

## Original Article

# Regulation by miR-210 on apoptosis and Bcl-2 in the oxidative stress-induced apoptosis in gastric cancer MKN45 cells

Tianxiang Xu<sup>1,2</sup>, Xiaoxia Wang<sup>3</sup>, Wu Ji<sup>1</sup>

<sup>1</sup>PLA Research Institute of General Surgery, Jinling Hospital, Southern Medical University, Nanjing 210002, Jiangsu, China; <sup>2</sup>Center of Tumor, <sup>3</sup>Intensive Care Unit, Inner Mongolia People's Hospital, Hohhot 010017, Inner Mongolia Autonomous Region, China

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**Abstract:** MicroRNAs (miRNAs) are recently recognized to involve in the gastric cancer tumorigenesis. The present study was to profile miRNA expression and investigate its role in gastric cancer MKN45 cells under oxidative stress. MKN45 cells were treated with H<sub>2</sub>O<sub>2</sub>, the cellular viability, cell cycle distribution and apoptosis were examined. Then miRNAs were profiled with miRNA microarray. Finally, the regulatory role of miR-210 (microRNA-210, miRNA-210) on the H<sub>2</sub>O<sub>2</sub>-induced apoptosis of MKN45 cells was investigated. Results demonstrated that H<sub>2</sub>O<sub>2</sub> treatment significantly reduced cell viability, deregulated the cell cycle and induced apoptosis in MKN45 cells. The miRNA microarray and quantitative PCR indicated 11 upregulated miRNAs and 14 downregulated miRNAs, which were associated with cell cycle, apoptosis, DNA replication and DNA repair, in the H<sub>2</sub>O<sub>2</sub>-treated cells. In addition, the upregulation of miR-210 markedly reduced the Bcl-2 expression, promoted the cleavage of caspase 3 and thus induced apoptosis in MKN45 cells. In conclusion, deregulated miRNAs, particularly miR-210 was associated with the oxidative stress-induced cell cycle deregulation and apoptosis in gastric cancer cells, probably via inhibiting Bcl-2. The present study implies the mediatory role of miRNAs, such as miR-210 in the oxidative stress-induced apoptosis in gastric cancer cells.

**Keywords:** Oxidative stress, miR-210, apoptosis, gastric cancer, Bcl-2

## Introduction

Gastric cancer (GC) is one of the most prevalent tumors with high morbidity and mortality [1]. Surgery at early stage is the most common treatment. However, most GC patients present with an advanced stage of the disease, for early-stage tumors are usually asymptomatic [2]. Previous studies have revealed that oxidative stress (OS) are implicated in gastric tumorigenesis, and modulate expression of microRNAs in tumor [3]. However, mechanisms underlying the relationship between OS and GC have not been elaborately studied.

Oxidant stress is believed to play a role in the pathogenesis of many gastric disorders [4]. Reactive oxygen species (ROS) are produced by cellular processes such as mitochondrial metabolism and protein folding [5]. Compared to healthy cells, cancer cells usually own higher levels of ROS and higher antioxidant activities in an uncontrolled status [6], and cancer cells

are unable to deal with additional oxidative stress and are sensitive to excessive ROS [7]. Over-expressed ROS destroys the intracellular redox balance and presents an oxidative stress on cancer cells that can ultimately cause cell aging or death [8]. In addition, oxidative stress was found to affect numerous signaling pathways through protein phosphorylation and selectively active a number of genes, thus chronic exposure to low levels of ROS may impact cell growth and differentiation [4].

MiR-210 (microRNA-210, miRNA-210) is generally recognized as a Hypoxia-inducible factors (HIF) target [9, 10]. miR-210 is specially over-expressed in renal clear cell carcinomas (RCCs) expressing high levels of HIF [11]. MiR-210 is up-regulated in most solid tumors and negatively affects the clinical outcome [12, 13]. In breast cancer, over-expressed miR-210 may be caused by decreased oxygen tension [14]. microRNA expression analysis by several independent groups showed that miR-210 was sig-

nificantly up-regulated in GC patients, and was conceived as a potential diagnostic marker of GC [22, 23]. It was reported that gene silencing of miR-210 upregulates target gene expression and promotes aberrant gastric epithelial cell proliferation during chronic *Helicobacter Pylori* infection [15]. However, to date, the mechanisms underlining the association of miR-210 with GC still remain elusive.

In this research, we tried to profile miRNA expression in gastric cancer MKN45 cells under oxidative stress, and then to investigate the role of deregulated miRNAs in the MKN45 cells. We demonstrated that miR-210 is a critical miRNA, which mediate the oxidative stress-induced apoptosis in gastric cancer cells probably via inhibiting Bcl-2. Our work provides more evidence for the possible involvement of miR-210 in development of gastric malignancies.

