Research Paper

Serum-Derived Exosomes MicroRNA-923 and MicroRNA-523 Expression to Regulate Autophagy in Esophageal Squamous Cell Carcinoma

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MicroRNA-923 and microRNA-523 have been shown to be involved in various malignancies in addition to other biological processes. There is growing evidence that oxidative stress plays a crucial role in cancer. At present their role in esophageal squamous cell carcinoma is unclear. Reverse transcription polymerase chain reaction was used to detect the expressions of microRNA-923 and microRNA-523, enzyme-linked immunosorbent assay was used to detect oxidative stress-related factors, and Western blotting was used to detect the expressions of autophagy related proteins, endoplasmic reticulum stress-related proteins and apoptosis-related proteins. The effects of microRNA-923 and microRNA-523 on cell proliferation were detected by cell counting kit 8, and the formation of intracellular autophagosomes was analyzed by immunofluorescence. The results showed that the expression of microRNA-923 and microRNA-523 was significantly enhanced in esophageal squamous cell carcinoma serum exosomes. Light-chain 3 II, autophagy-related 5, autophagy related 12, nitric oxide and hydrogen peroxide expression was increased and total antioxidant capacity expression was decreased in esophageal squamous cell carcinoma tissues. Hydrogen peroxide induced oxidative stress promoted the expression of microRNA-923 and microRNA-523 as well as cell proliferation, apoptosis, autophagy and endoplasmic reticulum stress. MicroRNA-923 and microRNA-523 could also promote cellular autophagy and endoplasmic reticulum stress. Unexpectedly, microRNA-923 and microRNA-523 inhibited apoptosis, indicating that apoptosis was not involved in hydrogen peroxide induced microRNA-923 and microRNA-523 regulated cell proliferation. It was confirmed that hydrogen peroxide induced microRNA-923 and microRNA-523 mediated cell proliferation, autophagy and endoplasmic reticulum stress. Collectively, our study showed that oxidative stress induced microRNA-923 and microRNA-523 expression regulates cellular autophagy in esophageal squamous cell carcinoma. This will provide some clues to gain insight into the molecular and biological mechanisms of esophageal squamous cell carcinogenesis.

Key words: Oxidative stress, autophagy, esophageal squamous cell carcinoma, microRNA-923, microRNA-523, exosomes

Esophageal cancer ranks as the 7th most common malignancy and the 6th leading cause of cancer-related death worldwide, with 604 000 new cases and 544 000 deaths in 2020[1]. Esophageal cancer is one of the most common malignancies of the digestive tract, with Esophageal Squamous Cell Carcinoma (ESCC) being the predominant pathological type of esophageal cancer worldwide, accounting for 90 % of all cases in China[2-4]. Despite advances in early diagnosis, surgery and radiotherapy, the prognosis of ESCC remains poor and remains a challenge[4-6]. Thereby, there is an urgent need to understand the occurrence of ESCC, which will support the development of diagnostic markers and new treatment strategies.

Oxidative stress as a key site of disease transformation can contribute to the progression of diseases and cancers by promoting phenomena such as oxidation of nucleic acids, proteins, lipids and other components, inflammatory responses

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and apoptosis through the increase of peroxides and free radicals[7-10]. In turn, Hydrogen peroxide (H_2O_2) can absorb large amounts of oxygen free radicals thereby inducing oxidative stress in cells, thus it is widely used as an apoptosis inducer[11]. Low concentrations of H_2O_2 function as signaling molecules to regulate signaling pathways at different levels thereby affecting cell division and growth[12,13]. Nevertheless, when H_2O_2 levels were excessive, the cell cycle would be blocked, which would further lead to apoptosis and autophagy[14,15]. The recent studies have shown that tumor oxidative stress can induce the Endoplasmic Reticulum Stress (ERS)[16]. In addition, the ERS can be inter-regulated with autophagy in several ways[17,18]. Therefore, research on the mechanisms of oxidative stress and related signaling pathways in cancer could help provide new strategies for cancer prevention and treatment.

It is well known that exosomes mediate communication across cells by transporting cell-derived proteins and nucleic acids, including various microRNA (miRNA)[19,20]. miRNAs are a class of small Ribonucleic Acid (RNA) of 18-25 nucleotides in length[21,22]. In part, miRNAs are dysregulated in response to oxidative stress[23,24]. These miRNAs respond to oxidative stress by regulating the expression of their target genes at the transcriptional and translational levels. The target genes can shift cells from proliferation to cellular arrest to prevent further cellular damage[25-27]. Studies have shown that abnormally high expression of miR-923 and miR-523 in breast cancer, liver cancer and melanoma is associated with poorer tumor prognosis[28-30]. Endoplasmic reticulum-based stress inducers induce miR-923 and miR-523 expression in human embryonic kidney HEK293 cells[31]. Currently, there are no relevant studies on the regulation of miR-923 and miR-523 expression by oxidative stress.

