P53 transcribes GSDMD to induce delayed pyroptosis in radiation-induced intestinal injury

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Research

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Abstract

Background

radiation-induced intestinal injury (RIII) is an important cause of death in nuclear accidents and common complication after radiotherapy in patients with pelvic, abdominal, or retroperitoneal tumors. Up to now there is no effective means to prevent or treat RIII due to its complex mechanism, in which the death of intestinal cells is the main reason. Recently, GSDMD-mediated pyroptosis was identified as an important type of cell death and play a role in many diseases. However, the effect of pyroptosis on RIII is still unclear.

Method

using GSDMD knockout mice, the role of pyroptosis in the RIII was investigated. By detecting the release of LDH, expression of GSDMD, Caspase-11, Caspase-1 and absorption rate of SYTOX Green, the pyroptosis of radiated Mode-k cells was determined, simultaneously the common pyroptosis induced by LPS was conducted as positive control. Further, the upstream of GSDMD were screened by predictive analysis of transcription factors combing RNA-seq.

Results

we showed that GSDMD-mediated pyroptosis is involved in the process of RIII, and unexpectedly found that radiation induced a delayed pyroptosis that is substantially different from common pyroptosis induced by such as LPS. Further investigation revealed that radiation-induced DNA damage up-regulated the expression of P53, which subsequently transcribes GSDMD. In addition, the up-regulated GSDMD led to pyroptosis simultaneously promoting Ca^{2+} influx that afterwards enhanced apoptosis induced by radiation. Finally, targeting GSDMD, disulfiram displayed a potential protection for RIII.

Conclusion

radiation could induce delayed pyroptosis in the intestinal epithelial cell that is greatly different from common pyroptosis. During that process, GSDMD was cleaved and had inducible high expression which was mainly mediated by P53 transcription.

Introduction

Radiation-induced intestinal injury (RIII) is the culprit of death in patients who are long-term exposed to large dose of nuclear radiation. In clinic, it is also common as a complication of radiotherapy for abdominal tumors, resulting in outcome limitation and decreasing quality of life in patients [1]. Generally, a radiation dose ≥ 10Gy can lead to intestinal tissue degeneration, necrosis and bleeding, which in turn

can cause the incidence of radiation disease, a type of disease associated with a short-term death time within 5–10 days and a mortality rate of 100% [2, 3]. Currently, great advance has been achieved in prevention and treatment of bone-marrow radiation diseases. However, there is still no ideal therapeutic strategy toward the RIII caused by large-dose radiation exposure. It, therefore, has become a crucial difficulty in the field of Radiation Medicine that needs to be addressed urgently.

The incidence of RIII is overly complicated. It has been identified that radiation acts on biomacromolecules, such as DNA and protein, in a direct or an indirect manner, which can cause death of intestinal crypt stem cells and epithelial cells. As a consequence, the intestinal epithelial barrier will be damaged to result in disorders of intestinal digestion and absorption in the body, hydropower acid-base imbalance and even bacterial translocation, eventually contributing to organic failure [4–6]. Cell death appears to play a core part in the incidence of RIII. Previous research believed that apoptosis is the predominant program in deaths induced by radiation. As more studies have been carried out, it fails to well interpret the mechanism underlying the radiation-induced injury [5], including the changes in plasma membrane permeability and the release of inflammatory factors. In this context, there is a growing need for new theory supporting the specific mechanism underlying cell death induced by radiation. _ENREF_9

For the past few years, the emerging of and growing research on pyroptosis have provided a new way into the mechanism under radiation-induced cell death. Pyroptosis is a controllable programmed cell death that can cause the formation of plasma membrane pores dependent on the Gasdermin family of proteins. Upon pyroptosis, there will be increasing cell swelling, and when cell membrane rupture occurs, the intracellular contents will be released to activate strong inflammatory responses [7, 8]. There are two common pathways involved in pyroptosis: canonical inflammasome or non-canonical inflammasome activation pathways. Under the canonical inflammasome pathway, exogenous substances (predominantly Gram-negative bacteria) invade cells with concomitant binding between inflammasome, a multi-protein complex assembled with the involvement of pattern recognition receptors (PRR), and apoptosis-associated speck-like protein containing (ASC) CARD. In that way, the inflammatory Caspase-1 will be subsequently activated to obtain cleavage of GSDMD proteins, and the N-terminal region will then be released to the cell membrane, which triggers the formation of membrane pores and the release of IL-1β and IL-18. Consequently, the cells gradually undergo lysis and swelling, and the final rupture leads to the incidence of pyroptosis. Under the non-canonical inflammasome activation pathways, the pyroptosis is also induced by the cleavage of GSDMD proteins, while the cleavage is performed by the activated Caspase-11/4/5 (Caspase-11 in mouse and Caspase-4/5 in human) under the actions of intracellular lipopolysaccharide (LPS). Between the two pathways, except the difference in the injured cell type, GSDMD is always the key executor that induces the incidence of cell pyroptosis [9].

