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## miR-202 Promotes Cell Apoptosis in Esophageal Squamous Cell Carcinoma by Targeting HSF2

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Esophageal squamous cell carcinoma (ESCC) is one of the most common malignant cancers with high mortality around the world. However, the regulatory mechanism of ESCC carcinogenesis is not completely known. Here we demonstrate the novel role of miR-202 in regulating ESCC cell apoptosis. The analysis of data obtained from the GEO database showed that the expression of miR-202 is aberrantly decreased in tumor tissue from ESCC patients and cultured ESCC cell lines. After transfection with miR-202 mimic or inhibitor, the apoptotic capacity of ESCC cells was significantly increased by miR-202 overexpression but reduced by miR-202 repression. We then identified HSF2 as a direct target of miR-202 with the binding site on the 3'-UTR of HSF2 mRNA in ESCC cells. The apoptosis of ESCC cells induced by the miR-202 mimic could be repressed by HSF2 overexpression. Further studies indicated that HSF2 overexpression strongly upregulated the expression of Hsp70 at both the mRNA and protein levels. In addition, HSF2/Hsp70 suppressed ESCC cell apoptosis by preventing caspase 3 activation. In conclusion, miR-202 is a potential tumor suppressor in human ESCC and acts by regulating the apoptosis of ESCC cells by targeting HSF2, in which caspase 3 activation is involved. This might provide a novel therapeutic target for human ESCC.

**Key words: miR-202; Esophageal squamous cell carcinoma (ESCC); Apoptosis; HSF2; Hsp70**

### INTRODUCTION

Esophageal cancer is one of the most common malignant cancers in the world and the sixth deadliest among all carcinomas. There are two predominant types, squamous cell carcinoma (ESCC) and adenocarcinoma (EAC), which differ in histological characteristics (1). ESCC accounts for approximately 90% of all esophageal cancer patients. Despite significant developments in surgery, radiotherapy, and chemotherapy, the efficiency of these therapeutic strategies in esophageal cancer remains low, with 5-year survival rates of only 15% to 25% due to its aggressive features and poor clinical prognosis (2). A greater understanding of the mechanisms underlying esophageal cancer progression is thus necessary to improve the diagnostic and therapeutic strategies in human esophageal cancer.

MicroRNAs (miRNAs) are crucial endogenous non-coding single-stranded RNAs comprising 21–23 nucleotides. By binding to target mRNAs, most miRNAs act as negative regulators of gene expression at the posttranscriptional level (3). miRNAs bind to diverse mRNAs with distinct degrees of complementarity and regulate

multiple targets to form a complicated regulatory network in gene expression and cellular activity involved in various biological processes, including cell proliferation, differentiation, and apoptosis (4,5).

Altered expression of miRNAs has been found in patients with different cancers, and this has been associated with the pathogenesis of human cancers (6). In terms of esophageal cancer, dysregulation of miRNAs has been widely reported, including that of miR-21, miR-203, miR-205, miR-25, miR-93, miR-145, miR-27b, miR-100, and miR-125b, and is involved in the pathogenesis of esophageal cancer with a prognostic signature role (7–10). In a previous study, we reported the downregulation of miR-202 in ESCC for the first time (11). Differential expression of miR-202 has also been reported to be associated with other cancers (12,13). In human osteosarcoma, hepatocellular carcinoma, and colorectal carcinoma, aberrant regulation of miR-202 is involved in the modulation of cell growth and apoptosis through different pathways (14,15). However, whether the downregulation of miR-202 is correlated with the pathogenesis of esophageal cancer is not yet clear.

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Heat shock proteins (Hsps) are a highly conserved protein family that are induced by stress. According to their molecular size, Hsps can be classified into five types: Hsp100, Hsp90, Hsp70, Hsp60, and the small Hsps (16). The induction of Hsps by damaging stress is regulated by specific heat shock transcriptional factors (HSFs) (17). As functional molecular chaperones, Hsps prevent the aggregation of damaged proteins and help cells to cope with stressful conditions. Hsps also participate in oncogenesis as inhibitors of cell apoptosis by disrupting the mitochondria and caspase pathway (17,18). In the present study, miR-202 was found to be aberrantly expressed in ESCC and negatively regulated cell apoptosis by directly targeting HSF2 and subsequently affecting Hsp70. Our results reveal a novel role of miR-202 dysregulation in ESCC pathogenesis, which might be a potential therapeutic target for ESCC.

## MATERIALS AND METHODS

### *Cell Cultures*

Human ESCC cell lines EC9706, EC109, and KYSE-510 were purchased from Shanghai Tiancheng Technology Co., Ltd and were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Invitrogen) at 37°C in a 5% CO<sub>2</sub> atmosphere. Normal esophageal epithelial cell line Het-1A was maintained in DMEM containing 10% FBS.

