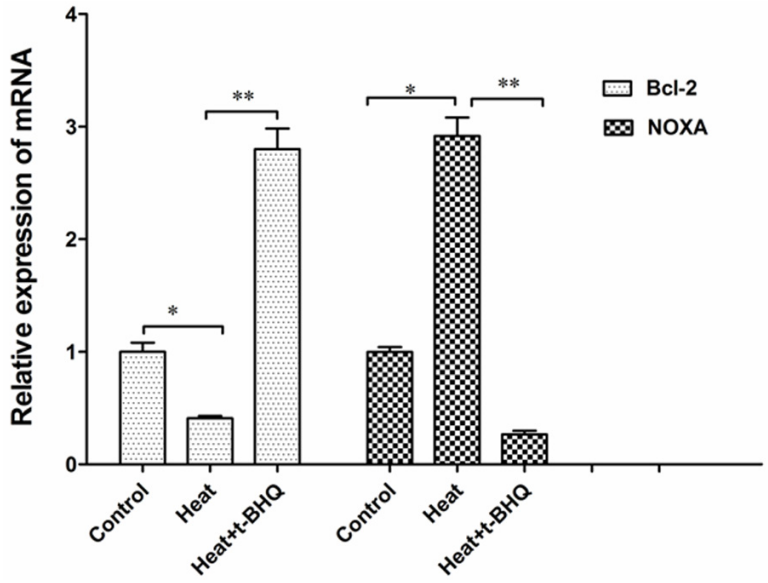

Figure 3. Hyperthermia down-regulated the Bcl-2 expression and up-regulated the XONA expression in mRNA level that was inhibited by the t-BHQ. (*P < 0.05, **P < 0.01).

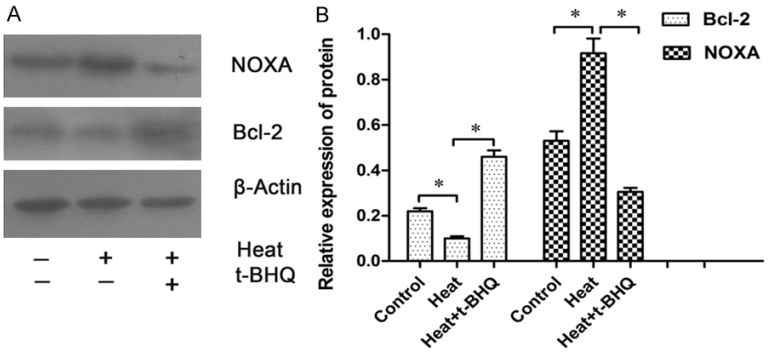

Figure 4. A. Hyperthermia down-regulated the Bcl-2 expression and up-regulated the XONA expression in protein level that was inhibited by the t-BHQ. B. The graphs showed the quantified data of Western blots. (*P < 0.05).

Results

Heat induced apoptosis of ESCC cell

The apoptosis rate and caspase activities were measured to ascertain the effect of heat treatment. The apoptosis rate and caspase-3, caspase-9 activity of heat group were significantly higher than those of control group (P < 0.05) (**Figures 1** and **2**). The expression of both Bcl-2 mRNA and protein was down-regulated in heat group compared to control group (P < 0.05 and P < 0.05) (**Figures 3** and **4**), on the contrary, the expression of both XONA mRNA and protein was up-regulated in heat group compared to control group (P < 0.05 and P < 0.05), (**Figures 3** and **4**). Thus, Hyperthermia could induce the apoptosis of ESCC cells.

T-BHQ suppressed the apoptosis of ESCC cell induced by heat treatment

The t-BHQ was one of activators of Nrf2. Eca109 cells were heated 43°C for 1 h, and then incubated witht-BHQ 50 μM for 24 h. The t-BHQ could inhibit the apoptosis rate, caspase-3 and caspase-9 activities induced by heat treatment (P < 0.05) (**Figures 1**, **2**), contemporary, it also could up-regulate the expression of Bcl-2 in mRNA and protein level (P < 0.01 and P < 0.05) (**Figures 3** and **4**). In addition, the t-BHQ can suppress the expression of both XONA mRNA and protein (P < 0.01 and P < 0.05) (**Figures 3** and **4**). These results suggest that T-BHQ suppressed the apoptosis induced by hyperthermia.

T-BHQ suppressed the nuclear translocation of Nrf2 induced by heat treatment

Nrf2 was one of the transcription factors, translocated to the nucleus, and transactivated the expression of various cytoprotective genes. Eca109 cells grew normally without any stimulus, Nrf2 was located in the cytoplasm and nucleus (**Figure 5A** and **5D**). When Eca109 cells were treated by heat, the Nrf2 scarcely existed in nucleus and shifted into cytoplasm (**Figure 5B** and **5D**), which was inhibited by t-BHQ (**Figure 5C** and **5D**). T-BHQ could sustain Nrf2 in nucleus of Eca109 cells. Thus, T-BHQ could reverse the nuclear translocation of Nrf2 induced by hyperthermia.

