Original Article

Hsa-let-7g miRNA regulates the anti-tumor effects of gastric cancer cells under oxidative stress through the expression of DDR genes

Haiqing Hu¹, Xuanzhong Zhao², Zhao Jin² and Mingxing Hou²

¹Department of Gastroenterology and Hepatology, the Affiliated Hospital of Inner Mongolia Medical University,
1 Tongdao North Road, Hohhot, Inner Mongolia, 010050, China

²Gastrointestinal surgery, the Affiliated Hospital of Inner Mongolia Medical University,
1 Tongdao North Road, Hohhot, Inner Mongolia, 010050, China

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ABSTRACT — Oxidative stress is linked to increased risk of gastric cancer (GC). Recent reports have found that hsa-let-7g microRNA (miRNA) has properties of anti-tumor and resistance to damages induced by oxidized low-density lipoprotein (ox-LDL). Dysregulation of hsa-let-7g was present in GC *in vivo* and *in vitro* under exogenous stress. However, we didn't know whether there are regulatory mechanisms of hsa-let-7g in GC under oxidative stress. This study was aimed at investigating the effects of hsa-let-7g microRNA (miRNA) on GC under oxidative stress. The results showed that H₂O₂ induced the increase of DNA damage response (DDR) genes (ATM, H2AX and Chk1) and downregulation of hsa-let-7g in GC cells. Further study confirmed Hsa-let-7g caused the apoptosis and loss of proliferation in GC cells exposed to H₂O₂ associated with repression of DDR system. Yet, we found let-7g didn't target DDR genes (ATM, H2AX and Chk1) directly. In addition, data revealed hsa-let-7g miRNA increased the sensitivity of GC to X-rays involving in ATM regulation as well according to application of X-rays (another DDR inducer). In conclusion, Hsa-let-7g miRNA increased the sensitivity of GC to oxidative stress by repression activation of DDR indirectly. Let-7g improved the effects of X-rays on GC cells involving in DDR regulation as well.

Key words: Gastric cancer, MicroRNA, Hsa-let-7g, Oxidative stress, DNA damage response

INTRODUCTION

The prevalence of gastric cancer (GC) is particularly serious in Asian countries (Leung *et al.*, 2008). Newly diagnosed GC cases in Asian countries accounted for about three-quarters of the world total in 2008 (Shen *et al.*, 2013). To date, the 5-year survival rate of GC patients is less than 15% (Correa, 2004). However, the pathogenesis of GC is largely unknown.

Nutritional, microbial, and genetic factors have been proposed to exert effects on the multistep and multifactorial process, where oxidative stress is involved (Nair *et al.*, 2000). The normal level of reactive oxygen species (ROS) is favorable to exhibit physiological functions (Valko *et al.*, 2006; D'Autreaux and Toledano, 2007). But, luxuriant ROS, which is denoted as oxidative stress, is harmful to the body (D'Autreaux and Toledano, 2007). It has been

confirmed that oxidative stress induced by free radicals is highly related to the development of several diseases, including cardiovascular, cancer, and chronic inflammation as causing DNA and protein modification and lipid peroxidation (Oberley, 2002; Hussain *et al.*, 2003; Sanders *et al.*, 2004). It is known that the gastric epithelium continuously undergoes oxidative stress owing to ingested food and cigarette smoke and inflammation induced by *Helicobacter pylori* infection, which could increase the risk of mucosal homeostasis collapsing (Gencer *et al.*, 2013; Farinati *et al.*, 2008). It happens that gene expression profiles and protein structure and function have been changed to result in tumor promotion once mucosaa homeostasis collapses (Tang *et al.*, 2010; Lowy *et al.*, 2006). So, it is inadvisable to ignore the relationships between oxidative stress and GC. Yet, the precise molecular mechanisms about the relationships remain

Correspondence: Mingxing Hou (E-mail: houmx1964@sohu.com)

unclear.