Current studies have revealed the association between pyroptosis and radiation-induced injury, yet the specific function of GSDMD remains elusive [10, 11]. In the present study, we established GSDMD knock-out mouse to deeply research into the role of GSDMD and pyroptosis in RIII. The findings showed that radiation could induce delayed pyroptosis in the intestinal epithelial cell. During that process, GSDMD was cleaved and had inducible high expression which was mainly mediated by P53 transcription. This

new-found pyroptosis form reported here helps improve the understanding on the mechanism of pyroptosis and provides a new idea for the prevention and treatment of RIII.

Materials And Methods

Experimental Animals

Wild-type C57BL/6 male mice (6–8 weeks) were purchased from Shanghai Jihui Experimental Animal Breeding Co., Ltd., and C57BL/GSDMD-/- mice were purchased from the Model Animal Research Center of Nanjing University. All experimental animals were raised and reproduced in accordance with the requirements of the Ethics Committee of Naval Medical University. Adult mice of the same age can be used for follow-up experimental research after co-raising and genotype identification.

Major materials and reagents

CCK-8 kit was purchased from Dongren Chemical Shanghai Co. Ltd (Dojindo Laboratories, Kumamoto, Japan No. CK04). 1640 medium was from Hyclone Co. Ltd (Hyclone, Los Angeles, America, No. SH30809.01B). Annexin V-FITC apoptosis kit was from TransGen Biotech (TransGen Biotech, Beijing, China, No. FA101-02). SYTOX™ Green Nucleic Acid Stain was purchased from Thermo Fisher Scientific (China) Co., Ltd, No. S7020. Lipopolysaccharide (LPS) was from Sigma-Aldrich Inc. (Sigma, L5293, USA). LDH Release Assay Kit was from Beyotime Co. Ltd (Beyotime, Haimen, Jiangsu, China, No. C0017). Peptide V5 was purchased from MCE (HY-P0081, MCE, China). Nigericin (S6653), Etoposide (S1225) and Pifithrin-α (PFTα, S2929) were purchased from Selleck Chemicals (Houston, TX, USA). Anti-GSDMD antibody (ab219800), Anti-Bax antibody (ab32503), Anti-Caspase1 antibody (ab179515), Anti-Caspase11(ab246496), Anti-Caspase4(ab238124), Anti-GAPDH (ab181602) were purchased from Abcam Co.Ltd (Abcam, Cambridge, England).

Radiation source

The radiation source used in this experiment is the ^{60}Co γ ionizing radiation source in Radiation Center of Naval Medical University. According to the experimental design, the whole-body radiation (12Gy) or abdominal local radiation (27Gy) was performed on each group of mice, and the dose rate was 1Gy/min. The radiation doses of 12Gy and 27Gy refer to Monica Mangoni' [12] and Jean-Victor Lacavé-Lapalun' [13] study.

Establishment of abdominal local radiation (ABI) in mice

Before experiment, mice were anesthetized by injection intraperitoneally with 1% sodium pentobarbital (50 mg/kg). After anesthesia, mice were fixed in a plastic box for radiation, by which the abdomen was exposed to ray and other parts were shielded by lead bricks (as shown in Fig. 1A). The mice received 27Gy radiation in the abdominal cavity, and the dose rate was 1Gy/min. After radiation, mice were transferred to a new cage and keep warm until they wake up. Next, survival period of 30 days was recorded continuously, or small intestine tissues were taken at the designed time point.

Cell lines

HEK293T cells were obtained from ATCC and were cultured with high-glucose DMEM supplemented (10% FBS + 1% tertiary antibody). MODE-K cell lines belong to small intestinal epithelial cells of mice, which was purchased from Shanghai Huzhen Biological Technology Co., Ltd. And were cultured with RPMI 1640 medium (10% FBS + 1% tertiary antibody). All the cells were placed at an incubator under a condition of 5% CO_2, 37℃.

Collection of mice feces

After abdominal radiation with 27 Gy of γ-rays, mice were randomly taken into the metabolic cage. On the 1st (Day 1), 2nd (Day 2), and 3rd (Day 3), the feces were collected. Then the amounts of feces were counted, the shape were observed, and weight were recorded after feces were air dry in a cool place.