### *Quantitative RT-PCR*

TRIzol reagent (Sigma-Aldrich) was used to isolate the total RNA from cultured cells. The cDNA of miR-202 was prepared using MicroRNA First-Strand Synthesis and miRNA Quantitation kits (Takara, Dalian, P.R. China) according to the manufacturer's instructions. The CellAmp Direct RNA Prep Kit for quantitative polymerase chain reaction (qPCR) and a protein analysis kit (Takara) were used to test the expression of HSF2 and Hsp70. Ct values of U6 and GAPDH were used as the internal control to normalize the relative expression of miR-202 as well as HSF2 and Hsp70. All PCRs were performed in triplicate. Relative expression levels were determined using the 2<sup>-ΔΔCt</sup> method.

### *Western Blotting*

Total proteins were isolated from cultured cells and separated by SDS-PAGE (Invitrogen), followed by electrophoretic transfer to PVDF membrane. Primary antibodies [rabbit anti-cleaved caspase 3, anti-HSF2, 1:1,000 dilution (Abcam); mouse anti-Hsp70, 1:1,000 dilution (Abcam); mouse anti-β-actin, 1:3,000 dilution (ABclonal)] were incubated with blots at 4°C overnight. The membrane was then incubated with secondary antibodies for 1 h at room temperature. Bands were

visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia, Piscataway, NJ, USA).

### *Cell Transfection*

miR-202 mimic, inhibitor, and negative control miRNA (NC) were purchased from RiboBio (Guangzhou, P.R. China). Cells were cultured to approximately 80% confluence and then transfected with 100 nM miR-202 mimic or inhibitor as well as NC miRNA using Lipofectamine 2000 (Invitrogen) twice for 48 h. Transfection of HSF2 siRNA and negative oligonucleotides (NO) (RiboBio) was also performed using the Lipofectamine 2000 kit.

The open reading frame (ORF) of HSF2 was amplified and cloned into the pAdTrack-CMV vector (Clontech). The recombinant adenovirus particles containing pAd-HSF2 were obtained according to the manufacturer's protocol. Cells were infected with adenoviruses at 20 multiplicity of infection (MOI) for 48 h without apparent cytotoxicity.

### *Cell Apoptosis*

A cell apoptosis assay was performed using the Annexin-V-Fluorescein Isothiocyanate Kit (Immunotech, Marseille, France). In brief, following culture in serum-free DMEM for 16 h, cells were harvested with ice-cold PBS and resuspended with binding buffer. Cell suspensions (5 × 10<sup>6</sup> cells/well) were then stained with 0.5 μg/ml annexin V-fluorescein isothiocyanate and 0.6 μg/ml propidium iodide (PI) for 15 min, followed by analysis using a FACSCalibur™ (Becton Dickinson).

### *Luciferase Reporter Assay*

The luciferase reporter assay was performed as described previously (13). Briefly, the 3'-UTR of HSF2 mRNA was cloned into the pGL3 luciferase vector (Promega, Madison, WI, USA) by PCR. The site-directed mutagenesis was introduced into the miR-202 binding site of HSF2 3'-UTR using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene). Recombinant pGL3 plasmid (200 ng) and miR-202 mimic (100 nM) were cotransfected into cultured cells (1 × 10<sup>5</sup> cells/well) in 96-well plates using Lipofectamine 2000. After 36 h of transfection, luciferase activity was analyzed using the Dual Luciferase Assay (Promega). Renilla activity was used as the internal control. Experiments were performed independently at least three times.

### *Statistical Analysis*

ESCC-related raw data including miRNA microarray data and gene expression microarray data were downloaded from the GEO database and log<sub>2</sub> transformed. A comprehensive miRNA analysis was performed to identify differential miR-202 expression using the miRNA profile (GSE13937), which included data from 44 ESCC cases and 44 adjacent noncancerous tissues. HSF2 gene

expression analysis was performed on the basis of the GDS3838 profiles containing 17 ESCC cases and 17 adjacent noncancerous tissues. Correlation analysis was used to evaluate the correlation between miR-202 and HSF2 expression in ESCC specimens. All data are expressed as mean  $\pm$  SEM. GraphPad Prism 6 (La Jolla, CA, USA) was used to analyze the differences between groups. A value of  $p < 0.05$  was considered as statistically significant.

## RESULTS

### *miR-202 Is Aberrantly Downregulated in Human ESCC Samples and Cells*

To determine the potential role of miR-202 in human ESCC, we first analyzed the expression levels of miR-202 in human ESCC tumor tissue of patients from the GEO database. The results showed that miR-202 expression was significantly downregulated in ESCC tumor tissues compared with adjacent noncancerous tissues (Fig. 1A). We also measured the expression of miR-202 in the human ESCC cell lines EC9706, EC109, and KYSE-510 using real-time qPCR. As shown in Figure 1B, the expression levels of miR-202 in all ESCC cells were remarkably lower than in the normal esophageal epithelial cell line Het-1A. These data suggest that miR-202 dysregulation might play a positive role in human ESCC tumorigenesis.