MicroRNAs (miRNAs), small single-stranded RNA molecules containing about 22 nucleotides, are noncoding RNAs molecules which can bind the 3'-untranslated region of target messenger RNA (mRNA) to regulate diverse gene expression. At present, the role of some miRNAs has been revealed to associate with oxidative stress in GC. Aberrant miRNA-21 was found under oxidative stress in GC tissues and GC cells. Further study revealed oxidative stress promoted gastric carcinogenesis according to upregulating miR-21 expression (Tu *et al.*, 2014). Excessive ROS induced by macrophages stimulated the promotion of GC growth according to suppression of miR-328 (Ishimoto *et al.*, 2014). Several recent studies have indicated that the dysregulation of hsa-let-7g was present in GC *in vivo* and *in vitro* under exogenous stress (Wu *et al.*, 2011; Hummel *et al.*, 2010). Moreover, a negative feedback modulation has been reported between Hsa-let-7g miRNA with oxidative stress in some diseases (Chen *et al.*, 2011). Thus, we are interested in whether there are regulatory mechanisms of hsa-let-7g in GC under oxidative stress.

ROS induced by oxidative stress stimulates DNA damage response (DDR), which is activated upon DNA damage (Chen *et al.*, 2012). Cancer cells often display significant modification at the DDR-associated factors and develop inhibition to DNA damage-inducing agents (Tessitore *et al.*, 2014). In the last decade, it has been documented that miRNAs were involved in the regulation of DNA damage/repair process in some cancers (Hu *et al.*, 2010; Wang *et al.*, 2011; Crosby *et al.*, 2009). We have found some miRNAs possess the regulatory effects on radiosensitivity of cancer (Zhao *et al.*, 2012). Reviews have concluded MiRNAs involved in the regulation of DNA damage/repair mechanisms can be considered markers to predict the response to radiotherapy (Zhao *et al.*, 2012; Tessitore *et al.*, 2014). In terms of GC, both miR-300 and miR-642 potentially regulated DNA damage and repair pathway-related genes by using miRNA microarray after two clinical GC samples exposed to X-rays (He *et al.*, 2014). Another report found that miR-375 targets p53 to regulate the response to ionizing radiation and etoposide treatment in several gastric cancer cell lines (Liu *et al.*, 2013). miR-221 and miR-222 regulate radiosensitivity of gastric carcinoma cells (SGC7901 cells) as well (Chun-Zhi *et al.*, 2010). It's interesting to explore whether there are other microRNAs involved in the regulation of DDR in GC.

In this study, we focused on the expression of DDR genes and miRNA hsa-let-7g in GC cells exposed to H₂O₂. Further study was to investigate the effect of hsa-

let-7g on GC and the crosstalk between hsa-let-7g with DDR genes under oxidative stress. Moreover, experiments were performed to assess potential application value of hsa-let-7g in anticancer therapeutics of GC.

MATERIALS AND METHODS

Cell lines, cell culture and treatments

Human GC cell line (AGS) was acquired from American Type Culture Collection (ATCC, Rockville, MD, USA). AGS cells were grown in DMEM (Gibco, Invitrogen, Carlsbad, CA, USA) with 10% FBS in a humidified incubator containing 5% CO₂ at 37°C. Cells were subcultured after reaching 80% confluence. H₂O₂ (Sigma-Aldrich, St. Louis, MO, USA) was diluted in serum-free DMEM. Cells were treated with H₂O₂ (100 µM or 200 µM) for 6, 12 or 24 hr when cells were confluent at 90%. Total RNA or proteins were extracted at indicated time points. Human embryonic kidney epithelial cells (293 T cells) were grown in DMEM with 10% FBS in a humidified incubator containing 5% CO₂ at 37°C. Hsa-let-7g mimics (miR mimics) and miR-control, purchased from GenePharma (GenePharma, Shanghai, China), were transfected into AGS cells at 50 nM concentration respectively. All cellular transfections were conducted using Lipofectamine 2000 (Invitrogen).