## Methods

### Cell culture

Human gastric cancer cell line MKN45 was purchased from the ATCC. The cells were routinely cultured in RPMI 1640 medium (Gibco, USA) containing 10% heat-inactivated fetal bovine serum (Hyclone, USA), 100 units/ml penicillin, and 100 µg/ml streptomycin in a humid cell incubator with an atmosphere of 5% CO<sub>2</sub> at 37°C.

### Transient transfection

Liposome-mediated transfection was performed using Lipofectamine TM (Invitrogen, Carlsbad, CA, USA). Briefly, MKN45 cells were plated onto 12-well plates overnight and transfections were performed on cells at 70-80% confluence. Briefly, lipofectamine (5 µl) and miRNA-210 mimics (1 ng) were diluted in 200 µl of RPMI1640 at room temperature for 30 min after mixing. The lipofectamine-DNA complex was added to MKN45 cells and incubated for 48 hours.

### MTT (methyl thiazolyl tetrazolium) assay

Cells were seeded into 96-well plates at a density of 8×10<sup>4</sup> per well and incubated overnight in RPMI 1640 containing 10% heat-inactivated FBS. H<sub>2</sub>O<sub>2</sub> was dissolved in DMSO and diluted with 1640 medium to final concentrations of 40 µM. The tumor cells were incubated with

H<sub>2</sub>O<sub>2</sub> for 24 h before the MTT assay. After removing the medium supernatant, 200 µl of DMSO was added into each well and mixed thoroughly. The plate was incubated at 37°C to dissolve air bubbles for 5 minutes, and OD570 value of each well was measured at 570 nm wavelengths using a microplate reader (Thermo scientific, USA). The results were calculated as (A570 of control wells-A570 of treated wells)/(A570 of control wells-A570 of blank wells) × 100%.

### Cell apoptosis assay

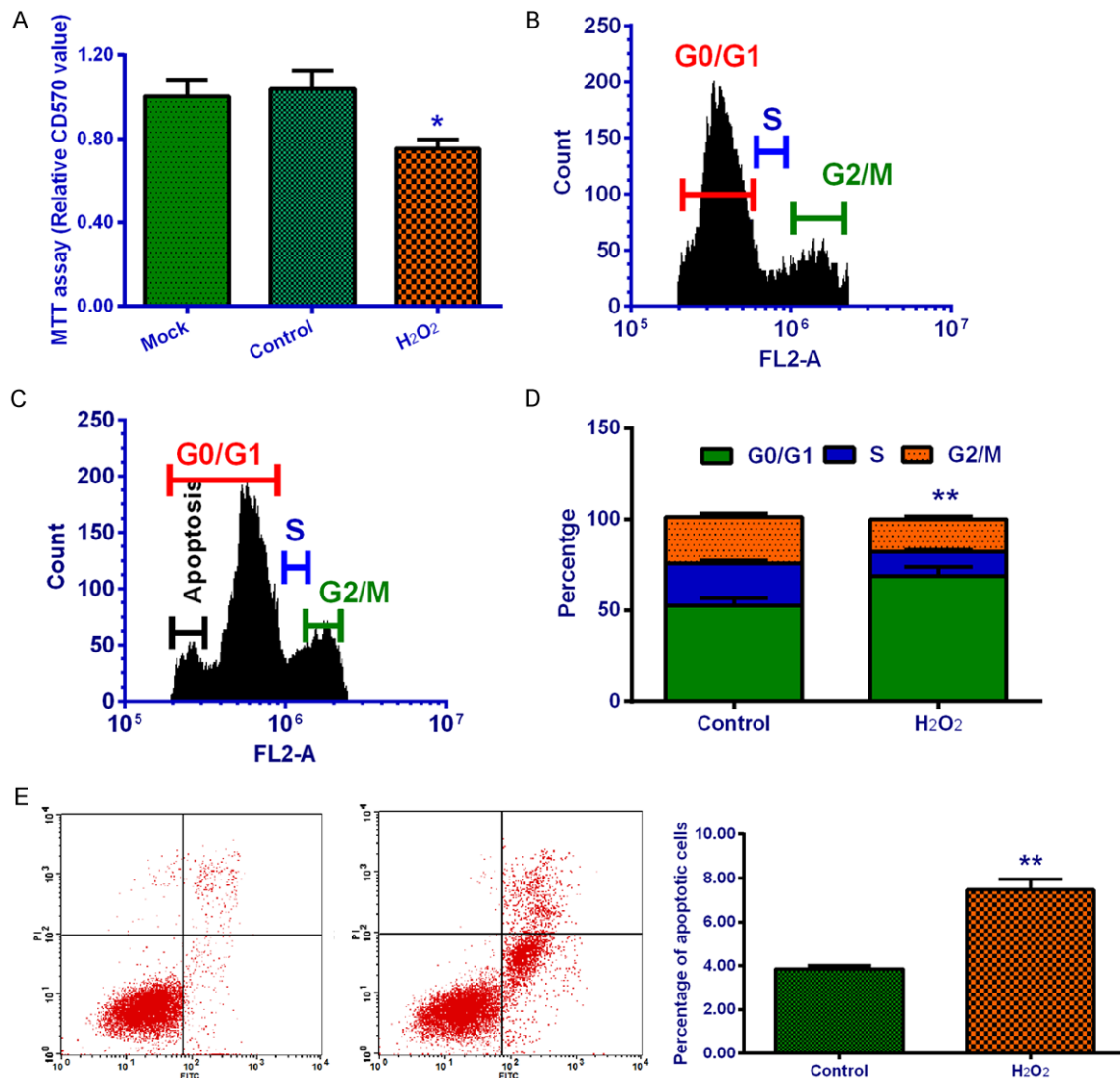
Cells apoptotic percentage was performed by Annexin V-FITC Apoptosis Detection Kit (Merk Company, USA) as the tutorial described [16]. Briefly, the H<sub>2</sub>O<sub>2</sub> treated MKN45 cells were centrifuged at 800×g for 5 minutes and washed twice with Dulbecco's Phosphate Buffer Solution (PBS), then the cells were suspended in the 400 µl 1× Binding Buffer at a concentration of 1×10<sup>5</sup> cells/ml, then each 5 µl of Annexin V-FITC and Propidium Iodide was added in turn and mixed, the treated cells were placed in the dark at RT for 5-15 minutes to perform flow cytometry analysis.

