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MiR-513a-3p promotes radiation-induced apoptosis of human lung cells by inhibiting glutathione S-transferase P1

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Radiotherapy is a common treatment for lung cancer. However, radiation pneumonitis caused by radiotherapy can affect the quality of life and prognosis of lung cancer patients. miR-513a-3p has been found to sensitize human lung adenocarcinoma cells to chemotherapy by targeting glutathione S-transferase P1 (GSTP1). Here, we found that x-ray induced the apoptosis of BEAS-2B and miR-513a-3p expression in a dose- and time-dependent manner, and miR-513a-3p-mimic significantly increased x-ray induced apoptosis, while miR-513a-3p-inhibitor significantly decreased x-ray induced apoptosis. Dual luciferase gene reporter system showed that miR-513a-3p targeted to inhibit the expression of GSTP1 in BEAS-2B cells. Moreover, knockdown of GSTP1 significantly increased, while overexpression of GSTP1 decreased the apoptosis of BEAS-2B induced by x-ray. Importantly, overexpression of GSTP1 significantly reduced miR-513a-3p-mimic elevated x-ray -induced apoptosis in BEAS-2B cells. In conclusion, x-ray caused increased expression of miR-513a-3p, and miR-513a-3p promoted x-ray-induced apoptosis of human lung cells by inhibiting GSTP1.

1. Introduction

Lung cancer is a malignant tumor that originates in the bronchial mucosa or glands of the lungs. In China, the incidence and mortality of lung cancer in men account for the first place in all malignancies (Chen et al. 2016; Siegel et al. 2017). Surgery, chemotherapy and radiotherapy are the most commonly used treatment options for lung cancer patients, and about 60% of lung cancer patients in China receive radiotherapy (Shi et al. 2019). Radiotherapy causes difficult-to-repair DNA double-strand breaks through high-energy X-ray irradiation of chest lesions, thereby inhibiting cell proliferation and promoting apoptosis. However, due to the limitations of irradiation technology, when the chest lesions receive radiotherapy, the surrounding normal lung cells will also receive partial or full dose irradiation, which causes damage to normal cells, that is, the toxic reaction of radiotherapy to normal cells (Bainbridge et al. 2018; Lu et al. 2005).

MicroRNA (miRNA) is a non-coding, single-stranded RNA encoded by 20-25 ribonucleotides that can regulate cell proliferation, differentiation, apoptosis, metabolism, and invasion/migration through targeted regulation of its target gene translation and transcription levels (Lu et al. 2005; Bartel 2009). And it is not only a biomarker for the diagnosis of multiple diseases, but also plays an important role in the development of the disease (Wang et al. 2016). miR-513a-3p has been found to promote the migration of A549 cells by inhibiting β-8 protein expression (da Silveira et al. 2018). And previous study also found that miR-513a-5p inhibited progesterone receptor expression and constitutes a risk factor for breast cancer (Muti et al. 2018). Importantly, miR-513a-3p regulated apoptosis of human lung adenocarcinoma cells induced by chemotherapy via targeting Glutathione S-transferase P1 (GSTP1) (Zhang et al. 2012).

GSTP1 is an important member of the glutathione S-transferase protein family, and GST plays an anti-oxidation and toxin removal effect in cells, and is considered to have an inhibitory effect on apoptosis caused by exogenous stimulation (Holley et al. 2007; Mutallip et al. 2011). However, the effect of miR-513a-3p and GSTP1 on apoptosis of human lung cells induced by x-ray is still unclear. In this study, we revealed that x-ray induced miR-513a-3p expression in a dose- and time-dependent manner, and miR-513a-3p promoted x-ray-induced apoptosis by inhibiting GSTP1.