This study was to investigate the mechanism of oxidative stress on cellular autophagy in ESCC. In this study, we examined the levels of oxidative stress in the serum exosomes of ESCC patients and the expression of miR-923 and miR-523 in ESCC tissues. This analyzes the correlation between miR-923 and miR-523 expression, and oxidative stress in esophageal squamous carcinoma, and to elucidate the mechanism of miR-923 and miR-523 regulation of autophagy in esophageal squamous carcinoma cells.

MATERIALS AND METHODS

Cell lines and culture:

ESCC cell line TE-13 was obtained from Zolgene Biotechnology Co., Ltd. (Fuzhou, China). TE-13 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)-high glucose medium (Gibco) containing 10 % Fetal Bovine Serum (FBS) (PAN biotech) at 37° in an incubator containing 5 % Carbon dioxide (CO_2).

Transfection:

TE-13 cells were inoculated at 1×10^5 cells/well for 24 h in 24-well plates followed by transfection of miR-923 and miR-523 mimic inhibitors and their corresponding negative controls (NC-mimics, NC inhibitors) for 48 h with riboFECT CP Reagent (Ribo) according to the manufacturer's instructions.

Treatment of ESCC cell with H_2O_2:

The TE-13 cells were inoculated in 96-well plates at 1×10^5 cells/well and incubated at 37° for 24 h in a 5 % CO_2 incubator, followed by replacing the medium with fresh medium containing H_2O_2 so that the final concentrations of H_2O_2 were 0, 10, 50, 100, 200 and 400 µM, respectively, and assayed after 24 h of incubation. In order to establish a model of oxidative stress cells were treated with the appropriate concentration of H_2O_2 that had the best effect on cell viability. The experiments were performed in triplicate at least three times.

Reverse Transcription Polymerase Chain Reaction (RT-PCR):

We performed quantitative RT-PCR (qRT-PCR) to validate miR-923 and miR-523 expression in 80 serum exosome samples each from healthy individuals and ESCC patients. Serum exosomes were extracted by ultrafast centrifugation. To determine miR-923, miR-523, Light Chain 3 (LC3-II), Autophagy Related 5 (ATG5) and Autophagy Related 12 (ATG5) expressions in H_2O_2-induced TE-13 cells. Reverse transcription reactions were performed using Reverse Transcription System (Promega). Quantitative PCR amplification was performed using SYBR Green Real-time PCR Master Mix (Promega) and the reactions were incubated in an ABI 7500 PCR System (ABI, United States of America (USA)). Primers were as follows, miR-923

5'-CCAGGATTCCCTCAGTAATGG-3' (forward) and 5'-AGTGCAGGGTCCGAGGTATT-3' (reverse); miR-523 5'-CGCTCTAGAGGGAAGCGC-3' (forward) and 5'-AGTGCAGGGTCCGAGGTATT-3' (reverse); U6 5'-CTCGCTTCGGCAGCACA-3' (forward) and 5'-AACGCTTCACGAATTTGCGT-3' (reverse); Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) 5'-AGAAGGCTGGGGCTCATTTG-3' (forward) and 5'- AGGGGCCATCCACAGTCTTC-3' (reverse); LC3-II 5'-GCGAGTTACCTCCCGCAG-3' (forward) and 5'-TCATGTTGACATGGTCCGGG-3' (reverse); ATG5 5'-GAGTAGTTGCCTGGAGGAGC-3' (forward) and 5'-CCACTGCAGAGGTGTTTCCA-3' (reverse); ATG12 5'-TGCTGGAGGGGAAGGACTTA-3' (forward) and 5'-CACGCCTGAGACTTGCAGTA-3' (reverse). The data were quantitatively analyzed by $2^{-\Delta\Delta Ct}$ method.

Western blot:

Total cellular proteins were extracted by adding protein lysate containing Phenylmethylsulfonyl Fluoride (PMSF) (P8340, Solarbio) after cell collection precipitation, and proteins were separated on 4 % Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS/PAGE) gels and transferred to 8.5×6.5 cm nitrocellulose membranes (BioTrace, Pall, USA). The transferred NC membranes were closed with 5 % Bovine Serum Albumin (BSA) for 2 h and then incubated overnight at 4° with a specific primary antibody shaker to further incubate the blots with Horseradish Peroxidase (HRP)-conjugated secondary antibodies and visualize them using a chemiluminescence instrument (JS-2012, Shanghai Peiqing Technology Co., Ltd.). Antibody information is as follows; anti-beclin 1 antibody (ab210498, Abcam), anti-LC3B antibody (ab192890, Abcam), anti-ATG5 antibody (ab2108327, Abcam), anti-ATG12 antibody (abab109491, Abcam), Inositol-Requiring Enzyme 1 (IRE1); Endoplasmic Reticulum to Nucleus Signalling 1 (ERN1) polyclonal antibody (27528-1-AP, Proteintech), anti-ATF6 (ab227830, Abcam) and antibody anti-Protein kinase-like Endoplasmic Reticulum Kinase (PERK) antibody (ab229912, Abcam).