### Cell cycle analysis

Flow cytometric analysis of cells was performed using a Propidium Iodide ReadyProbes® Reagentkit (Thermofisher, USA) [17]. Briefly, treated MKN45 cells were washed twice with PBS solution. Then the cell suspension was centrifuged at 1,500 rpm, for 5 min at room temperature. Discarding all the supernatant and 1 ml of 70% methanol was added to the cell pellet. After incubation at -20°C for 24 hr, 1 ml of cold propidium iodide (PI) stain solution (20 µg/ml PI, 20 µg/ml RNase A and 0.1% Triton X-100) was added and it was incubated for 15 min in darkness at room temperature. PI was activated at 488 nm and fluorescence signal was amplified with PI fluorescence being measured at 617 nm. Cell cycle distribution is presented as the number of cells versus the amount of DNA, and apoptotic level was determined by counting cells of DNA content below G0/G1, S and G2/M phases with Cell Quest software (Becton Dickinson, San Jose, CA) and Flowjo software (Treestar, USA).

### MicroRNA array analysis

To identify the Oxidative Stress-specific miRNAs, we used the ABI TaqMan MicroRNA Array



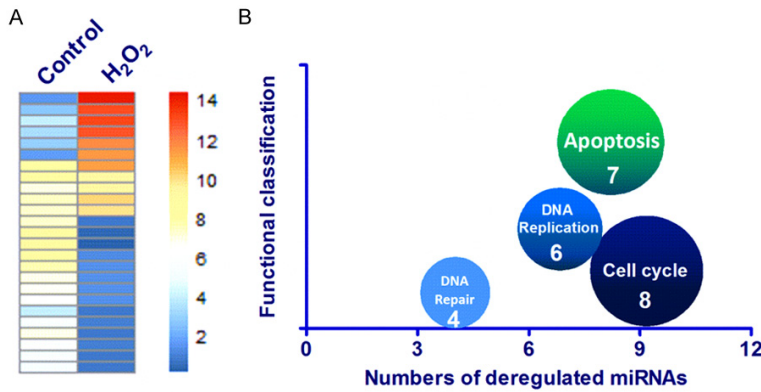
**Figure 1.** Cell viability, cell cycle distribution and apoptosis in the H<sub>2</sub>O<sub>2</sub>-treated gastric cancer MKN45 cells. (A) MTT assay for the cellular viability of control or the MKN45 cells, which were treated with 40  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 hours; (B and C) Flow cytometry analysis of the cell cycle distribution in the control (B) or H<sub>2</sub>O<sub>2</sub>-treated (C) MKN45 cells; (D) The percentages of cells in the G0/G1, S, G2/M phases in the two groups of cells were measured by cellQuest Pro software; (E) The percentage for apoptotic MKN45 cells, assessing by flow cytometry using Annexin V-FITC/PI Apoptosis Detection Kit. \*P<0.05, \*\*P<0.01, ns: no significance.

kit (Applied Biosystems) according to the manufacturer's protocol for profiling the production of miRNAs [18]. The heat map of miRNA was drawn by HeatmapBuider (<http://msdn.microsoft.com/netframework/downloads/redist.aspx>).