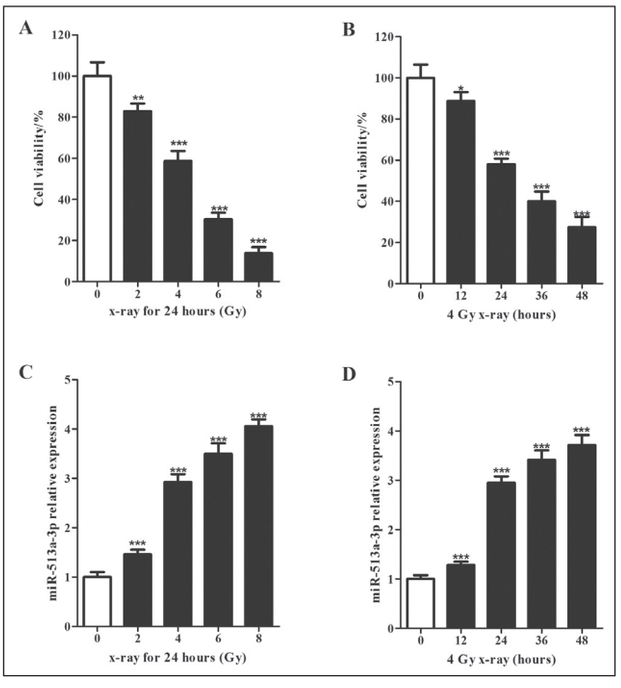

Fig. 1: Effect of x-ray on miR-513a-3p expression in human lung cell. A, BEAS-2B was exposed to different doses of x-ray for 24 hours (A) or 4 Gy x-ray for different time (B), and we assessed the cell viability using a CCK8 kit; C-D, BEAS-2B was exposed to different doses of x-ray for 24 hours (C) or 4 Gy x-ray for different time (D), and we detected the expression of miR-513a-3p using qPCR. Repeat each test independently at least 3 times, and Data was expressed as (mean±SD). * was P<0.05, ** was P<0.01 and *** was P<0.001 vs 0 group.

2. Investigations and results

2.1. X-ray induced miR-513a-3p expression in a dose- and time-dependent manner

At first, we evaluated the damage of human lung cells (BEAS-2B) induced by x-ray using a CCK8 kit, and found that the viability of BEAS-2B gradually decreased as the dose of x-ray increased under the same exposure time (**Fig. 1A**). Similarly, the viability of BEAS-2B gradually decreased as the exposure time of x-ray increased under the same exposure dose of x-ray (Fig. 1B). miR-513a-3p was found to regulate the apoptosis of human lung cancer cells (Zhang et al. 2012). Here, we detected the expression of miR-513a-3p in BEAS-2B after being exposed to x-ray. As showed in Figure 1C, the expression of miR-513a-3p in BEAS-2B gradually increased as the dose of x-ray increased under the same exposure time (**Fig. 1C**), and the expression of miR-513a-3p in BEAS-2B gradually increased as the exposure time of x-ray increased under the same exposure dose of x-ray (**Fig. 1D**). Therefore, miR-513a-3p was related to the damage of BEAS-2B induced by x-ray. In addition, we set the exposure condition of x-ray was 4Gy for 24 hours in the following research.

2.2. miR-513a-3p promoted x-ray-induced apoptosis

To investigate the expression of miR-513a-3p on the apoptosis of BEAS-2B induced by x-ray, we regulated miR-513a-3p expression by transferring miR-513a-3p-mimic/miR-513a-3p-inhibitor into BEAS-2B cells, and transferred miR-513a-3p-NC into BEAS-2B cells as negative control. The results of qPCR showed (**Fig. 2A**) that no matter with or without the exposure of x-ray, compared with control group, miR-513a-3p-NC could not significantly change the expression of miR-513a-3p in BEAS-2B cells, and miR-513a-3p-mimic could significantly increase the expression of miR-513a-3p, while miR-513a-3p-inhibitor could significantly decreased the expression of miR-513a-3p. Next, we investigated the expression of miR-513a-3p on the apoptosis of BEAS-2B induced by x-ray using flow cytometry, and the results showed that (**Figs. 2B and 2C**) transferring miR-513a-3p-NC, miR-513a-3p-mimic or miR-513a-3p-inhibitor into BEAS-2B does not cause apoptosis, while miR-513a-3p-mimic increased the apoptosis of BEAS-2B induced by x-ray, and miR-513a-3p-inhibitor decreased the apoptosis of BEAS-2B induced by x-ray.