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells with RNeasy kit (Qiagen, Valencia, CA, USA). qRT-PCR was conducted on ABI 7500 with SYBR green PCR kits (Applied Biosystems). GAPDH gene was named as endogenous control. Cellular miRNA was isolated using the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA). MirVana qRT-PCR miRNA Detection Kit (Ambion) and qRT-PCR Primer Sets (Ambion) was used. The U6 small nuclear RNA was internal control of miRNAs. The data were normalized by 2^{-ΔΔCt} method as relative quantification. The primers used were as follows. ATM (GenBank accession number NM_000051.3): F: 5'-TGG GCT CTG GAA TCA TAC GGC-3', R: 5'-TAA CGC TCA CGA GTG CTC ACC AC-3'. Chk1 (GenBank accession number AF016582.1): F: 5'- AAC GCC TTC CTT GAT GGA AT -3' and R: 5'-GCGACTTGAACG-GAATATCTGT -3'. H2AX (GenBank accession number NM_002105.2): F: 5'- CCC TCG GGC GGC AAG AA -3' and R: 5'- GGA GGG CGG ACG GCG GAC AGG -3'. GAPDH: F: 5'-TTT TCC CTC TTC TTG ACT CAC CC-3' and R: 5'- GTG CCT TTC ATT CCA TCC AGC-3'.

Apoptosis and viability assay

Cellular apoptosis was assessed using Annexin-V-fluorescein isothiocyanate (Annexin V-FITC) apoptosis detection kit (Sigma-Aldrich) according to the manufacturer's instructions (KeyGen Biotech., Nanjing, China). Briefly, cells were harvested after indicated treatments. Then cell suspensions were fixed overnight with ice-cold 70% ethanol. Next, the fixed cells were stained with propidium iodide or annexin V-FITC after centrifugation and resuspensions. Analyses were performed with flow cytometer (BD FACScan). Data were expressed as the percentage of apoptotic cells from the total cells. For viability assay, cells with indicated treatments were incubated in CCK-8 (Dojindo, Kumamoto, Japan). The 450 nm absorbance of each well was assayed after visual color occurrence at indicated time points. All experiments were performed in triplicate.

Clonogenic cell assay

AGS cells were grown to about 80% confluence in 35 mm dishes. Then, cells were transfected with miR mimics or miR-control at 50nM concentration. Following, cells were exposed to 0, 2, 4 and 8 Gy X-ray irradiation (6 MV, the dose rate was 200 Gy/min) by a PRIMUS accelerator (SIEMENS Medical Solutions, Erlangen, Germany). Cells (100 for cells irradiated with 0 or 2 Gy, 200 for 4 Gy and 2000 for 8 Gy) were counted and cultured for clonogenic assay. The colony was formed in 7 days. Then, we stained the cells with crystal violet (0.005%) for 20 min and recorded the colony numbers using Alpha Innotech (San Leandro, CA, USA) imaging software. Plating efficiency (PE) was calculated as: $PE = (\text{colony number}/\text{plating cell number}) \times 100\%$, in triplicate.

Western blotting

Cells were lysed in lysis buffer (20 mM Tris HCL pH 8, 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA, protease and phosphatase inhibitors) followed by sonication on ice. Supernatants were fractionated by 10% SDS-PAGE gels and electro-transferred onto nitrocellulose membranes (Millipore) after centrifuged at 14,000 g for 30 min at 4°C. The membranes were incubated by primary antibodies. Then, the signals were acquired by using the ECL detection systems (Super Signal West Femto, Pierce) after incubated with horseradish-peroxidase-conjugated secondary antibodies (Pierce). Primary antibodies were used as follows. Rabbit antibodies against human H2AX, phospho-ATM (S1981), ATM, and Chk1 were purchased from Cell Signaling Technologies, Beverly, MA, USA. Anti- β -actin (Sigma) was used as internal con-

trol. The Western blotting images of three independent experiments were scanned and quantified by Image J software (National Institutes of Health). Band intensities were assessed and normalized to the β -actin bands as indicated experiments.