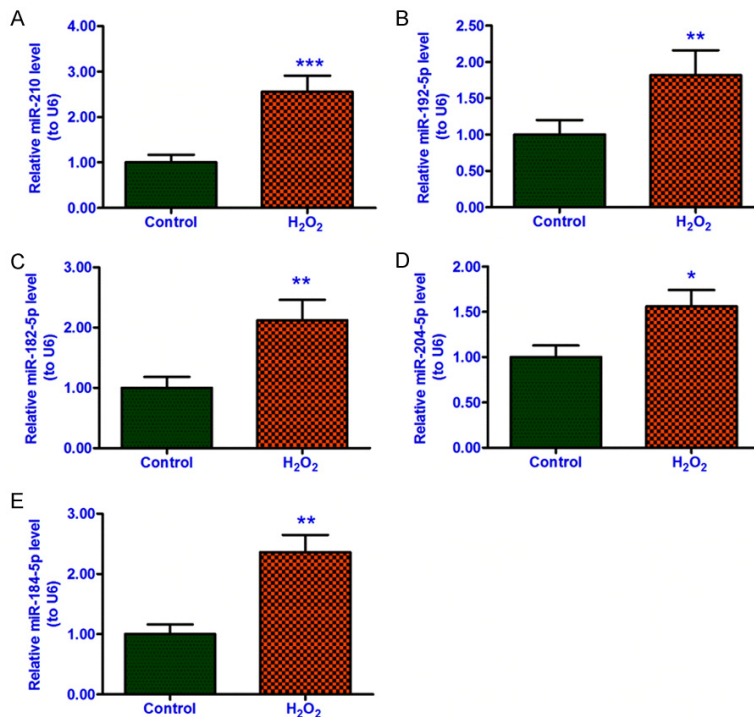
#### Western blotting assay

H<sub>2</sub>O<sub>2</sub> or/and miRNA treated MKN45 cells were lysed with lysis buffer (Invitrogen, USA) on ice for 20 min, and the cell lysates were centrifuged at 13,000 g at 4°C for 30 min, then the supernatant was collected as the total cellular

protein extract. After determining protein concentration using the BCA Protein Assay Kit (Bio-rad, USA). Equal amount of each cellular protein was loaded onto 10% SDS polyacrylamide gel. The separated proteins were electrophoretically transferred to PVDF membranes (Bio-rad, USA). The membrane was blocked overnight in blocking buffer containing PBS-T and 5% non-fatty milk. Then the membrane was incubated with primary mouse antibodies against cleaved caspase 3, Bcl-2, and  $\beta$ -actin for 1 hour separately and was washed with PBST for 4 times subsequently. Following incubating



**Figure 2.** Heat map representation and classification for the deregulated miRNAs in the  $H_2O_2$ -treated MKN45 cells. MKN45 cells were treated with  $40 \mu M H_2O_2$  for 24 hours, then the cellular miRNAs were profiled with miRNA microarray. A: miRNA expression is represented in the heat map in the color scale of -2 to +14 in green-red color scheme; B: Deregulated miRNAs were classified in function into cell cycle-, Apoptosis-, DNA replication- and DNA repair-associated miRNAs.



**Figure 3.** Relative levels of miR-210, miR-192-5p, miR-182-5p, miR-204-5p and miR-184-5p in the  $H_2O_2$ -treated MKN45 cells. The relative levels of miR-210 (A), miR-192-5p (B), miR-182-5p (C), miR-204-5p (D) and miR-184-5p (E) were examined by RT-qPCR in the control or  $H_2O_2$ -treated MKN45 cells for 24 hours. The relative miRNA level was presented as a relative value of  $H_2O_2$ -treated to control sample, with U6 as internal control. Statistical significance were shown respectively.  $P < 0.05$  (\*),  $P < 0.01$  (\*\*),  $P < 0.001$  (\*\*\*).

with the secondary HRP-conjugated antibody for 1 hour, the PVDF membrane was washed for 4 times and was treated with ECL reagent (Pierce, USA) and subjected to X-ray film. Each

band was quantified using Image software, according to the grayscale value of each band.