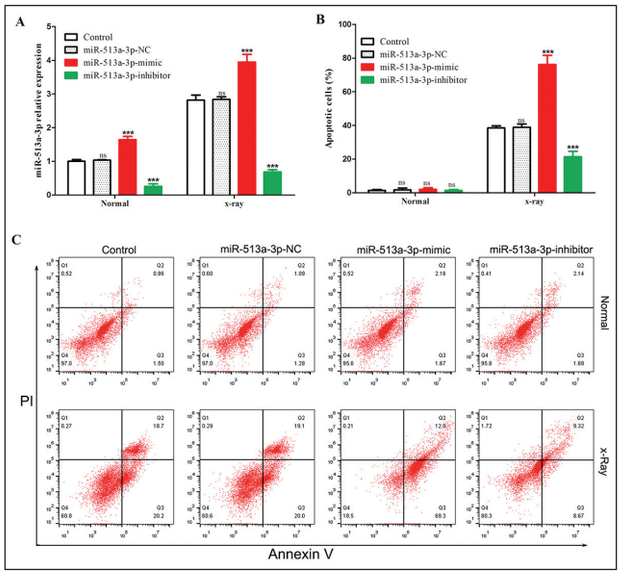

Fig. 2: Effect of miR-513a-3p expression on the apoptosis of BEAS-2B induced by x-ray. A-C, After being transferred with miR-513a-3p-NC, mimic and inhibitor into BEAS-2B, we detected the expression of miR-513a-3p using qPCR with or without inducing by x-ray (A), and we detected the apoptosis of BEAS-2B using Flow cytometry with or without inducing by x-ray (B and C). Repeat each test independently at least 3 times, and data were expressed as mean±SD. ns was P>0.05 vs Control group, *** was P<0.001 vs miR-513a-3p-NC group.

2.3. GSTP1, a target gene of miR-513a-3p, reduced x-ray-induced apoptosis

miR-513a-3p, a miRNA, does not code protein, so it must regulate x-ray-induced apoptosis by regulating downstream target genes. According to the prediction of the bioinformatics website (www.mirbase.org and www.targetscan.org) and a previous study (Zhang et al. 2012), GSTP1 was selected as the object of continued research not only because GSTP1 is the target gene of miR-513a-3p, but also because GSTP1 has been found to be involved in regulating apoptosis (Holley et al. 2007). Firstly, to study GSTP1 was a target gene of miR-513a-3p, we synthesized wild-type (WT) and mutant (MUT) GSTP1 sequences, and then recombined them into a plasmid carrying the fluorescein gene to transfer into BEAS-2B cells, the results of luciferase activity detected by a Luciferase Reporter Assay Kit showed that (**Fig. 3A**) miR-513a-3p-mimic significantly decreased and miR-513a-inhibitor significantly increased the luciferase activity in BEAS-2B which was transferred with WT GSTP1, while miR-513a-3p-mimic could not significantly decrease and miR-513a-inhibitor also could not significantly increase the luciferase activity in BEAS-2B which was transferred with MUT GSTP1. At the same time, we also detected the expression of GSTP1 protein in BEAS-2B using immunoblotting (**Fig. 3B**) and immunofluorescence (**Fig. 3C**), and found that miR-513a-3p-mimic significantly decreased the expression of GSTP1 protein, while miR-513a-3p-inhibitor significantly increased the expression of GSTP1 protein in BEAS-2B. All in all, the results showed that GSTP1 was a target gene of miR-513a-3p, and miR-513a-3p inhibited the expression of GSTP1 protein in BEAS-2B.