Discussion

Esophageal cancer is the 8-th most common cancer and the 6-th leading cause of cancer death in the world, survive is very low, because

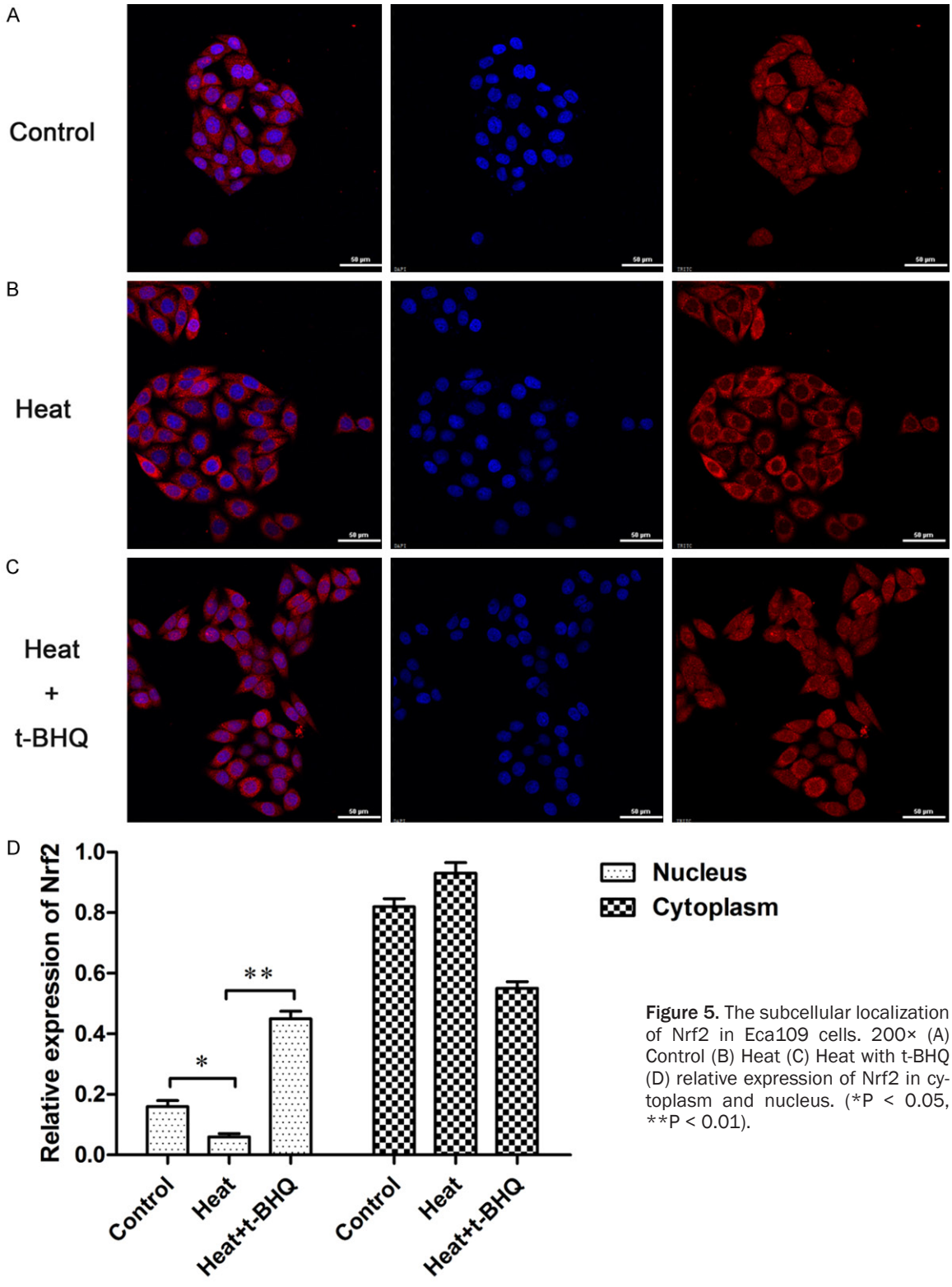

Figure 5. The subcellular localization of Nrf2 in Eca109 cells. 200× (A) Control (B) Heat (C) Heat with t-BHQ (D) relative expression of Nrf2 in cytoplasm and nucleus. (*P < 0.05, **P < 0.01).

of its aggressive nature, distant metastasis at diagnosis, and most unknown molecular mechanism of its progression. Eendoscopic resection, surgery, radiotherapy, and chemotherapy contributed to improvements in prognosis of esophageal cancer patients [4, 20, 21]. How-

ever, chemotherapy and radiation resistance had limited the therapy of esophageal cancer. Hyperthermia is currently regarded as the fourth line of therapy and is mainly applied as an adjunct, ranked below surgery, chemotherapy and radiotherapy [14]. Nevertheless, hyperthermia has the effect of radiosensitivity and chemosensitivity, inhibiting the tumormetastasis, promoting the immunity of the organism [22, 23]. More than 20 randomized clinical trials demonstrated the significant improvement in clinical prognosis through standard treatment regimens of radiation and/or chemotherapy with hyperthermia [24].