#### Quantitative real time PCR assays for mRNA expression

Total RNA was extracted from the  $H_2O_2$  treated MKN45 cells using the RNeasy Mini-kit (Qiagen Inc.) according to the manufacturer's recommendation and was reverse transcribed with SuperScript II RT (Invitrogen-Gibco). Quantitative real-time PCR was then conducted with SYBR® Green mastermix (Life tech, USA) in a 7500 Fast PCR instrument (Applied Biosystems, USA) using the special primers for miRNAs [19-21], separately. The cycle threshold (Ct) values of the target gene were normalized to  $\beta$ -actin from the same sample as relative mRNA levels. All samples were run in triplicate in the 96-well reaction plates.

#### Statistical analysis

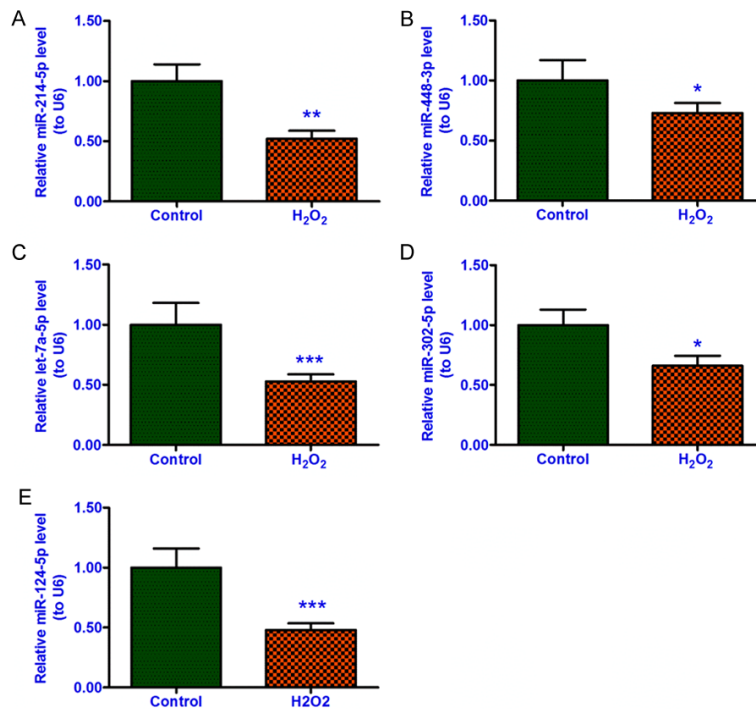
All experiments were assayed in triplicate. Data are expressed as means  $\pm$  SD. All statistical analyses were performed using GraphPad Pro. Prism 5.0 (GraphPad, SanDiego, CA). Statistical differences between two groups were assessed by Student's t test. A  $p$  value  $< 0.05$  was considered statistically significant.

#### Results

##### $H_2O_2$ treatment disturbed the cell cycle

As shown in **Figure 1A**, the cellular viability in  $H_2O_2$ -treated group was down-regulated by 20% compared with that of mock or control group with a statistical difference. We also checked the cell cycle distribution in the control group and the





**Figure 4.** Relative levels of miR-214-5p, miR-448-3p, let-7a-5p, miR-302-5p and miR-124-5p in the H<sub>2</sub>O<sub>2</sub>-treated MKN45 cells. The relative levels of miR-214-5p (A), miR-448-3p (B), let-7a-5p (C), miR-302-5p (D) and miR-124-5p (E) were examined by RT-qPCR in the control or H<sub>2</sub>O<sub>2</sub>-treated MKN45 cells for 24 hours. The relative miRNA level was presented as a relative value of H<sub>2</sub>O<sub>2</sub>-treated to control sample, with U6 as internal control. Statistical significance was shown respectively. P<0.05 (\*), P<0.01 (\*\*), P<0.01 (\*\*\*).

H<sub>2</sub>O<sub>2</sub>-treated group, as indicated in **Figure 1B** and **1C**, H<sub>2</sub>O<sub>2</sub> treated cells presented a higher apoptotic crest. Then, the percentages of cells in the G0/G1, S, G2/M phases in the two groups of cells were measured by cell Quest Pro software, the constituent ratio of each phase is 50% (G0/G1 phase), 25% (G2/M phase), and 25% (S phase), respectively. Regarding the H<sub>2</sub>O<sub>2</sub> treated cells, the constituent ratio of each phase is 70% for G0/G1 phase, 17% for G2/M phase, and 13% for S phase (**Figure 1D**). A statistical difference was observed between two groups. In addition, H<sub>2</sub>O<sub>2</sub> treatment increased the apoptotic rate by 75% compared with the control group.