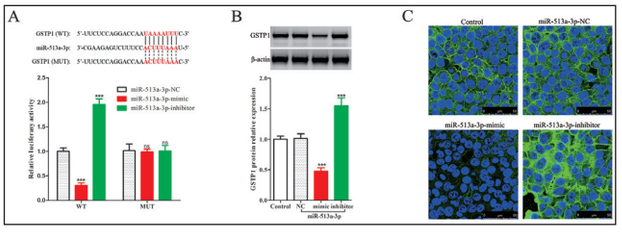

Fig. 3: GSTP1 was a target gene of miR-513a-3p. A, Wild-type (WT) or mutant (MUT) sequence of GSTP1 bound to miR-513a-3p (up), WT or MUT sequence of GSTP1 bound to miR-513a-3p were co-transferred into BEAS-2B cells with miR-513a-3p-NC, miR-513a-3p-mimic or miR-513a-3p-inhibitor, and then measured the luciferase activity (down); B-C, Immunoblotting (B) and cellular immunofluorescence (C) was used to detected the expression of GSTP1 protein in BEAS-2B cells after being transferred with miR-513a-3p-NC, mimic and inhibitor. Repeat each test independently at least 3 times, and data were expressed as mean±SD. ns was P>0.05 and *** was P<0.001 vs miR-513a-3p-NC.

2.4. miR-513a-3p promoted x-ray-induced apoptosis by inhibiting GSTP1

To test the expression of GSTP1 on the apoptosis of BEAS-2B induced by x-ray, we established GSTP1 knockdown (si-GSTP1) and GSTP1 overexpression (up-GSTP1) BEAS-2B cells, and verified their successful establishment by qPCR and immunoblotting (**Fig. 4A**). At the same time, we also detected the expression of GSTP1 protein by immunofluorescence, and found that (**Fig. 4B**) si-GSTP1 decreased the expression of GSTP1 protein and up-GSTP1 increased the expression of GSTP1 protein. Additionally, we found that (**Figs. 4C and 4D**) knockdown of GSTP1 or overexpression of GSTP1 did not induce the apoptosis of BEAS-2B under normal condition, while knockdown of GSTP1 significantly increased the apoptosis of BEAS-2B induced by x-ray, and overexpression of GSTP1 significantly decreased the apoptosis of BEAS-2B induced by x-ray. Importantly, overexpression of GSTP1 could decrease the apoptosis of BEAS-2B induced by x-ray after being transferred with miR-513a-3p-mimic (**Fig. 5**). The results indicated that miR-513a-3p promoted x-ray-induced apoptosis by inhibiting GSTP1.

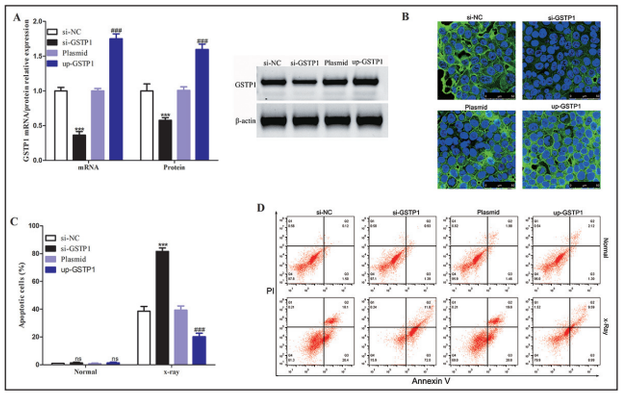

Fig. 4: Effect of GSTP1 expression on the apoptosis of BEAS-2B induced by x-ray. A, After being transferred with si-GSTP1 or up-GSTP1 into BEAS-2B, we detected the expression of miR-513a-3p using qPCR, we detected the expression of GSTP1 mRNA using qPCR and GSTP1 protein using immunoblotting; B, Cellular immunofluorescence was used to detected the expression of GSTP1 protein in BEAS-2B cells after being transferred with si-GSTP1 or up-GSTP1; C-D, After being transferred with si-GSTP1 or up-GSTP1 into BEAS-2B, we detected the apoptosis of BEAS-2B using Flow cytometry with or without inducing by x-ray. Repeat each test independently at least 3 times, and data were expressed as mean±SD. ns was P>0.05 and *** was P<0.001 vs si-NC group; n was P>0.05 and ### was P<0.001 vs plasmid group.