Enzyme-Linked Immunosorbent Assay (ELISA):

The caspase-3, caspase-8 and caspase-9 ELISA kits (Solarbio, USA, Cat: KHC4021 and Cat: BC3830, BC3850 and BC3890) were used according to the manufacturer's guidelines to measure the TE-13 cells produced caspase-3, caspase-8 and caspase-9 concentrations.

Immunohistochemical analysis:

To begin with, paraffin sections were dewaxed and hydrated then rinsed with Phosphate Buffer Solution (PBS) followed by a peroxidase blocking agent to block endogenous peroxidase activity. Anti-LC3B antibody was added and incubated overnight at 4° followed by the addition of HRP enzyme-labeled secondary antibody for 30 min at 37°. After 3 washes in PBS, SABC HRP-streptavidin, 1:200-400 PBS dilution was added and incubated for 30 min at 37°. After incubation, 3,3' Diaminobenzidine (DAB) was added dropwise for color development, hematoxylin was re-stained, and the slices were routinely dehydrated and sealed before microscopic examination and photography.

Total Antioxidant Capacity (TAC), H_2O_2 and Nitric Oxide (NO) detection:

TAC, H_2O_2 and NO in sera of healthy individuals and ESCC were assayed according to the TAC assay kit (Solarbio), H_2O_2 content assay kit (Solarbio) and H_2O_2 content assay kit (Solarbio). The ratio of 0.9 ml of reagent I was added to each 100 µl of serum and mixed thoroughly; the supernatant was centrifuged at 8000 g for 10 min at 4°, and the supernatant was extracted and assayed using an enzyme marker (DNM-9602, Beijing Plantronix Technology Co., Ltd.,).

Statistical analysis:

All experimental data were analyzed using Statistical Package for the Social Sciences (SPSS) 20.0 statistical software. For normally distributed data, t-test was used for two-by-two comparisons and paired t-test for paired data, one-way Analysis of Variance (ANOVA) was used for comparison of multiple groups of data. Data did not obey normal distribution, and non-parametric tests were used Spearman's analysis of correlation. $p<0.05$ indicates a statistically significant difference.

RESULTS AND DISCUSSION

To investigate the expression of miR-923 and miR-523, oxidative stress and cellular autophagy levels in clinical samples into ESCC, we verified the expression of miR-523 and miR-932 in exosomes which were found to be significantly increased in the serum of patients with ESCC (fig. 1A and fig. 1B). The levels of oxidative stress (NO, H_2O_2 and TAC) in serum were examined in 80 pairs of healthy individuals and ESCC patients. As shown in fig. 1C, NO and H_2O_2 levels were significantly higher and TAC expression was significantly lower in serum of ESCC patients (fig. 1D). Since H_2O_2 could elicit cell damage thereby inducing cell death, to investigate whether oxidative stress induced cellular autophagy in esophageal cancer. We examined the expression levels of cellular autophagy-associated proteins LC3-II, ATG5 and ATG12 in tissue samples by IHC and RT-PCR. The results showed that the expression of LC3-II, ATG5 and ATG12 showed a significant increase in cancer patients (fig. 1A-fig. 1C). Above results suggest that miR-923 and miR-523 are abnormally highly expressed, oxidative stress and cellular autophagy levels increased in ESCC, yet whether there is an interrelationship between the three needs to be further investigated.

To investigate the further mechanism of action of oxidative stress on ESCC cells, we tested the effect of increased oxidative stress on ESCC cell viability by incubating the cells in different concentrations of H_2O_2 (0, 10, 50, 100, 200 and 400 μM). It was shown that cell viability was altered in a dose and time dependent manner in all groups (fig. 2A). As in the control group, cell viability increased with time in the 10 and 50 μM H_2O_2 groups. In contrast, in the group with higher H_2O_2 concentrations (100, 200 and 400 μM), cell viability was significantly reduced compared to cells treated with control and low concentrations of H_2O_2 (10 and 50 μM; $p<0.05$). In the high concentration H_2O_2-treated group, cell viability was consistently reduced for 72 h after H_2O_2 treatment, indicating that cell growth or apoptosis was affected by high oxidative stress. This indicates that oxidative stress induces cell damage thereby affecting cell viability.