#### Relative levels of miRNAs in the H<sub>2</sub>O<sub>2</sub>-treated MKN45 cells

After MKN45 cells were treated with 40  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 hours, then the cellular miRNAs were profiled with miRNA microarray. Then miRNA expression is represented in the heat map in the color scale of -2 to +14 in green-

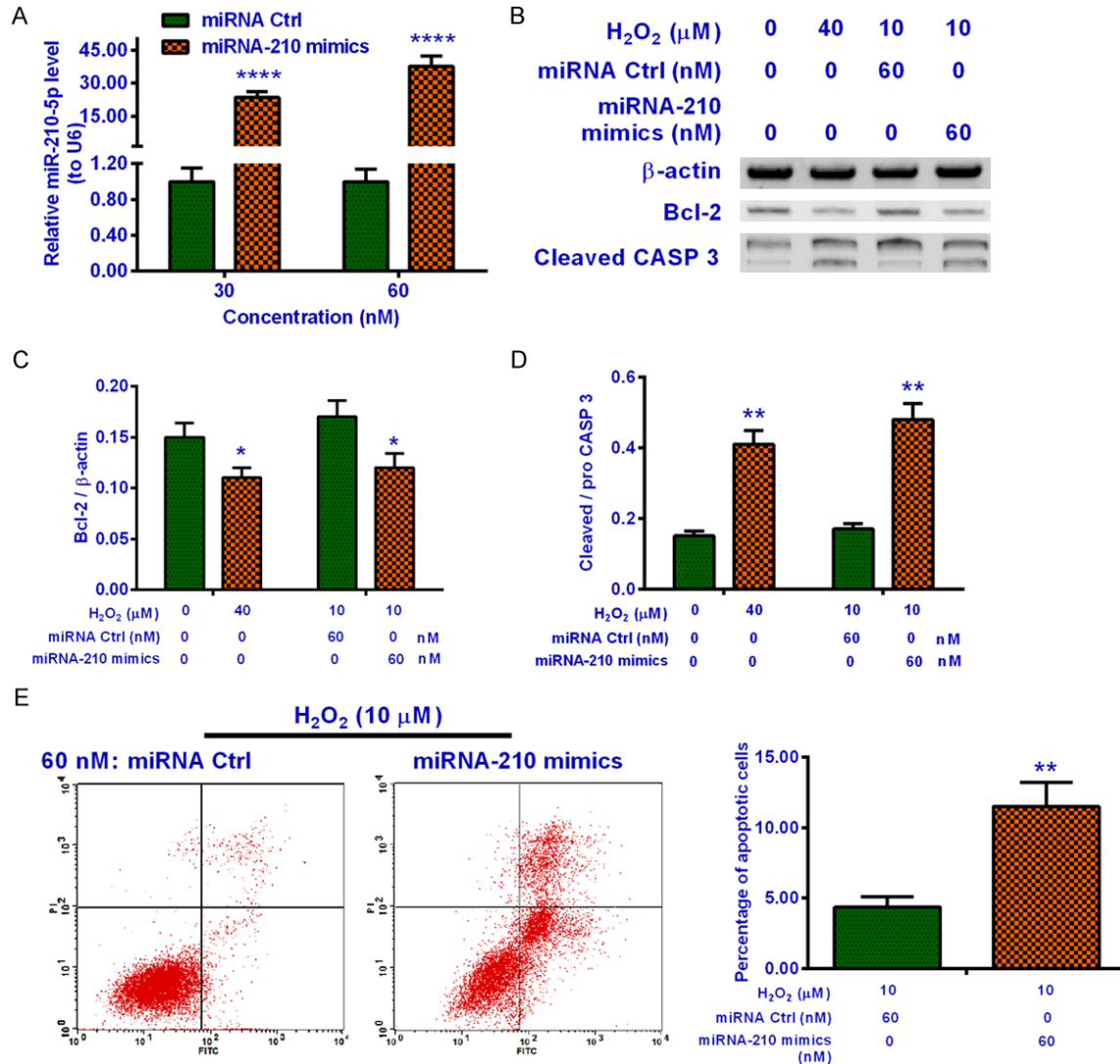
red color scheme (**Figure 2A**). Deregulated miRNAs were classified in function into eight cell cycle-, seven Apoptosis-, six DNA replication-, and four DNA repair-associated miRNAs (**Figure 2B**).