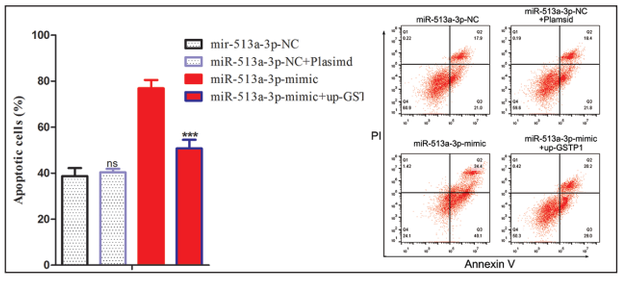

Fig. 5: MiR-513a-3p regulated x-ray-induced apoptosis by regulating GSTP1 expression in BEAS-2B. After being transferred with miR-513a-mimic or miR-513a-mimic/up-GSTP1 into BEAS-2B, we detected the apoptosis of BEAS-2B using flow cytometry with inducing by x-ray. Repeat each test independently at least 3 times, and data were expressed as mean±SD. ns was P>0.05 vs miR-513a-3p-NC group, *** was P<0.001 vs miR-513a-3p-mimic group.

3. Discussion

Lung cancer is one of the most common malignant tumors in the world, and it is also the most common disease that causes tumor-related deaths. Radiotherapy is one of the main methods for the treatment of tumors, and plays an important role in the treatment of most lung cancers. However, at the same time, chest radiotherapy can easily cause related complications, radiation pneumonia is one of them. Statistics showed that 5%-36% of patients would develop grade 2-5 radiation pneumonia after conventional chest radiotherapy (Ochiai et al. 2015; Wang et al. 2017). Therefore, it is of great significance to study the molecular mechanism of normal human lung cell damage caused by radiation to protect normal cytotoxicity caused by radiotherapy.

miR-513a-3p is located on human chromosome Xq27.3, and is reportedly to be involved in the regulation of tumor cell proliferation (Ma et al. 2020), migration (da Silveira et al. 2018), chemotherapeutic drug sensitivity (Zhang et al. 2012) and apoptosis (Li et al. 2020). In the present study, we found that x-ray induced miR-513a-3p expression in a dose- and time-dependent manner, and miR-513a-3p-mimic significantly increased x-ray induced apoptosis, while miR-513a-3p-inhibitor significantly decreased x-ray induced apoptosis, which suggested that miR-513a-3p was a miRNA related to x-ray-induced apoptosis in human lung cells. Importantly, previous studies have found that miR-513a-3p was also a miRNA related to apoptosis, such as Shin et al. (2012) found that miR-513a-5p mediated TNF-α and LPS induced apoptosis via downregulation of X-linked inhibitor of apoptotic protein in endothelial cells; It was found that miR-513a-3p promoted the apoptosis of HK-2 cells induced by dichlorvos by targeting the expression of Bcl2 protein (Li et al. 2018).

As we all know, miR-513a-3p, as a non-coding miRNA, must be involved in the regulation of cell biological characteristics through regulation of target gene expression. To study the molecular mechanism of miR-513a-3p promoting x-ray-induced apoptosis in human lung cells, we analyzed the gene sequence of miR-513a-3p and referred to a previous study (Zhang et al. 2012), GSTP1 was selected as the object of continued research not only because GSTP1is the target gene of miR-513a-3p, but also because GSTP1 has been found to be involved in regulating apoptosis (Holley et al. 2007; Mutallip et al. 2011). GSTP1 is mainly located at the cytoplasm and mitochondria and consists of 210 amino acids. The gene encoding GSTP1 protein in humans is located on chromosome 11q13, which is the subgroup with the highest content of GSTs, accounting for about 83% of the total GSTs (Moyer et al. 2008). Importantly, GSTP1 was found to be related to many lung diseases, such as decreased lung function after stem cell transplantation in children (Stark et al. 2017), chronic obstructive pulmonary disease (Ihii et al. 1999) and childhood asthma (Salam et al. 2007; Lee et al. 2010).