### *Overexpression of miR-202 Promotes Cell Apoptosis in ESCC Cells*

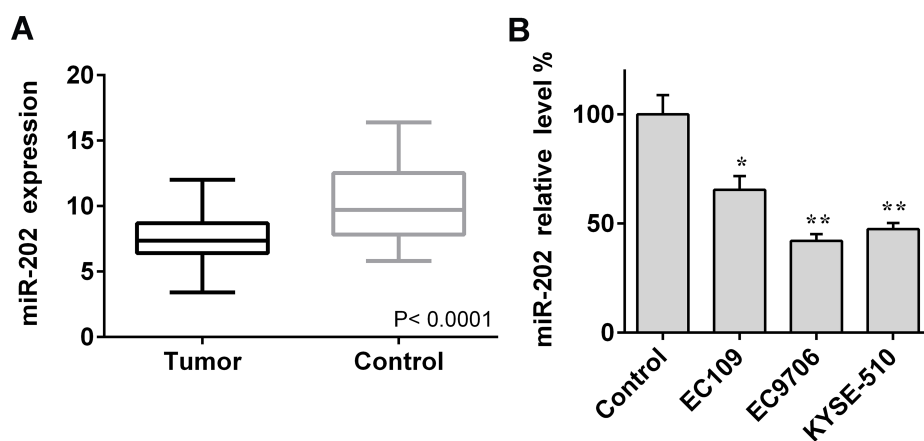
To further determine the possible effect of low miR-202 expression on human ESCC progression using cell lines EC9706 and KYSE-510, we employed a specific mimic and inhibitor. The efficiency of the miR-202 mimic and inhibitor was confirmed by RT-PCR. As seen in Figure 2A, miR-202

expression was significantly enhanced by the mimic and decreased by the inhibitor in comparison with the control.

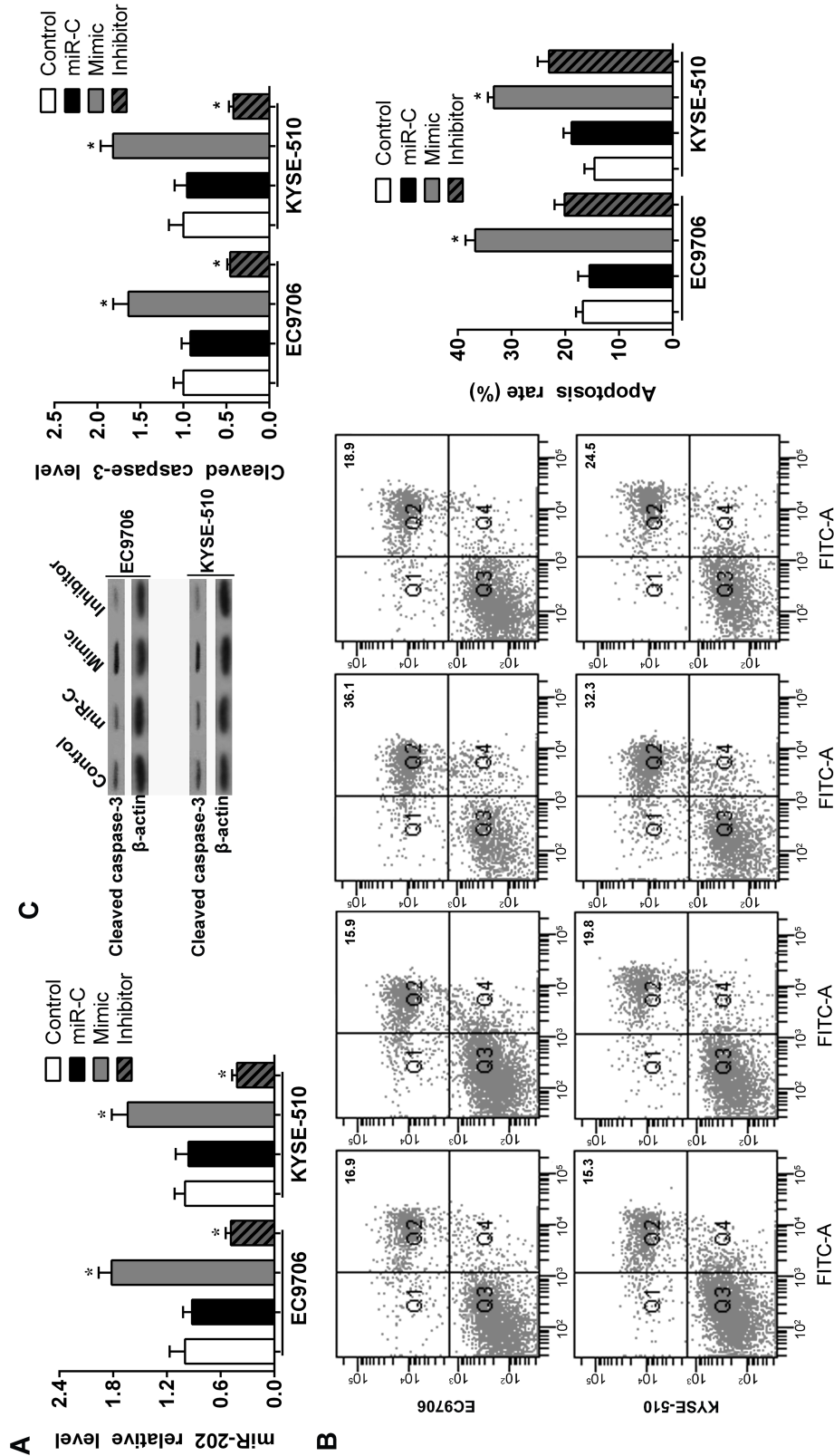
The apoptosis of ESCC cells was then measured by flow cytometric analysis. The results showed that miR-202 overexpression remarkably increased the rate of apoptosis of both EC9706 and KYSE-510 cells compared with the control (Fig. 2B). However, the enhanced apoptosis of ESCC cells was significantly reduced by the miR-202 inhibitor. A Western blotting assay further revealed that cleaved caspase 3 was upregulated by miR-202 overexpression but decreased by miR-202 suppression (Fig. 2C). These results indicate that miR-202 plays a positive role in the regulation of cell apoptosis during ESCC progression.