We checked the relative expression level of miR-210, miR-192-5p, miR-182-5p, miR-204-5p, and miR184-5p, each gene was found presenting a higher level compared with the control group with statistical differences. In detail, miR-210 (P<0.001, **Figure 3A**), miR-192-5p (P<0.01, **Figure 3B**), miR-182-5p (P<0.01, **Figure 3C**), miR204-5p (P<0.05, **Figure 3D**), and miR184-5p (P<0.01, **Figure 3E**) were improved by 2.5 times, 1.8 times, 2.0 times, 1.5 times, and 2.3 times compared with the control group, respectively.

The relative levels of miR-214-5p, miR-448-3p, let-7a-5p, miR-302-5p, and miR-124-5p were examined by RT-qPCR in the control or H<sub>2</sub>O<sub>2</sub>-treated MKN45 cells for 24 hours. It was found that the relative levels of miR-214-5p (P<0.01, **Figure 4A**), miR-448-3p (P<0.05, **Figure 4B**), let-7a-5p (P<0.001, **Figure 4C**), miR-302-5p (P<0.05, **Figure 4D**), and miR-124-5p (P<0.001, **Figure 4E**) were down-regulated by 50%, 40%, 50%, 45%, and 52%, respectively.

#### miR-210-5p promotes apoptosis in MKN45 cells via inhibiting the expression of Bcl-2

MKN45 cells were transfected with 30 or 60 nM control miRNA or miR-210-5p mimics, and then were cultured in the medium supplemented with 10 or 40  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 hours, then the cellular miR-210 level, the apoptosis-associated proteins and apoptosis were examined respectively. As shown in **Figure 5A**, miRNA-210 mimic promoted the miRNA-210 levels 20- to 30-time higher than miRNA control (P<0.0001). In the next step, we measured the  $\beta$ -actin, Bcl-2, and cleaved caspase 3 expres-



**Figure 5.** miR-210-5p promotes apoptosis in MKN45 cells via inhibiting the expression of Bcl-2. MKN45 cells were transfected with 30 or 60 nM control miRNA (miRNA Ctrl) or miR-210-5p mimics, and then were cultured in the medium supplemented with 10 or 40 μM H<sub>2</sub>O<sub>2</sub> for 24 hours, then the cellular miR-210 level, the apoptosis-associated proteins and apoptosis were examined respectively. (A) Relative miR-210 level to U6 in the MKN45 cells, which were transfected with 30 or 60 nM miRNA Ctrl or miR-210-5p mimics for 24 hours; (B-D) Western blotting assay (B) for or the level (ratio to internal control, β-actin) of B-cell lymphoma-2 (Bcl-2) (C) and cleaved caspase 3 (cleaved CASP 3) (D), the active form of caspase 3, in the MKN45 cells, which were treated with 0, 10 or 40 μM H<sub>2</sub>O<sub>2</sub> for 24 hours, with or without the transfection with 60 nM miRNA Ctrl or miR-210-5p mimics. (E) Flow cytometry analysis and the percentage for apoptotic MKN45 cells, which were treated with 10 μM H<sub>2</sub>O<sub>2</sub> for 24 hours, and were transfected with 60 nM miRNA Ctrl or miR-210-5p mimics. All experiments were performed in triplicate independently. And statistical significance was showed as \*P<0.05, \*\*P<0.01 OR \*\*\*\*P<0.0001.

sion level in the different concentrations of miRNA and H<sub>2</sub>O<sub>2</sub> (Figure 5B). In detail, 40 μM H<sub>2</sub>O<sub>2</sub> treatment decreased the Bcl-2/β-actin level by 33%, and cells treated by 10 μM H<sub>2</sub>O<sub>2</sub> plus 60 nM miRNA-210 mimic present a Bcl-2/β-actin level 25% lower than those treated by 10 μM H<sub>2</sub>O<sub>2</sub> plus 60 nM miRNA control (P<0.05, Figure 5C). In addition, cells treated by 40 μM H<sub>2</sub>O<sub>2</sub> have a higher percentage of cleaved/pro caspase 3 (P<0.01, Figure 5D), similar results

were also observed between the 10 μM H<sub>2</sub>O<sub>2</sub> plus 60 nM miRNA-210 mimic-treated group and 10 μM H<sub>2</sub>O<sub>2</sub> plus 60 nM miRNA control-treated group, treatment by 10 μM H<sub>2</sub>O<sub>2</sub> plus 60 nM miRNA-210 decreased the cleaved/pro caspase 3 by 2.5 times compared with the control.