Luciferase reporter assay

3'-UTR of H2AX (position -19 to 1046) and Chk1 was amplified by PCR and cloned into pGL3 (Promega) to obtain pGL3-H2AXUTR plasmid and pGL3-Chk1UTR plasmid (Lezina *et al.*, 2013; Wang *et al.*, 2011). The luciferase-UTR reporter plasmid that contains ATM 3'-UTR carrying a putative let-7g miRNA binding site (2210-2569) was constructed and inserted into pGL3-Luc vector named as pUTR (Yan *et al.*, 2010). The constructs and the internal control vector pRL-TK (Promega) were co-transfected into 293T cells. Cells were transfected with 50 nM of miR-let-7g mimics or miR-control 6 hr later. Luciferase reporter assays were performed after 24-hr transfection by the Dual-Luciferase Assay System (Promega) according to the manufacturer's instructions.

Data analysis

All data were calculated as mean $\pm$ S.E.M. Student t-test was used to compare two groups. Comparison among multiple samples was made by ANOVA. The SPSS 18.0 was used for statistical analysis. $P < 0.05$ was considered a statistically significant difference.

RESULTS

H₂O₂ resulted in the dysregulation of Hsa-Let-7g miRNA and DNA repair genes in GC cells

Reactive Oxygen Species (ROS) induced by oxidative stress, including H₂O₂, have been known to stimulate DNA damage response (DDR) (Chen *et al.*, 2012). To survey the possible mechanism of how H₂O₂ induced DDR in GC cells, we assayed the induction of DDR genes (ATM, H2AX and Chk1), as these agents are considered the key members of the DDR pathway (Shrivastav *et al.*, 2008). The three DDR members were measured in mRNA level and protein level. It was found that both 100 μ M and 200 μ M H₂O₂ promoted significant upregulation of ATM mRNA expression at 6 hr ($P = 0.011$ in 200 μ M H₂O₂ group) and 12 hr ($P = 0.012$ and $P = 0.001$ respectively), compared to the C group (without treatment of H₂O₂) (Fig. 1A). Moreover, the levels of H2AX (Fig. 1B) and Chk1 (Fig. 1C) were significantly high in mRNA expression under 200 μ M H₂O₂ exposure at 6 hr ($P = 0.047$ for H2AX) and 12 hr ($P = 0.011$ for H2AX and $P = 0.004$ for

Fig. 1. H₂O₂ resulted in the dysregulation of hsa-let-7g miRNA and DNA repair genes in AGS cells. Cells were treated with H₂O₂ at concentrations of 100 μM and 200 μM for 6 hr or 12 hr. Total RNA or proteins were extracted at the indicated time points. Expression of ATM mRNA (A), H2AX mRNA (B) and Chk1 mRNA (C) was assayed with qRT-PCR. GAPDH gene was denoted as endogenous control. Let-7g miRNA was tested with MirVana qRT-PCR miRNA Detection Kit and the U6 small nuclear RNA was internal control of miRNAs (E). The data were normalized by 2^{-ΔΔCt} method as relative quantification. Results were indicated as mean ± S.E.M. The ATM, H2AX, and Chk1 proteins and phosphorylated ATM proteins (pATM) were measured by Western blotting (D). *P < 0.05 was considered a statistically significant difference.

Chk1 respectively), when compared with the C groups. In addition, there were obvious changes in ATM, H2AX and Chk1 protein expression between cells exposed to H₂O₂ with normal cells (without treatment of H₂O₂). Our investigation of phosphorylated ATM (pATM) showed the level of pATM in cells exposed to H₂O₂ was dramatically higher than that in AGS cells without exposure, as shown in Fig. 1D. To observe the effects of H₂O₂ on hsa-let-7g in GC cells, the expression of let-7g was measured by qRT-PCR (Fig. 1E). The hsa-let-7g expression in AGS after H₂O₂ exposure for 12 hr was significantly lower than in AGS cells without treatment ($P = 0.004$ and $P = 0.001$ for 100 μ M and 200 μ M respectively), as shown in Fig. 1E.