### *HSF2 Is the Direct Target of miR-202*

We predicted the potential target of miR-202 by searching the TargetScan and miRDB databases to explore the underlying mechanism of action of miR-202 in ESCC. Our analysis indicated that there was a possible binding site for miR-202 in the 3'-UTR of HSF2 mRNA (Fig. 3A). The expression of HSF2 in EC9706 and KYSE-510 cells was then determined by Western blotting. As predicted, the protein level of HSF2 was significantly downregulated by miR-202 overexpression but upregulated by the miR-202 inhibitor in EC9706 and KYSE-510 cells (Fig. 3B). To further validate the prediction of HSF2 as the target of miR-202, luciferase reporter analysis was performed. The wild and mutant 3'-UTRs of the HSF2 mRNA were cloned into the pGL3 luciferase vector (Fig. 3A). As shown in Figure 3C, miR-202 mimic decreased the luciferase activity of the pGL3-HSF2-3'-UTR-wt reporter in both EC9706 and KYSE-510 cells. On the contrary, the miR-202 mimic had no inhibitory effect on the luciferase activity of the pGL3-HSF2-3'-UTR-mut reporter.

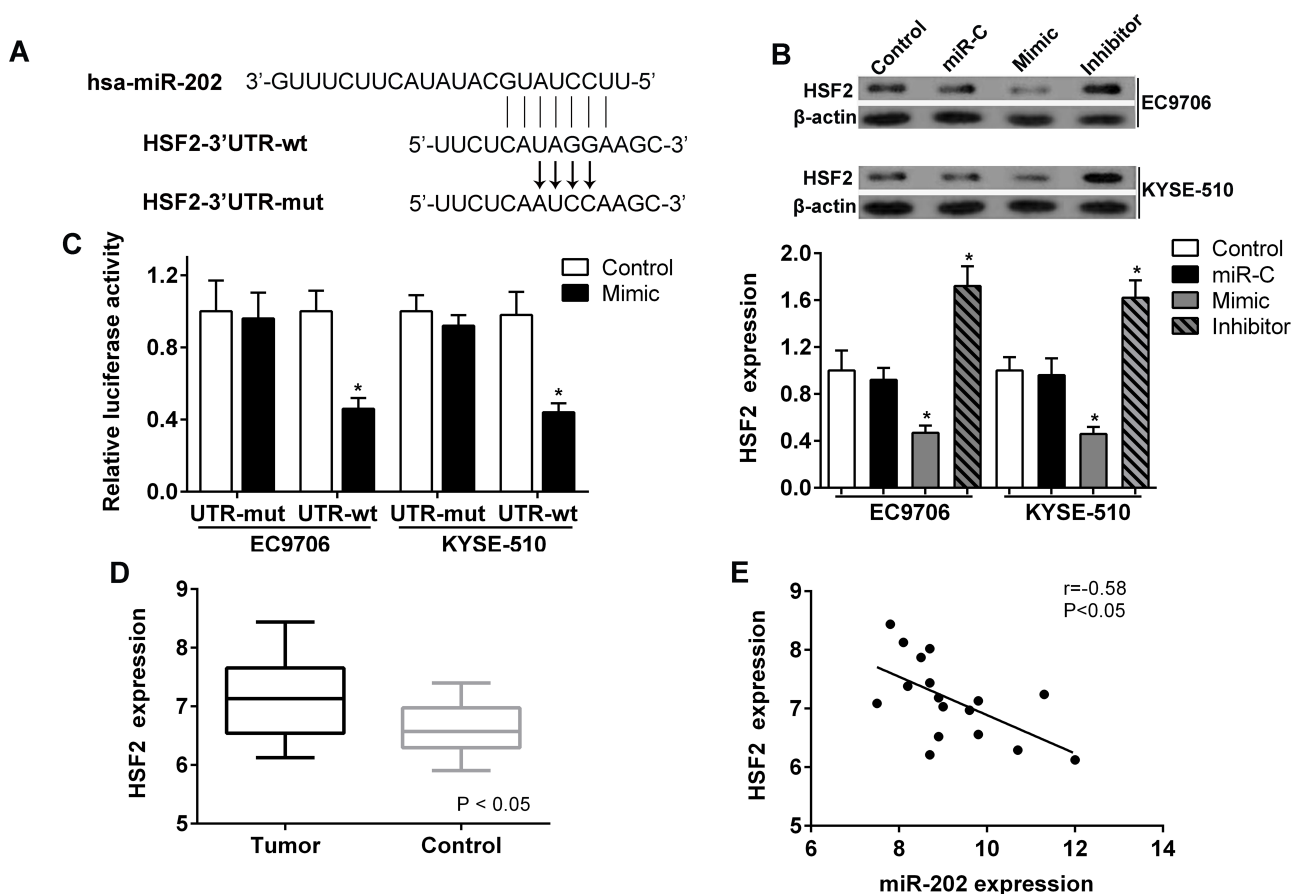


**Figure 1.** The expression of miR-202 is downregulated in human ESCC. (A) Expression levels of miR-202 in human ESCC tumor tissue and adjacent noncancerous tissues (Control) of patients from the GEO database. (B) The levels of miR-202 in human ESC cell lines EC9706, EC109, and KYSE-510 as well as the normal esophageal epithelial cell line Het-1A (Control) were examined using RT-PCR (\*\* $p < 0.01$ , \* $p < 0.05$  vs. Control).