Concretely, hyperthermia at 42-43°C cloud enhance effects of numerous cytotoxic drugs such as taxane, paclitaxel, and docetaxel [25]. However, the synergistic mechanism of hyperthermia with radiation and/or chemotherapy has not been sufficiently understood. In addition, a few studies corroborated that Nrf2 was related to the occurrence and progress of many tumors, including lung cancer, head and neck squamous cancer, liver cancer, ESCC, colon cancer, pancreatic cancer, gastric cancer and gallbladder carcinoma [15, 26]. Sustained accumulation or activation of Nrf2 has shown to tolerate chemotherapeutic agents and radiation [18]. Thus, the complete understanding of the role of Nrf2 is very important for elucidating the synergistic mechanism of multiple therapy of tumor.

In this study, we evaluated the effect of heat treatment of Eca109 cells. The apoptosis activities were analyzed after heat treatment, our findings showed that the apoptosis rate, caspase-3 and caspase-9 activities were induced, the expression of Bcl-2 was down-regulated, and the NOXA expression wasup-regulated. Hyperthermia also can activate other proteins of mitochondria pathway, such as AIF, Smac/Diablo [27, 28]. Moreover, hyperthermia induced apoptosis by increasing the sensitivity of death receptors [29], including DR4, DR5, TNFR1 and FAS [30]. In addition, heat induced cell apoptosis through the autocrine and paracrine systems and non-caspase-dependent apoptosis [28, 30]. Hyperthermia had a direct cytotoxic effect that was mainly based on denaturation of cytoplasmatic and membrane proteins [14]. Hyperthermia had effect on the cell cycle, it resulted in inefficient mitosis and consecutive polyploidy by damaging the mitotic apparatus at M-phase, chromosomal damage at S-phase, and abrogated G2/M checkpoint activation [14, 31, 32]. Radiosensitivity and chemosensitivity were strengthened through the cell cycle impacted by hyperthermia. The precise mechanisms of hyperthermia resulted in tumor cell death are complicated and highly dependent on the heating profile.

Previous research suggested that nuclear proteins were most sensitive and a high degree of correlation between nuclear protein aggregation and heat-induced cell kill [33-35]. Hyperthermia could loosen the combining of some nuclear proteins and make them out of the nucleus. The DNA repair protein, Mre11 drove out of the nucleusunder heat treatment, leading to asub sequent sensitization to ionizing radiation. This process could be concerned with heat isolating Mre11 from its functional complex with other DNA repair proteins [36, 37]. Malignant melanoma cell was subjected to heat shock, the nucleolar protein, nucleolin was dissociated from its functional partner nucleophosmin and an association with the DNA replication protein, RPA. Then, this complex was exported from the nucleus [38]. Our finding showed that heat induced Nrf2 out of the nucleus in Eca109 cell, the t-BHQ suppressed this procedure and apoptosis induced by heat. This result was similar as reported earlier for Nrf2 [15, 17]. Niture and Jaiswal reported that Nrf2 was induced into the nucleus the t-BHQ, combined to AREr3 promoter region and promoted Bcl-2 gene expression which lowered cellular apoptosis and increased cell survival [39, 40]. Hep-G2 cell was treated by t-BHQ, resulting in the nuclear accumulation of Nrf2 that was known to regulate the expression of detoxifying enzymes [41]. Accumulation of Nrf2 protein was observed in the nucleus inmethylated HT29 cells, and NQO-1 and AKR1C1 were overexpression after t-BHQ stimulation [42]. Thus, the nuclear aggregation of Nrf2 by t-BHQ is important for tumor cell survival. Our results showed that hyperthermia made the Nrf2 shift out of the nucleus and accelerated cellular apoptosis. As mention before, the validity radiation and/or chemotherapy with hyperthermia may attribute to the nuclear translocation of Nrf2.

In conclusion, the present study showed hyperthermia induced the apoptosis of ESCC cell and

impacted on the nuclear translocation of Nrf2. However, we do not investigate the definite mechanism of the nuclear translocation of Nrf2 induced by hyperthermia, there needed further researches. Radiation and/or chemotherapy with hyperthermia could be a promising comprehensive therapy of tumor.

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Disclosure of conflict of interest

None.

Address correspondence to: Daoke Yang, Department III of Radiation Oncology, The First Affiliated Hospital of Zhengzhou University, 1 Jianshe East Road, Zhengzhou 450000, Henan, China. Tel: +86 15903650068; E-mail: 15903650068@163.com

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