Hsa-let-7g promoted apoptosis and inhibited cellular viability in the presence of H₂O₂

To further study the effects of let-7g on GC cells upon oxidative stress, the hsa-let-7g mimics (50 nM) were performed to increase the amount of let-7g by transfection.

The expression of let-7g increased to 973 fold and 643 fold post 24-hr transfection and 48-hr transfection respectively (Fig. 2A). Of note, addition of let-7g miRNA mimics significantly increased the apoptotic rate in the presence of 100 μ M H₂O₂, in contrast with that in the miR-control group ($P = 0.017$ for 24 hr, $P = 0.028$ for 48 hr), as shown in Fig. 2B. The cellular proliferation was measured with MTT assay. It was found that cells with let-7g miRNA mimics had higher proliferation than that with miR-control not only at 24 hr but also 48 hr under 100 μ M H₂O₂ exposure (Fig. 2C). These findings illustrated hsa-let-7g miRNA contributed to the apoptosis and loss of proliferation in GC cells exposed to H₂O₂.

Hsa-let-7g repressed activation of DDR upon oxidative stress indirectly.

Given the effect of H₂O₂ on DDR genes and the regulatory effect of let-7g on cellular apoptosis and proliferation upon oxidative stress, we supposed whether let-7g

Fig. 2. Let-7g miRNA increased the damages induced by H₂O₂ including stimulation of apoptosis and loss of cellular viability. AGS cells underwent 100 μ M H₂O₂ treatments for 24 or 48 hr after transfection with miR-let-7g mimics (miR-mimics) or miR-control at 50 nM concentration. The efficiency of transfection was assessed with qRT-PCR (A). Then, the apoptosis rate was assayed with Annexin V-FITC apoptosis detection kit. Data were expressed as the percentage of apoptotic cells among the total cells (B). The viability was measured by CCK-8 assay (C). All experiments were performed in triplicate. * $P < 0.05$ was considered a statistically significant difference.

Fig. 3. Let-7g miRNA repressed the expression of DNA genes (ATM, H2AX and Chk1) in mRNA level and protein level. AGS cells underwent the indicated treatments in the presence of 100 μ M H₂O₂. The expression of ATM (A), H2AX (B) and Chk1 (C) mRNA was assayed by qRT-PCR. Results were normalized by 2^{- $\Delta\Delta C_t$} method and indicated as mean $\pm$ S.E.M. The ATM, H2AX and Chk1 proteins were compared between the miR-mimics group and the miR-control group (D). Band intensities of Chk1 protein were assessed by image J software and normalized to the β -actin bands (E). All experiments were performed in triplicate. The statistical results were indicated by asterisks ($P < 0.05$) and ** represents $P < 0.01$.

was related to the DDR genes upon oxidative stress. To confirm this hypothesis, the DDR genes including ATM, H2AX and Chk1 were measured. Let-7g miRNA (50 nM) decreased the expression of DDR genes (ATM, H2AX and Chk1) significantly at 24 hr ($P = 0.001$, $P = 0.049$ and $P = 0.014$ respectively) (Figs. 3A-3C). Moreover, let-7g also downregulated the level of ATM and H2AX in protein expression under H₂O₂ exposure for 24 hr (Fig. 3D). We also found significant differences in Chk1 pro-

tein between the miR-control group and the miR-mimics group, as shown in Fig. 3D and Fig. 3E ($P = 0.02$).

To further investigate the relationship between let-7g and DDR genes, we provided four databases (TargetScan, microcosm, Pictar, mirtar) for let-7g candidates which could targeted 3' UTR of these genes (ATM, H2AX and Chk1). Unfortunately, there was no seed sequence to be acquired (data not shown). To confirm the prediction, the 3' UTR of three genes were