**Figure 2.** Effects of miR-202 on ESCC cell apoptosis. EC9706 and KYSE-510 cells were cultured in medium (Control), followed by transfection with miR-202 mimic, inhibitor, or negative control miRNA (miR-C). (A) After 48 h of transfection, the expression levels of miR-202 were determined using RT-PCR. (B) Cell apoptosis was tested by flow cytometric analysis, and the rate of apoptosis was quantified. (C) The levels of cleaved caspase 3 were measured by Western blotting assay (\* $p < 0.05$  vs. Control; # $p < 0.05$  vs. Mimic).





**Figure 3.** miR-202 directly targets the HSF2 mRNA. (A) miR-202 target sequences in the 3'-UTR of HSF2 mRNA predicted by searching the TargetScan and miRDB databases. (B) The protein expression of HSF2 in EC9706 and KYSE-510 cells was evaluated by Western blotting. (C) Luciferase activity was measured in cells transfected with HSF2-3'-UTR-wt (UTR-wt) or HSF2-3'-UTR-mut (UTR-mut). (D) Expression of HSF2 in human ESCC tumor tissue and adjacent noncancerous tissues (Control) of patients from the GEO database. (E) Correlation between HSF2 and miR-202 expression in ESCC tumor tissues (\* $p < 0.05$  vs. Control).