To explore whether oxidative stress regulates miR-923 expression, miR-923 expression levels in cells were examined after treatment with different concentrations of H_2O_2. In fig. 2B, miR-923 expression was significant elevated after H_2O_2 treatment of cells, where 50 μM H_2O_2-treated miR-923 was consistently elevated for 72 h after treatment, while miR-923 expression decreased to normal level after 72 h after high concentration of H_2O_2 treatment.

As above indicated that oxidative stress induces miR-923 expression, and then we further explore the impact of miR-923 on H_2O_2-induced proliferation, apoptosis, and autophagy in TE-13 cells. miR-923 mimic, miR-932 inhibitor, negative control mimic NC and inhibitor NC were transfected into H_2O_2-treated TE-13 cells (fig. 2C). As shown in fig. 2D, the cell proliferation after miR-923 overexpression and H_2O_2 treatment was higher than that of the control mimic NC, and the cell proliferation after miR-932 mimic and H_2O_2 co-treatment was higher than that of the other groups. The cell proliferation after miR-932 downregulation was significantly lower than that of control inhibitor NC, while the inhibition of cell proliferation by miR-923 inhibitor could be suppressed after H_2O_2 treatment. Results suggest that H_2O_2 induces miR-923 to promote TE-13 proliferation.

To determine whether apoptosis is involved in miR-932 mediated proliferation of TE-13 after activation of H_2O_2, we found that H_2O_2 promoted the expression of apoptosis-related proteins (caspase-3, caspase-8 and caspase-9) compared with control mimic NC, whereas miR-923 overexpression inhibited apoptosis and then co-treatment with H_2O_2 treatment counteracted the effect on apoptosis. Apoptosis was promoted when miR-923 was down-regulated and further increased by co-treatment with H_2O_2 (fig. 2E). Those results indicated that miR-932 could inhibit H_2O_2 mediated apoptosis of TE-13 cells and that apoptosis was not involved in H_2O_2 activation after miR-932 mediated proliferation of TE-13.

Western blotting was used to detect the expression of autophagy-related factors (Beclin 1, LC3-II, ATG5 and ATG12) and ERS related proteins (IRE, ATF6 and PERK). In fig. 2F, Beclin 1 expression was significantly decreased and LC3-II, ATG5, ATG12, IRE, ATF6 and PERK expression were significantly increased upon H_2O_2 treatment compared to control mimic NC, indicated that H_2O_2 induced oxidative stress induced ERS and further promoted cellular autophagy. In miR-932 overexpression, LC3-II, ATG5, ATG12 and

ATF6 expression was significantly increased and Beclin 1 expression was significantly decreased, while IRE and PERK protein expression levels were not significantly different (fig. 2F). In contrast, the expression levels of Beclin 1, LC3-II, ATG5, ATG12, IRE, ATF6 and PERK showed a significant decrease after miR-932 downregulation (fig. 2G), suggested that miR-932 was involved in regulating cellular autophagy and ERS. Whereas either miR-932 overexpression or miR-932 downregulation was accompanied by H_2O_2 treatment, Beclin 1 expression was significantly reduced and LC3-II, ATG5, ATG12, IRE, ATF6 and PERK expression were significantly increased compared to miR-932 mimic or inhibitor groups. Since ERS can induce LC3-II expression and promote the formation of autophagosomes, we detected the formation of LC3-II autophagic spots by cellular immunofluorescence. As shown in fig. 2H and fig. 2I, H_2O_2 and miR-932 promote LC3-II (green) accumulation in cells. The results indicate that H_2O_2 promotes ERS as well as autophagy of miR-932 in TE-13 cells. Above results suggest that H_2O_2 induces miR-923 mediated proliferation and autophagy in ESCC cells.

Since ERS inducers can induce miR-923 and miR-523 expression[6]. And we have previously verified that oxidative stress can induce ERS and regulate miR-923 expression. Therefore we further explored whether oxidative stress also has the same regulatory effect on miR-523. As shown in fig. 3A, miR-523 expression showed a significant increase after H_2O_2 treatment of cells, while miR-923 expression decreased to normal level after 72 h after high concentration of H_2O_2 treatment. And miR-523 in 50 µM H_2O_2 treated continued to be elevated at 72 h post-treatment (fig. 3B). The results confirm that oxidative stress promotes miR-523 expression.