Cell viability detection

Cell viability was analyzed using CCK-8 method. Cells in the logarithmic growth phase ere seeded into 96-well plates at 5000 cells/well. 450nm absorbance were measured 24 hrs. after radiation.

Cell Apoptosis Detection

Cell apoptosis was detected using Annexin V/PI kit. 24 hours after radiation, cells were stained with annexin V-FITC and propidium iodide (PI) according to the manufacturer's instruction. The apoptosis rate was analyzed by flow cytometry.

SYTOX Green flow cytometry detection

After cells were digested and resuspended, SYTOX Green dye was added at a ratio of 1:1000, and then allowed to stand for 20 minutes and then detected by flow cytometry. The excitation wavelength was 488nM.

LDH release detection

Lactate dehydrogenase (LDH) can be used as an indicator to determine the number of dead and damaged cells. The cells are radiated with γ-rays and then conducted in accordance with the method of the LDH detection kit. The calculation formula of cell damage rate is cell damage rate (%)= [(AC)/(BC)]×100% [A: absorbance of sample (sample hole-sample Blank hole); B: absorbance of high control (high control Hole-high control Blank well); C: absorbance of low control (low control hole-background Blank well)], calculate the average value of n = 3.

Western Blotting (WB)

Cells were plated in medium dishes with a density of 1×10^6 cells per dish. After corresponding treatments, proteins were extracted at corresponding time points. Culture medium was discarded, and cells were washed twice with PBS. Protease inhibitor and phosphatase inhibitor were added 1:100 into RIPA Lysis and Extraction Buffer (Product # 89900, Thermo Scientific™) for protein lysate preparation. 150μl of

lysate was added to each sample and cells were lysed on ice for 10 min. Lysate was scraped off and cells were fully lysed with an ultrasonic cell disruption instrument. Lysate was centrifuged at 12,000 rpm for 15 min at 4°C, 150μl of supernatant was acquired for each sample and 37.5μl of 5× Protein Sample Loading Buffer (Product # LT103, Epizyme Biotech, China) was added, mixture was boiled at 100°C for 10 min.

Construction of GSDMD Overexpression Cell Lines

GSDMD overexpression and control lentiviral vectors were purchased from OBiO Technology (Shanghai) Corp., Ltd. The plasmid vectors and Gsdmd element were pRlenti-CMV-EGFP-3FLAG-PGK-puro and pRlenti-CMV-EGFP-Gsdmd-PGK-puro representing control and overexpression, respectively. Transfected cells were seeded in 12-well plates in a density of 1×10^5 cells per well. 500μl of culture medium was added, GSDMD overexpression virus were added with a MOI of 20. Polybrene was added with a final concentration of 1:1000. After 24 hours of virus infection, 500μl of culture medium was added and cells were incubated for another 24 hours.

Luciferase reporter gene experiment

The plasmids were constructed as information: pGL4.10-Gsdmd Promoter (H15164) ;

pLenti-EF1a-EGFP-P2A-Puro-CMV-MCS-3Flag (H149). 293T cells were firstly seeded in 96-well plates. After fusion to 70%, cells were co-transfected with luciferase reporter gene plasmid and trans-acting factor gene plasmid. 48 hours later, culture medium was removed, and cells were incubated with PLB for 15min. 5μL supernate was mixed with LAR II, and 2 seconds later, the absorbancy was detected by microplate reader. According to the absorbancy, the transcriptional activity between P53 and GSDMD was determined.

RNA isolation and real-time quantitative PCR

Total RNA was isolated from MODE-k using Total RNA Isolation Kit (Beyotime, Haimen, Jiangsu, China) according to the manufacturer's instructions. Total RNA (500 ng) was transcribed using the PrimeScriptTM RT Master Mix Kit (TAKAR, Dalian, China). Real-time reverse-transcriptase PCR was carried out in triplicate using a TaKaRa EpiTaq HS (Takara). The sequences of the primers used are shown in β-Actin was used as internal reference gene and $2-\Delta\Delta Ct$ method was applied to analyze data. All the primer sequences were displayed in the table1.

RNA-Seq Analysis

No-radiated Mode-k and radiated Mode-k were seeded in dishes of 60mm diameter at a density of 1×10^6 cells per dish. Each kind of cells were seeded in a total of 3 dishes and incubated for 24hs. Medium was removed and cells were washed with sterilized PBS for once and then lysated with 1 ml/dish TRIzol™ Reagent (Thermo Fisher Scientific (China) Corp., Ltd). Cell lysate was kept frozen with dry ice and sent to Guangzhou Kedio Biotechnology Co., Ltd. for RNA-Seq analysis. The deferentially expressed genes (DEGs) were screened as threshold of $P<0.05$, and $|log2FC|>0.58$.