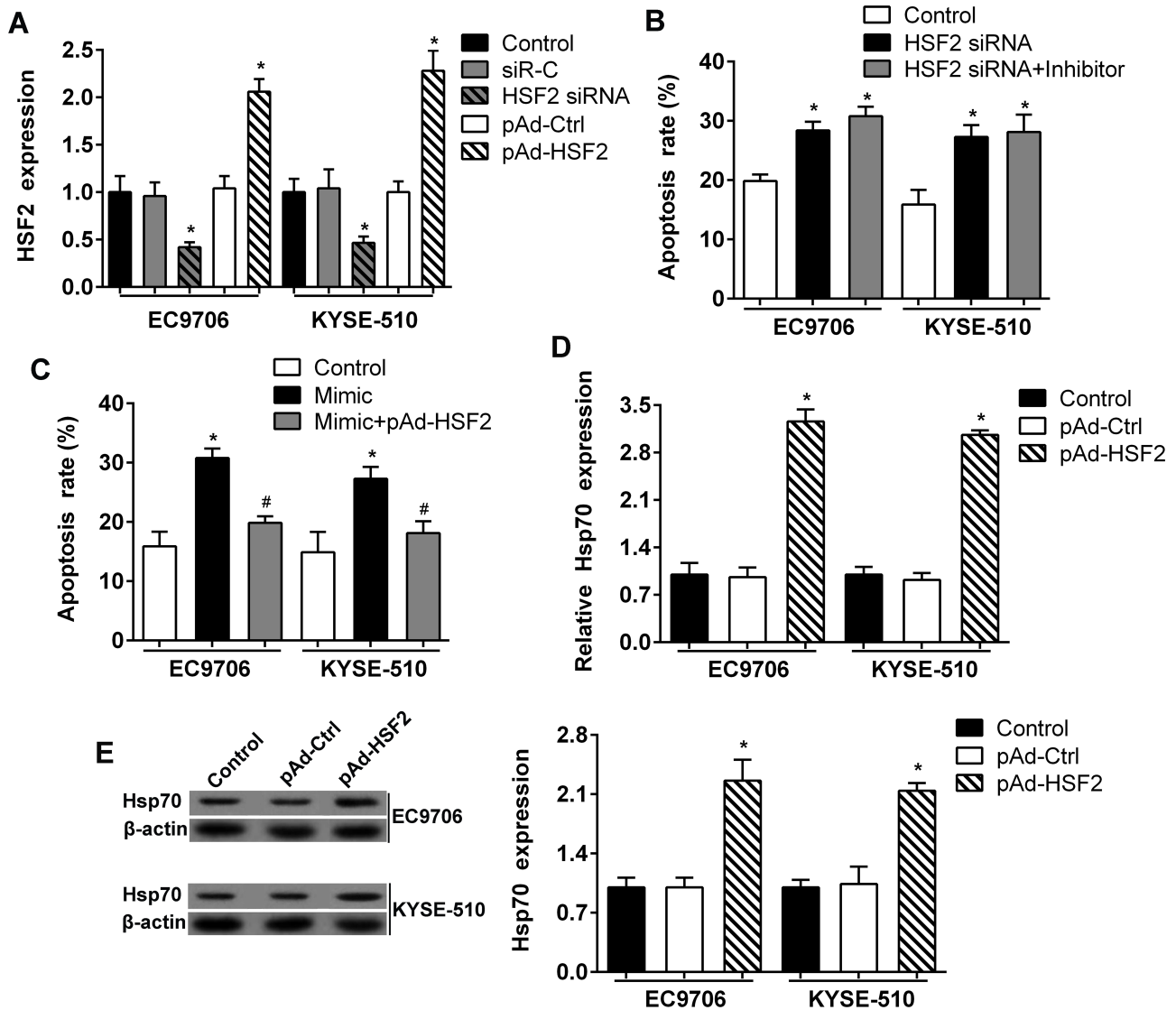
We also analyzed the expression levels of HSF2 in human ESCC tumor tissue of patients from the GEO database. The results showed that HSF2 expression was significantly increased in ESCC tumor tissues compared with adjacent noncancerous tissues (Fig. 3D). Furthermore, the ectopic expression of HSF2 exhibited a negative correlation with miR-202 levels in ESCC tumor tissues ( $p < 0.05$ ) (Fig. 3E). Taken together, our data suggest that HSF2 mRNA is a direct target of miR-202 in ESCC cells and that the dysregulation of miR-202 and HSF2 expression is involved in ESCC carcinogenesis.

#### *HSF2 Inhibits miR-202-Mediated Cell Apoptosis by Upregulating Hsp70*

To further evaluate whether HSF2 plays a role in miR-202-mediated ESCC cell apoptosis, we conducted a series of assays in EC9706 and KYSE-510 cells. First, we employed HSF2 siRNA and adenovirus transduction to inhibit and overexpress HSF2, respectively, as

seen in Figure 4A. The results showed that HSF2 silencing increased the rate of apoptosis of both EC9706 and KYSE-510 cells (Fig. 4B). However, the miR-202 inhibitor did not reduce the rate of apoptosis of HSF2-depleted ESCC cells (Fig. 4B). Additionally, the apoptosis of cells induced by the miR-202 mimic could be repressed by HSF2 overexpression (ORF without 3'-UTR) (Fig. 4C), suggesting that miR-202 regulates the apoptosis of ESCC cells by targeting HSF2 expression.

Hsps are effective inhibitors of oncogenic apoptosis. HSF2 is one of the main transcriptional regulators of Hsp expression and is able to modulate Hsp70 expression by binding the Hsp70 promoter (17,19). To further explore the mechanism involved in the inhibitory effect of HSF2 on ESCC cell apoptosis, the expression levels of Hsp70 in EC9706 and KYSE-510 cells were evaluated. As shown in Figure 4D and E, gene expression of Hsp70 at both the mRNA and protein levels was strongly upregulated by HSF2 overexpression. The above results indicate that