Then, we further explored the effects of miR-523 on H_2O_2 induced proliferation, apoptosis, and

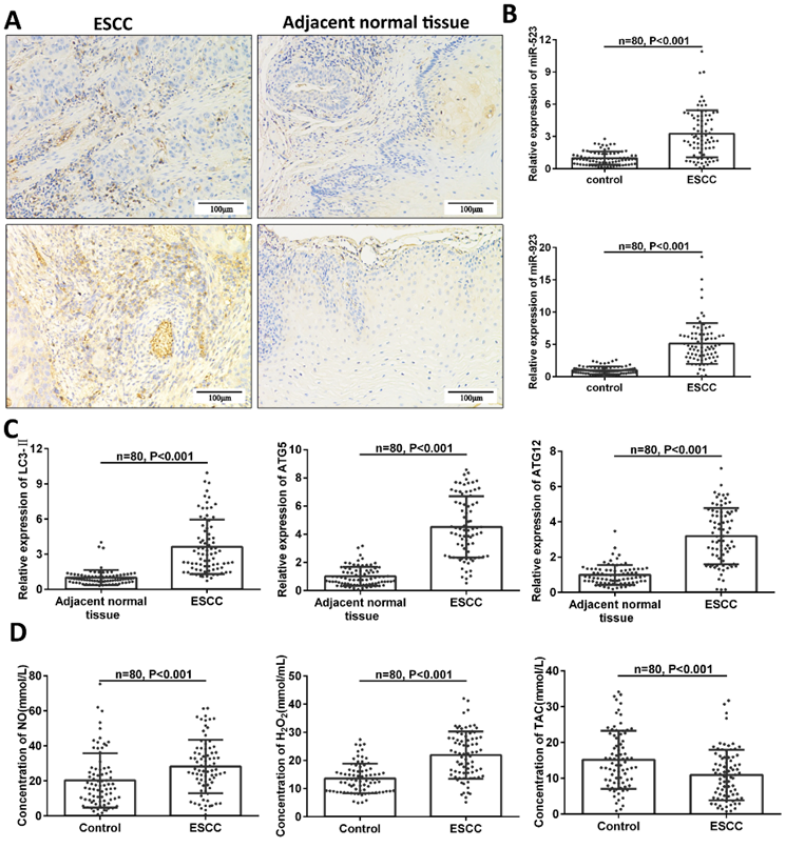

Fig. 1: Expression of miR-923 and miR-523, oxidative stress-related factors and autophagy-related proteins in ESCC, (A): IHC assays for LC3-II expression in ESCC and healthy human tissues; (B): Expression levels of miR-923 and miR-523 in ESCC and healthy human serum exosomes (n=80); (C): Autophagy-related proteins in ESCC and healthy human tissues and (D): Expression levels of oxidative stress-related factors in ESCC and healthy human sera

Note: The results of each experiment were repeated three times. Results are shown as mean±SD, *p<0.05, **p<0.01 and ***p<0.001 (n=80)