Although the mechanism of normal lung cell damage caused by radiotherapy is still unclear, researchers generally agree that the damage of DNA strands caused by reactive oxygen species (ROS) produced by radiation on cells is one of the causes of cell damage (He et al. 2015; Sonveaux 2017). Radiation acts on water molecules in cells through high-energy ionizing radiation to generate a large amount of ROS, and ROS causes apoptosis by causing double-stranded DNA breaks (He et al. 2015; Sonveaux 2017). As an important member of the glutathione S-transferase protein family, GSTP1 plays an anti-oxidation effect in cells. In this study, we found that knockdown of GSTP1 significantly increased, while overexpression of GSTP1 decreased the apoptosis of BEAS-2B induced by x-ray. Previous studies have found that GSTP1 was a protein related to apoptosis. On one hand, GSTP1 can exist alone or partially combine with Jun-JNK to form a complex, inhibit the phosphorylation of JNK and thereby inhibit cell apoptosis under non-stress conditions (Okamura et al. 2015; Li et al. 2015); On the other hand, under stress conditions, such as increased ROS content in cells, GSTP1 forms multimers, reduces the dimers formed with JNK to release JNK activity, reduces MAPK pathway inhibition, and increases cell apoptosis (Ziegler et al. 2001; Kim 2006).

More importantly that overexpression of GSTP1 could decrease the apoptosis of BEAS-2B induced by x-ray after being transferred with miR-513a-3p-mimic which indicated that miR-513a-3p promoted x-ray-induced apoptosis by inhibiting GSTP1.

4. Experimental

4.1. Cell culture and cell transfection

BEAS-2B (CRL-9609, ATCC, USA) was cultured in DMEM medium which was added 10% fetal bovine serum at 37 °C with 5% CO_2. si-NC (5'-CUUUUAGCUGAGGAAAAUAUG-3'), si-GSTP1 (5'- AGAAACUGGCGACAAAUCCUC -3'), miR-513a-3p-NC (5'-CUUGUGGAACUUUACGGGAU-3), miR-513a-3p-mimic (5'-UAAAUUUCACCUUUCUGAGAAGC-3') and miR-513a-3p-inhibitor (5'-AUUUAAAGUGGAAAGACUCUUCG-3') were transfected into 2.5 x 10^6 BEAS-2B cells using Lipofectamine 2000 (11668019, thermo fisher, USA) according to the manufacturer's protocols. For wild type (WT) or mutated (MUT) versions of the 3'-UTR of GSTP1 were cloned into pisCHECK2 (97157, Addgene, USA), and then began transfection into cells as si-RNA, and used a Dual-Lucy Assay kit (D00100, Solarbio, China) to detect luciferase activity following the manufacturer's protocol. For overexpression of GSTP1, we first cloned the gene sequence of GSTP1 into pcDNA3.1 plasmid (VT1001, Youbao Biotechnology Co., Ltd., China), and transferred the recombinant plasmid into BEAS-2B cells using Lipofectamine 2000 according to the manufacturer's protocols. 72 h later, cells were cultured with 200 μg/ml G418 for 7 days, puromycin resistant cells were set for experiments. At last, we determined GSTP1 protein expression by Real-time Quantitative Polymerase Chain Reaction (qPCR), immunoblotting and immunofluorescence.