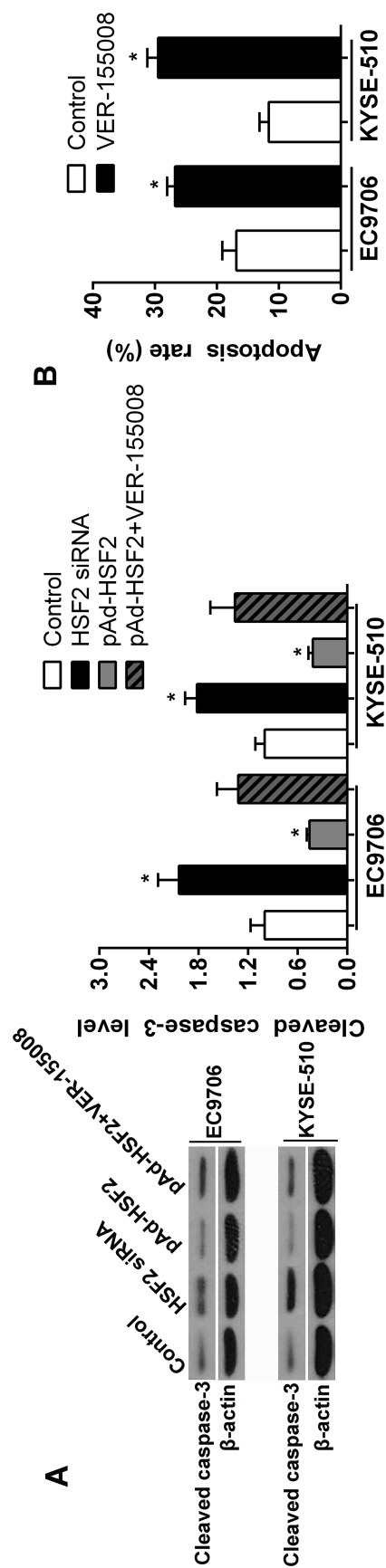
**Figure 4.** HSF2 inhibits cell apoptosis by upregulating Hsp70. (A) The protein levels of HSF2 in EC9706 and KYSE-510 cells transfected with specific HSF2 siRNA or adenoviruses pAd-HSF2 were detected by RT-PCR. (B) The apoptosis of EC9706 and KYSE-510 cells was investigated after transfection with HSF2 siRNA in the presence or absence of the miR-202 inhibitor. (C) The apoptosis of cells was then tested after treatment with adenovirus pAd-HSF2 transduction in the presence or absence of the miR-202 mimic. (D) RT-PCR and (E) Western blotting were used to determine the expression of Hsp70 in EC9706 and KYSE-510 cells transfected with adenoviruses (pAd-HSF2) or not (\* $p < 0.05$  vs. Control; # $p < 0.05$  vs. miR-202 mimic-treated cells).

miR-202 dysregulation-mediated upregulation of HSF2 contributes to the inhibition of oncogenic apoptosis of ESCC cells, in which Hsp70 is involved.

#### *HSF2/Hsp70 Suppresses ESCC Cell Apoptosis by Preventing Caspase 3 Activation*

Previous studies have demonstrated that Hsp70 confers an antiapoptotic effect by disrupting the mitochondria and caspase pathway in human cervical carcinoma cells and histiocytic lymphoma cells (18,20). To clarify whether the caspase pathway is implicated in HSF2/Hsp70-inhibited

ESCC cell apoptosis, caspase 3 activation was evaluated in EC9706 and KYSE-510 cells. Consistent with the results in Figure 2C, cleaved caspase 3 expression was down-regulated by HSF2 overexpression and increased by HSF2 siRNA (Fig. 5A). Further analysis confirmed that VER-155008, a specific molecular inhibitor of Hsp70, significantly increased the cleaved caspase 3 levels that were repressed by HSF2 overexpression (Fig. 5A) as well as the rate of apoptosis of ESCC cells (Fig. 5B). This suggests that HSF2/Hsp70 might suppress miR-202-mediated ESCC cell apoptosis by preventing caspase 3 activation.



**Figure 5.** Effects of HSF2/Hsp70 on caspase 3 activation in ESCC cells. (A) The levels of cleaved caspase 3 were detected by Western blotting in EC9706 and KYSE-510 cells transfected with specific HSF2 siRNA or adenoviruses pAd-HSF2 in the presence or absence of Hsp70 inhibitor VER-155008. (B) The apoptosis of EC9706 and KYSE-510 cells was investigated after treatment with VER-155008 in the presence or absence of adenoviruses (pAd-HSF2) (\* $p < 0.05$  vs. Control).

## DISCUSSION

Aberrant expression of miRNAs has been reported in various human cancers and is associated with disease progression (21). Dysregulation of miR-202 has also been identified in several carcinomas, where it exerts a tumor suppressor function or oncogenic function during carcinogenesis. Lin et al. reported that miR-202 is induced by transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) in human osteosarcoma and contributes to drug resistance by inhibiting cell apoptosis via the tumor suppressor PDCD4 (15). In pancreatic cancer, downregulated miR-202 induces cell apoptosis by regulating Mxd1 and Sin3A repressor complexes (14). miR-202 has also been proven to inhibit cell proliferation in human hepatocellular cancer and colorectal carcinoma by targeting low-density lipoprotein receptor-related protein 6 (LRP6) and ADP-ribosylation factor-like 5A (ARL5A), respectively (12,13). However, the function of miR-202 in ESCC development is not clear. In the present study, we determined a novel role of miR-202 in ESCC. Analysis of data from the GEO database revealed that miR-202 expression was strongly downregulated in ESCC tumor tissues. The expression of miR-202 was also decreased in cultured human ESCC cell lines. Further study found that ectopic expression of miR-202 promotes ESCC cell apoptosis, indicating that miR-202 dysregulation might play a positive role in human ESCC tumorigenesis.