At last, we checked the apoptotic rate of treated cells, as indicated in Figure 5E, 40 μM H<sub>2</sub>O<sub>2</sub>

treated cells increased the apoptotic rate by 2.5 times compared with the control, and treatment by 10  $\mu$ M  $H_2O_2$  plus 60 nM miRNA-210 improved the cleaved/pro caspase 3 by 3 times compared with the control (treated by 10  $\mu$ M  $H_2O_2$  plus 60 nM miRNA control). Interestingly, we firstly examined the regulatory role of miR-210 on the  $H_2O_2$ -induced apoptosis with 40  $\mu$ M  $H_2O_2$ . However, no significant difference was found between the miRNA control and miRNA-210 mimic groups (either 60 nM) ( $P < 0.01$ , **Figure 5E**). It implies that the regulatory role of miR-210 on the  $H_2O_2$ -induced apoptosis was only detectable when under a lower  $H_2O_2$  concentration.

## Discussion

In this research, we found that  $H_2O_2$  treatment significantly reduced cell viability, deregulated the cell cycle and induced apoptosis in the gastric cancer MKN45 cells. The miRNA microarray indicated that miR-210, miR-192-5p, miR-182-5p, miR-204-5p and miR-184-5p were up-regulated; miR-214-5p, miR-448-3p, let-7a-5p, miR-302-5p and miR-124-5p were down-regulated, in the  $H_2O_2$ -treated MKN45 cells. miR-210 was significantly up-regulated in GC patients, and was conceived as a potential diagnostic marker of GC [22, 23]. In the present study, we chose miR-210 as a important biomarker for deep exploration. The upregulation of miR-210 markedly reduced the Bcl-2 expression, promoted the cleavage of caspase 3 and thus induced apoptosis in MKN45 cells. The up-regulated miR-210 might mediate the oxidative stress-induced apoptosis in gastric cancer cells, probably via inhibiting Bcl-2. The present study implies the mediatory role of miRNAs, such as miR-210 in the oxidative stress-induced apoptosis in gastric cancer cells.

Many signals have been reported to induce ER stress [24, 25]. Additionally, it has been demonstrated that ROS plays a role in ER-stress-mediated apoptosis in a variety of cell types [26]. Recent evidence indicates that miR-210 directly antagonizes an apoptotic component, CASP8AP2 [27]. In our study, we also confirmed that up-regulation of miR-210 markedly reduced the Bcl-2 expression, promoted the cleavage of caspase 3 and thus induced apoptosis in MKN45 cells. This result is in accordance with previous reports [28, 29]. However, wheth-

er the miR-210 antagonizes apoptosis through inhibiting ER-stress is waiting to be elucidated.

Compared with other solid tumors, miR-210 expression is down-regulated in ovarian and esophageal squamous cell carcinomas. miR-210's over-expression in cancer cell lines got around hypoxia-induced cell cycle arrest and partially reversed the hypoxic gene expression signature. In our experiments, we also found that the  $H_2O_2$  treatment prolonged the G0/G1 period, and shortened the S period and G2/M period subsequently. Specially, an apoptosis-related wave in the G0/G1 period was induced by the  $H_2O_2$  treatment. The net impact of miR-210 on cell proliferation seems to be worthy of further studying.

miR-210 is a highly conserved, small, noncoding RNA that participates in apoptosis, inflammation, and tumorigenesis [30]. Our results indicate that miR-210 expression was up-regulated following  $H_2O_2$  treatment. Furthermore, the up-regulation of miR-210 was positively correlated with the cellular apoptotic level. These results suggest that miR-210 is involved in the  $H_2O_2$ -mediated apoptosis. Although the results of the miRNA array analysis indicated that there are other involved endogenous miRNAs, we chose to focus on miR-210, which emerged as a major potential regulator, additional investigations of other miRNAs are required in the future.

## Conclusion

Taken together, we found that oxidative stress reduced the cell viability, disturbed the cell cycle and induced apoptosis in gastric cancer cells, along with the deregulated miRNA expression in gastric cancer MKN45 cells. And the up-regulated miR-210 might mediate the oxidative stress-induced apoptosis in gastric cancer cells, probably via inhibiting Bcl-2. The present study implies the miRNAs, such as miR-210 play a role in the oxidative stress-induced apoptosis in gastric cancer cells.

## Acknowledgements

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## Disclosure of conflict of interest

None.

**Address correspondence to:** Wu Ji, PLA Research Institute of General Surgery, Jinling Hospital, Southern Medical University, 301-2, Zhongshan East Road, Xuanwu District, Nanjing 210002, Jiangsu Province, China. Tel: 008680860114; Fax: 008-680860114; E-mail: wjhheg17\_85@163.com

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