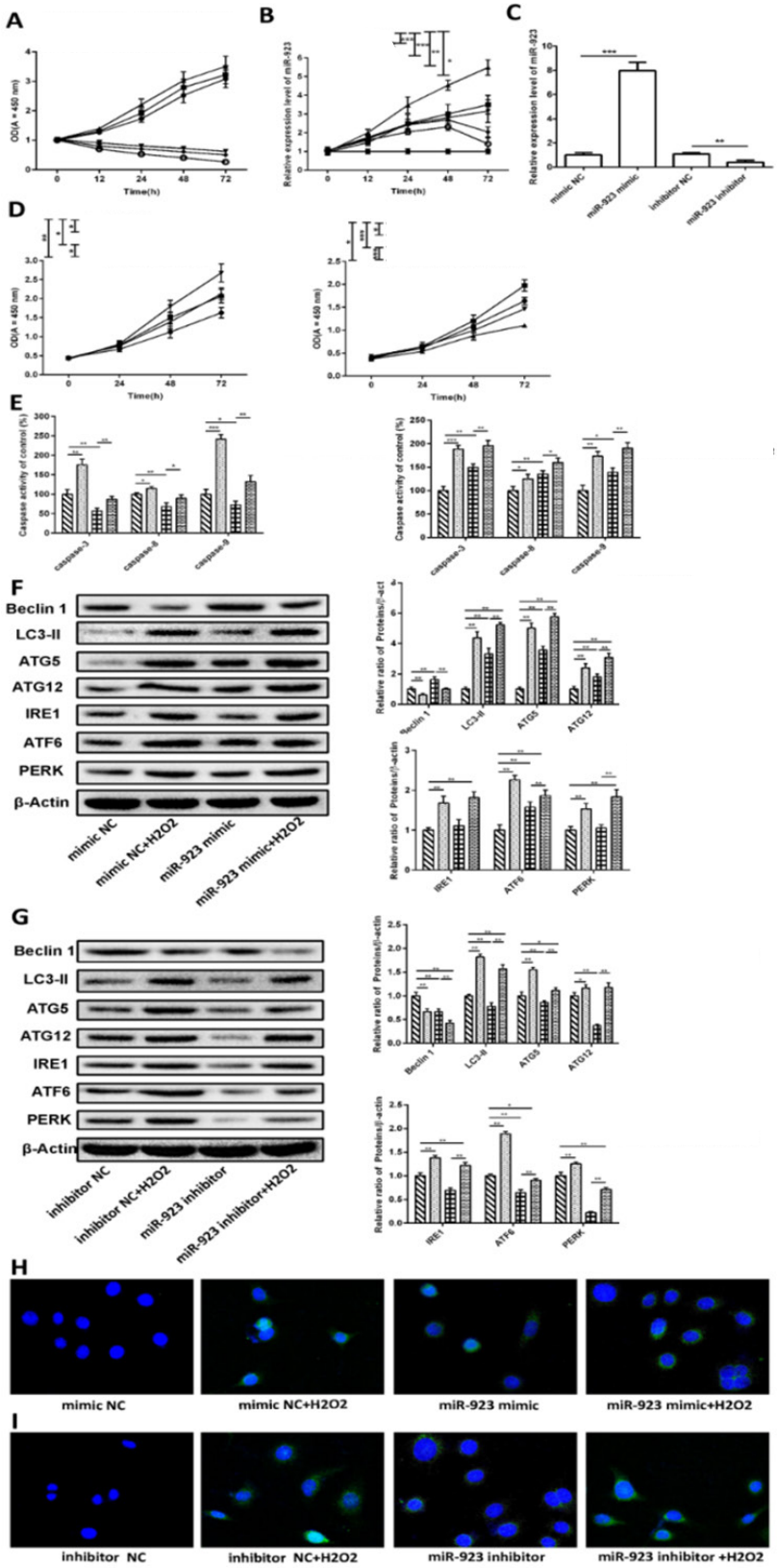

Fig. 2: Effect of miR-923 on H_2O_2 in ESCC (TE-13) cells, (A): The effects of different concentrations of H_2O_2 on cell viability; (B): Expression level of miR-923 by RT-PCR; (C): The miR-923 expression was confirmed in ESCC cells (TE-13) by RT-PCR after transfection; (D): The proliferation capacity was detected by CCK8; (E): The expression of apoptosis-associated proteins was detected by ELISA; (F and G): Western blot analysis; (H): miR-923 mimics and (I): miR-923 inhibitors for 24 h

Note: Results are shown as mean±SD, *p<0.05, **p<0.01 and ***p<0.001, (A and B) ( ): 0 µM; ( ): 10 µM; ( ): 50 µM; ( ): 100 µM; ( ): 200 µM and ( ): 400 µM (D) ( ): mimic NC; ( ): mimic NC+H_2O_2; ( ): miR-923 mimic; ( ): miR-923 mimic H_2O_2; (E-G) ( ): Inhibitor NC; ( ): Inhibitor NC+H_2O_2; ( ): miR-923 inhibitor and ( ): miR-923 inhibitor+ H_2O_2