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Fig. 4. Let-7g increased the sensitivity of GC cells to X-ray involving in activation of ATM gene. Cellular colony formation was assayed by clonogenic cell assay after exposure to 0, 2, 4 and 8 Gy X-ray irradiation. Plating efficiency (PE) was calculated as: $PE = (\text{colony number}/\text{plating cell number}) \times 100\%$. Error bars indicate the standard error of the mean of three individual experiments (A). Cells were cultured for 24 hr after exposure to 0, 2, 4 and 8 Gy X-ray irradiation. Next, the relative expression of ATM mRNA was measured (B). The ATM protein was assessed by Western blotting as well (C). All experiments were performed in triplicate. The statistical results were indicated by asterisks and ** represents $P < 0.01$.

inserted to pGL vector and luciferase reporter systems were constructed as described in Materials and Methods. There was no difference in the luciferase activity between constructs (pGL3-H2AXUTR and pGL3-Chk1UTR) and pGL3, when let-7 mimics were applied (data not shown). Next, let-7g miRNA putative binding sites with most complementary base pairing of ATM 3'UTR were cloned into luciferase reporter constructs (pUTR) (Supplementary Fig. 1A). Luciferase reporter gene activity in pUTR was no significantly different from that in pGL in the presence/absence of let-7 mimics (Supplementary Fig. 1B). This further suggested that let-7g might not target the DDR genes (ATM, H2AX and Chk1). However, let-7g significant repressed the activation of DDR indirectly.

Let-7g increased the sensitivity of GC cells to X-rays

Clonogenic assay was performed to detect the cell survival fractions after treatments with 0, 2, 4, and 8 Gy X-ray irradiation. Plating efficiency of AGS was calculated as shown in Fig. 4A. The plating efficiency of AGS was 85.0%, 66%, 50% and 27.7% respectively in the miR-control group when exposed to 0, 2, 4, and 8 Gy. PE was 84%, 47%, 28% and 15% respectively, in AGS cells with miR-mimics when exposed to 0, 2, 4, and 8 Gy (Fig. 4A). These data showed that let-7g increased the sensitivity of GC cells to X-rays dramatically. To determine whether the protective effects of let-7g against damage induced by X-rays were related to the DDR system, the ATM gene and ATM protein were measured in AGS cells exposed to X-rays for 24 hr. We found there were significant differences of expression of ATM gene mRNA between AGS cells with miR-control and cells with miR

mimics ($P = 0.038$ under 4 Gy X-ray irradiation and $P = 0.022$ under 8 Gy X-ray irradiation respectively) (Fig. 4B). Let-7g mimics application decreased the level of ATM protein in AGS under X-ray stress (Fig. 4C). This implied that let-7g increased the sensitivity of GC cells to X-ray involving in DDR system regulation.

DISCUSSION

Oxidative stress may cause chronic inflammation, which in turn induces the occurrence of chronic diseases, including cancers (Coussens and Werb, 2002). Extensive research has shown that oxidative stress is highly associated with the process of carcinogenesis, including survival, proliferation, angiogenesis and metastasis (Coussens and Werb, 2002; Mantovani, 2005). GC might be the product of oxidative stress as high risks of exposure that the gastric tissue suffers from the continuous oxidative stress and chronic inflammation (Farinati *et al.*, 2008). To investigate the effects of oxidative stress on GC, we applied H_2O_2 to mimic the oxidative stress. Oxidative stress mediated the DNA damage to engage DNA damage response (DDR) systems, which are characterized by double-strand DNA breaks (DSBs) repair (Hoeijmakers, 2001). This mechanism is denoted as a signaling cascade referring to activation of ATM/ATR, the deposition of H2AX and Chk1/2 (Shrivastav *et al.*, 2008; Mah *et al.*, 2010).