4.2. Cell viability assay

Cells (0.2 x 10^5) were seed into 96 cell culture plate at 37 °C with 5% CO^2. Twelve hours later, cells were exposure to different dose of x-ray (0, 2, 4, 6 and 8 Gy) for different time (0, 12, 24, 36 and 48 h). After being exposed, we added 10 μL CCK8 solution (C0009, Beyotime Scientific, China) into 100 μL cell culture medium for 1 h at 37 °C with 5% CO_2. At last, we detected absorbance rate at 450 nm.

4.3. Real-time quantitative polymerase chain reaction

We harvested the cell and extracted the total RNA from cells. RNA was reverse transcribed into cDNA by using PrimeScript™RT Master Mix reverse transcription kit (RR036B, Takara, Beijing, China). 20 μl Real-time fluorescence quantitative PCR (RT-qPCR) system was prepared according to the SYBR Green qPCR Master Mix kit instructions (638320, TakaRa, Beijing, China). The relative expression of miR-513a-3p and GSTP1 was calculated by 2^{-DDCt} method, and β-actin or U6 was used as a loading control. The PCR primers were showed as follows: miR513a-3p-F: 5'- TAAATTTCACCTTTCTGAGAAGG-3', miR-513a-3p-R: 5'- GCGAGCA-CAGAATTAATACGAC-3'; GSTP1-F: 5'-ACCTAGGGGATGGGCTTAGG-3', GSTP1-R: 5'-GCCATTGATGGGGAGGTTCA-3'; U6-F: 5'- AUAAAUCCCU-UUACACCUCTT-3', U6-R: 5'-AAUAAAUCCCUUUACACCUCTT-3'; β-actin-F: 5'- AGCCCATCCTTCGAGTACAAA-3', β-actin-R: 5'-TCTTGGTGCGATAACT-GGTGG-3'.

4.4. Apoptosis assay

We seeded 1.0 x 10^6 cells / well in a 6-well plate, and cells were exposed to 4 Gy x-ray for 24 hours. And then we harvested the cell to analyze the apoptosis using Annexin V-FITC/PI Double Stain Apoptosis Detection Kit (E606336, Sangon Biotech, China) as manufacturer's protocol.

4.5. Cellular immunofluorescence

Cells (1 x 10^5) were seeded into Lab-Tek chambered (155411, Thermo Scientific, USA) to be cultured for 24 h at 37 °C with 5% CO_2. Next day, we removed the cell culture medium and fixed cell with 4% paraformaldehyde for 10 min at room temperature. And then cells were blocked with 5% BSA for 1 h at room temperature. After fixing and blocking, cells were incubated with primary antibody against GSTP1 (3369S, Cell Signaling Technology, USA) overnight at 4 °C. And then cells were incubated with Goat Anti-Mouse IgG H&L (Alexa Fluor® 488) (ab150113, abcam, UK). At last, it should be counterstained the nucleus with 5 μg/mL DAPI for 5 min at room temperature. At last, all samples were analyzed by confocal microscopy.

4.6. Immunoblotting

Cells were lysed and were extracted total protein by a RIPA lysis buffer (P0013C, Beytime, China), and 50 ug total protein were analyzed using a 10% SDS-PAGE gel. After being blocked with 5% BSA for one hour at room temperature, we incubated primary antibody against GSTP1 (3369S, Cell Signaling Technology, USA) overnight at 4 °C. And then Goat Anti-Mouse IgG H&L (HRP) (ab6789, abcam, UK) was incubated at room temperature for 2 h. After washing three times with phosphate buffer saline-tween 20, ECL solution (WBKLS0100, Beijing Xinjingke Biotechnologies Co., Ltd-China) was added for detection. In addition, the gray value of protein brands was analyzed by Imag J 3.0 (IBM, USA) and β-actin was loading as control.

4.7. Statistical analysis

Data in the present study were showed as mean±SD, and were analyze by GraphPad Prism (v8.0.2.263, IBM, USA). Student's t-test was used to compare the difference between two group, and one-way ANOVA followed by turkey test was used to compare the difference between multiple group. And $P<0.05$ indicated significant difference.

Confilcts of interest: None declared.

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