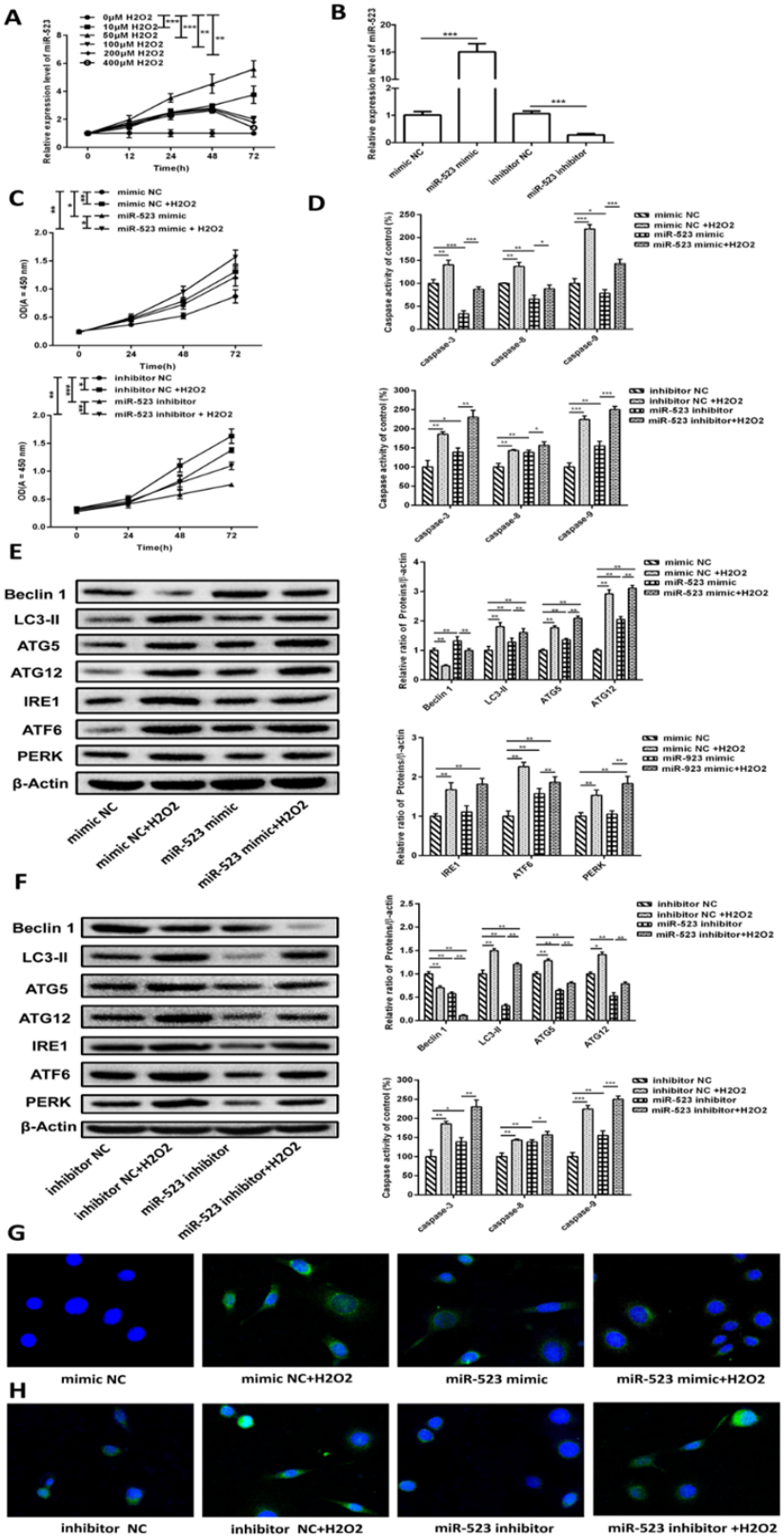

Fig. 3: Effect of miR-523 on H_2O_2 in ESCC (TE-13) cells, (A): Analysis of the effect of different concentrations of H_2O_2 on the expression level of miR-923 by RT-PCR; (B): The miR-523 expression was confirmed in ESCC cells (TE-13) by RT-PCR; (C): The proliferation capacity was detected by CCK8; (D): The expression of apoptosis-associated proteins was detected by ELISA; (E and F): Western blot analysis; (G): miR-523 mimics and (H): miR-523 inhibitors for 24 h and treated with or without H_2O_2

Note: Results are shown as mean±SD. *p<0.05, **p<0.01 and ***p<0.001, (A) ( ): 0 μM; ( ): 10 μM; ( ): 50 μM; ( ): 100 μM; ( ): 200 μM and ( ): 400 μM and (C) ( ): mimic NC; ( ): mimic NC+H_2O_2; ( ): miR-923 mimic; ( ): miR-923 mimic H_2O_2; (D) ( ): Inhibitor NC; ( ): Inhibitor NC+H_2O_2; ( ): miR-923 inhibitor and ( ): miR-523 inhibitor+H_2O_2

autophagy in TE-13 cells. miR-523 mimic, miR-523 inhibitor and negative control mimic NC, inhibitor NC were transfected into H_2O_2 treated TE-13 cells (fig. 3B). In fig. 3C, cell proliferation was higher in the miR-523 overexpression and H_2O_2 group than in the control mimic NC, and cell proliferation was higher in the miR-532 overexpression and H_2O_2 co-treatment than in the other groups. Cell proliferation was significantly lower in the miR-523 downregulated group than in the control inhibitor NC, while when treated with H_2O_2 it inhibited the inhibition of cell proliferation by miR-523 inhibitor. The results suggest that H_2O_2 induces miR-523 to promote the proliferation of TE-13.

Also to determine whether apoptosis is involved in H_2O_2 activation after miR-523 mediated proliferation of TE-13, we performed apoptosis related protein assays (caspase-3, caspase-8 and caspase-9) after H_2O_2 and miR-523 treatment. In fig. 3D, H_2O_2 promotes apoptosis compared to control mimic NC, while miR-523 overexpression inhibits apoptosis. Caspase-3, caspase-8 and caspase-9 expression were significantly increased after miR-523 overexpression followed by H_2O_2 treatment in contrast to miR-523 mimic group. When miR-523 were promoted apoptosis after downregulation and then further promoted apoptosis after H_2O_2 treatment. This suggests that apoptosis is not involved in miR-523 mediated proliferation of TE-13 after activation of H_2O_2.