HSFs comprise a family of transcriptional regulators of Hsps and modulate the expression of Hsps by binding to the heat shock elements on the Hsp genes (22). A previous study showed that altered expression of HSFs is associated with cell apoptosis (17). By bioinformatics analysis and luciferase reporter assay, we proved that HSF2 mRNA is a direct target of miR-202 in ESCC cells, with a binding site on the 3'-UTR. The expression level of HSF2 was significantly increased in human ESCC tumor tissue and showed a negative correlation with miR-202 levels, suggesting the involvement of miR-202 and HSF2 in human ESCC carcinogenesis.

Another approach we took to clarify the role of miR-202 was to investigate the interaction between the target HSF2 and downstream Hsps in ESCC cells. Hsps have been identified as effective regulators of tumor cell proliferation, metastasis, differentiation, and apoptosis. Over-expressed Hsp proteins in multiple human cancers play roles in cancer diagnosis, prognosis, and treatment (23). Hsp70 is one of the main members of the Hsp family and is abundantly expressed in many malignant tissues and cells (24). It has been reported that Hsp70 has an oncogenic apoptosis inhibitor function due to its capacity to disable apoptosis (25,26). HSF2 is the main transcriptional regulator of HSP expression and has been found to be able to modulate Hsp70 expression by binding the hsp70 promoter (22). We herein determined that HSF2 increases the expression of Hsp70 in cultured ESCC cells.

Disruption of cell apoptosis is one of the basic pathologies involved in the carcinogenesis of human carcinomas, including ESCC. Emerging evidence indicates that dysfunction of miRNAs plays a diverse role in the regulation of ESCC cell apoptosis. Zhu et al. reported that miR-16 expression is aberrantly increased in ESCC tumor tissues and could induce the suppression of ESCC cell apoptosis by directly targeting RECK and SOX6 mRNA (27). Furthermore, miR-141 was proven to be highly expressed and has a resistant role in cisplatin-induced apoptosis by downregulating YAP1 in human ESCC (28). In the present study, we found that the apoptotic capacity of ESCC cells was significantly increased by miR-202 overexpression but decreased by miR-202 repression. Notably, the overexpression of HSF2 attenuated the promoting effect of miR-202 on the rate of apoptosis of ESCC cells.

Additionally, we observed that caspase 3 activation in cultured ESCC cells was affected by miR-202 and downstream HSF2/Hsp70. The caspase 3-dependent pathway is involved in the cell apoptosis induced by heat shock, tumorigenesis factors, chemotherapeutic drugs, and other apoptosis signals. The protective role of Hsp70 against cell apoptosis by affecting caspase 3 activation has been reported in previous studies (18). Hsp70 might bind to caspase substrates to inhibit their cleavage to functional peptides. Li et al. found that Hsp70 prevents apoptosis induced by heat shock by acting as a suppressor of apoptosis downstream of cytochrome c release and upstream of caspase 3 activation (18). It was also reported that caspase 3-like protease-mediated apoptosis is inhibited by Hsp70 in cells treated with TNF, staurosporine, and doxorubicin (20). In this study, we found that caspase 3 activation was significantly downregulated by HSF2 overexpression, but aberrantly increased by HSF2 siRNA in ESCC cells. The Hsp70 inhibitor also significantly increased the rate of apoptosis of ESCC cells as well as the cleaved caspase 3 level. This indicates that HSF2/Hsp70 might suppress miR-202-mediated ESCC cell apoptosis by preventing caspase 3 activation.

Our study is not without its limitations. The regulation of miRNA expression and the interaction between miRNAs and target genes are very complicated. In terms of ESCC, there are various differentially expressed miRNAs that are involved in cancer-related signal transduction pathways (7,29). Therefore, interactive studies on different miRNAs may be requisite to clearly elucidate the role of dysregulated miRNAs in ESCC. Additionally, HSF2/Hsp70 is just one of the targets regulated by miR-202. Exploring the interactions between the dysfunction of miRNAs and the functional categories of target genes will be an important part of our next study to identify the potential mechanism involved in the pathological process of ESCC.

In summary, the present study revealed that miR-202 is a potential tumor suppressor in human ESCC and acts by regulating the apoptosis of ESCC cells by targeting HSF2. This might provide a novel therapeutic target for human ESCC.

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