The DNA damage response (DDR) is a kinase-based functional network primarily activated in response to different types of DNA lesions (Shrivastav *et al.*, 2008). It initiates signaling cascades to activate cell cycle checkpoints according to triggered by early phosphorylation-driven signaling cascades including phosphorylation of ATM (ATM activation) followed by a delayed response that acted as activation and upregulatory expression of DDR genes (Matsuoka *et al.*, 2007). Thereby, in our work, we identified the effects of oxidative stress and let-7g on DDR according to examining the expression levels of DDR genes as well as the phosphorylated ATM. The expression of DDR genes and miRNA hsa-let-7g was surveyed to assess the effect of oxidative stress in GC cells. The DDR genes (ATM, H2AX and Chk1) were significantly increased at the level of proteins and mRNAs in the presence of H_2O_2 (Figs. 1A-1D). H_2O_2 stimulated the activation of DDR genes (overexpression of pATM, H2AX and Chk1) as well (Fig. 1D). These data illustrated H_2O_2 resulted in the DNA damages of GC cells and stimulated activation of the DDR systems. Further, the expression of hsa-let-7g was assayed to observe the effects of H_2O_2 on hsa-let-7g in GC cells. The hsa-let-7g expression

in AGS after H_2O_2 exposure was significantly lower than in AGS cells without treatment ($p < 0.05$), as shown in Fig. 1E. The deregulation of let-7g under oxidative stress was similar to that under hydroxycamptothecin-induced stress (Wu *et al.*, 2011).

Cellular apoptosis and viability possesses key effects on the mechanism for neoplastic progression (Evan and Vousden, 2001). We increased the let-7g expression level to study the effects of let-7g on apoptosis and viability of GC cells upon oxidative stress. Data revealed hsa-let-7g miRNA contributed to the apoptosis and loss of proliferation in GC cells exposed to H_2O_2 . This meant hsa-Let-7g miRNA promoted the sensitivity of GC cells to oxidative stress. A previous study confirmed that let-7g regulated apoptosis to increase chemosensitivity of GC (Kim *et al.*, 2011). Our study advances the understanding of the effect of let-7g on GC.

Hsa-let-7g miRNA has been described to inhibit proliferation and migration of hepatocellular carcinoma by targeting diverse genes (Lan *et al.*, 2011; Chen *et al.*, 2014). Instead, let-7g induced the increase of proliferation and reduction of apoptosis in normal cells by targeting antiangiogenic genes or apoptotic genes (Zhang *et al.*, 2013). Thus, the effects exerted by let-7g as an inhibitor or promoter might be associated with microenvironment and its target genes. In this study, accumulating evidences may cast new light on the crosstalk between let-7g with DDR genes as previous results displayed upregulation of DDR genes and downregulation of let-7g under oxidative stress. Experiments proved let-7g was negative feedback link to expression of DDR genes according to exogenous addition of let-7g (Fig. 3). Moreover, there were no putative targets of let-7g in 3' UTR of ATM, H2AX and Chk1 by bioinformatics and experiments. We experimentally reconfirmed that let-7g didn't regulate putative binding sites with most complementary base pairing in 3' UTR of ATM directly according to the luciferase reporter system (Supplementary Fig. 1). Above all, let-7g did not directly target the DDR genes (ATM, H2AX and Chk1). However, let-7g significant repressed the activation of DDR indirectly. Of note, whether let-7g will target other DDR genes need to be further investigated as DDR systems contain lots of genes besides three main genes (ATM, H2AX and Chk1).

Ionizing radiation such as X-rays resulted in double-strand DNA breaks (DSBs) is similar mechanism to free radicals (including ROS) caused (Hoeijmakers, 2001). Thus we concentrated on whether let-7g had the similar effects on GC cells under X-rays. Interestingly, hsa-let-7g miRNA increased sensitivity of GC cells to X-rays (Fig. 4A). Simultaneously, downregulation of main DDR

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gene (ATM) was observed in GC with excessive let-7g amount under X-rays (Figs. 4B and 4C).

In summary, hsa-let-7g miRNA increased the sensitivity of GC to oxidative stress by repression activation of DDR indirectly. In addition, our data illustrated that let-7g improved the effects of X-rays on GC cells involved in ATM regulation. Nevertheless, the direct target genes of let-7g need to be further investigated upon stress in GC. Our work provided available evidence for the anti-GC mechanism of hsa-let-7g miRNA. We propose application of hsa-let-7g might be a promising therapeutic target for GC.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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