When treated with H_2O_2, Beclin 1 expression were significantly decreased and LC3-II, ATG5, ATG12, IRE, ATF6 and PERK expression were significantly increased compared to the mimic NC group, indicating that H_2O_2 induced oxidative stress induces ERS and further promotes the formation of cellular autophagosomes. In miR-523 overexpression, Beclin 1, LC3-II, ATG5, ATG12 and ATF6 expression was significantly increased, while IRE and PERK protein expression levels were not significantly different (fig. 3E). When miR-532 was down-regulated, the expression levels of Beclin 1, LC3-II, ATG5, ATG12IRE, ATF6 and PERK showed a significant decrease (fig. 3F), indicating that miR-523 regulates cellular autophagy and ERS. Whereas either miR-523 overexpression or miR-523 downregulation were accompanied by H_2O_2 treatment, Beclin 1 expression was significantly reduced and LC3-II, ATG5, ATG12, IRE, ATF6 and PERK expression were significantly elevated compared with miR-523 mimic or inhibitor groups. As shown in fig. 3G and fig. 3H, H_2O_2 and miR-523 promote LC3-II accumulation in cells. The results indicate the role of H_2O_2 induced miR-523 on autophagy in TE-13 cells and the regulation of ERS. Taken together, H_2O_2 induced miR-523 mediated proliferation and autophagy in ESCC cells.

This study investigated the relationship between oxidative stress, miR-923 and miR-523 and cellular autophagy to determine the effects of miR-923 and miR-523 on H_2O_2 induced oxidative damage in the ESCC cell line TE-13 with its mechanism. In this study, it was demonstrated that H_2O_2 induced oxidative damage could promote miR-923 and miR-523 expression, and increased cell proliferation, apoptosis, autophagy and ERS. Besides, miR-923 and miR-523 could also promote cell proliferation, autophagy and ERS. However, miR-923 and miR-523 inhibited the expression of apoptosis-related proteins (caspase-3, caspase-8 and caspase-9), which suggested that apoptosis was not involved in H_2O_2 induced miR-923 and miR-523 regulated cell proliferation. It was confirmed that H_2O_2 induced miR-923 and miR-523 mediated cell proliferation, autophagy and ERS.

ESCC is a fatal disease with a poor prognosis, which can be partly explained by the fact that many patients are diagnosed at an advanced stage of the disease[32,33]. Although ESCC has been extensively researched, the underlying mechanisms still remain unclear. Oxidative stress occurs in various cancers owing to increased oxidative and nitro radical cycling and disruption of cellular redox homeostasis leading to tumorigenesis. Previous studies have shown that oxidative stress affects ESCC occurrence and progression[34]. It was confirmed by our data that NO and H_2O_2 expressions were significantly higher and TAC levels were significantly lower in ESCC serum, suggesting that oxidative stress is a potential factor in esophageal carcinogenesis.

Oxidative stress has been reported to induce ERS pathways leading to cancer development. However, excessive activation of ERS could increase cellular damage and even lead to cell death[35-37]. Indeed, ERS can induce autophagy in multiple ways, and the interregulation of ERS and autophagy was a highly conserved process[18,19]. With the validation

of the expression of LC3-II, ATG5 and ATG12 proteins in ESCC tissues, we found that they showed high expression in ESCC tissues, further illustrating the relevance of autophagy on ESCC progression. There is growing evidence that miRNAs have a critical role in oxidative stress response and carcinogenesis. It has been shown that miR-155 can protect endothelial cells from damage caused by intracellular oxidative stress[28]. The progression of ESCC is promoted by miR-126 through inhibition of apoptosis and autophagy[38].

Studies have shown that abnormally high expression of miR-923 and miR-523 in breast cancer, liver cancer and melanoma is associated with poorer tumor prognosis[29,30]. Yet, endoplasmic reticulum-based stress inducers induce miR-923 and miR-523 expression in human embryonic kidney HEK293 cells[32]. For our results, we also found abnormally high expression of miR-923 and miR-523 in ESCC serum exosomes. Hence, we further explored the mechanisms by which miR-923 and miR-523 regulate autophagy in ESCC cells in a cellular model. We tested the cell viability of TE-13 cells treated with H_2O_2 with the finding that low concentrations of H_2O_2 did increase cell viability. However, when the concentration of H_2O_2 exceeded 100 μM, cell viability was inhibited. Also, miR-923 and miR-523 expression was examined and H_2O_2 was found to promote miR-923 and miR-523 expression. Previous studies have reported that H_2O_2 induced release of Reactive Oxygen Species (ROS) from cells can cause cellular damage. One of them, the LC3-I will be converted to LC3-II when conjugated with phosphatidylethanolamine, thus the LC3-II/I ratio was used to identify the activation of autophagy[39,40]. Hence, we examined the expression of apoptosis-related proteins, autophagy-related proteins, and ERS related proteins. The final results confirmed our hypothesis that H_2O_2 induced oxidative stress could induce miR-923 and miR-523 to mediate ERS and cellular autophagy, thereby promoting ESCC progression. This provides new insights into miRNA as a potential therapy for ESCC.

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Conflict of interests:

The authors declared no conflict of interests.

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