Statistical Analysis

Statistical analysis was conducted using SPSS19.0 software. Data is expressed as mean ± SEM. All experiments were repeated for three times. Student's t test was used for comparing data between two groups. Analysis of Variance (ANOVA) was used when there were multiple groups and SNK-q test was used for further multiple comparison between groups. Difference was considered as statistically significant when $P<0.05$.

Results

Pyroptosis is induced in radiation-induced intestinal injury

Mouse model of RIII was initially established via abdominal radiation (27Gy) (Figure 1A). Under radiation exposure, the height of the villi in the small intestine was greatly reduced and the depth of crypts were sharply decreased (Figure 1B-D). Additionally, the feces number and mass exhibited an evident downward trend, with concomitant hydration and a trend with no particles (Figure 1E-F). TUNEL assay revealed large numbers of brown positive cells in the intestinal villi and crypts, indicative of cell death (Figure 1G-H). These suggest severe intestinal injury after abdominal radiation at 27Gy.

Subsequently, the expression of pysroptosis-related proteins in intestinal tissue was detected. It was found that the expression of intestinal NLRP3, Caspase-1, Caspase-11 and GSDMD was increased after 24h of radiation exposure, with concomitant increasing cleavage of Caspase-1, Caspase-11 and GSDMD. Notably, the increase in villi was the most significant (Figure 1I). Further immunohistochemical assay for GSDMD confirmed the above findings (Figure 1J-K). Collectively the results identified the incidence of pyroptosis in the intestinal tissue following radiation, and the villous epithelium is involved predominantly.

Despite local radiation, total body radiation was performed at a dose of 12Gy (Figure S1A). The intestinal tissue was damaged remarkably, along with decreased length of villi and depth of crypts (Figure S1B-D). In the meantime, the GSDMD expression on villous epithelium was up-related as well (Figure S1E-F). All together concluded that radiation exposure could induce the incidence of pyroptosis in intestinal tissue.

GSDMD knock-out can alleviate the radiation-induced intestinal injury

It is established that GSDMD is the key executor involved in incidence of pyroptosis [14]. To identify the association between pyroptosis and RIII, GSDMD knock-out was obtained in C57BL/6 mice and radiation was performed. As compared to wild-type, the mice of GSDMD knock-out had significantly prolonged survival after radiation (Figure 2A). Moreover, there was mitigatory intestinal injury, increasing length of villi and depth of crypt (Figure 2B-D) as well as elevating number and mass in feces (Figure 2E-G). In the TUNEL assay, radiation exposure led to reduced number of brown positive cells in mice with GSDMD deficiency (H-I). This indicated that GSDMD deficiency could decrease the radiation-induced intestinal injury, and the mediated pyroptosis is crucial in that process.

Radiation exposure induces the incidence of delayed pyroptosis and up-regulates GSDMD expression

We had proved that the intestinal villous epithelium was the predominant tissues with pyroptosis by radiation. Next, Mode-k cells, a type of intestinal epithelial cell line was used to further confirm the relationship between radiation and pyroptosis. With radiation, the cleavage of Caspase-1, Caspase-11 and GSDMD in Mode-k cells was noted (Figure 3A), which led to obvious cell swelling (Figure 3B), and subsequent increased release of LDH (Figure 3C), higher absorption of SYTOX Green (Figure 3D-E) and aggregation of GSDMD on cell membrane (Figure 3F). Concomitantly, Caspase-1, Caspase-11, GSDMD expression levels were highly up-regulated following radiation, especially the GSDMD expression (Figure 3A). This further proved that radiation could induce the pyroptosis in intestinal epithelial cells.

However, it was noting that radiation-induced pyroptosis occurred mainly after 12 h of radiation. It conventional cases, pyroptosis, such as the type induced by LPS, occurs acutely, and has a rapid onset, generally without significant up-regulation of pyroptosis-related proteins including GSDMD. To gain more insight into the phenomenon, a comparative study on pyroptosis induced by radiation and LPS+Nigericin (representing the canonical inflammasome activation) was performed. It was found that LPS+Nigericin led to significant cleavage of GSDMD within 1 h without evident increasing expression (Figure 3G-I). In addition, the release and absorption of LDH and SYTOX Green advanced prominently and acutely (Figure 3C-E), resulting in cell swelling earlier in time and more frequent (Figure 3B). Consistent finding was also noted in HIEC cells, a type of human intestinal epithelial cell lines (Figure S2).

This demonstrated that, the pyroptosis in intestinal epithelial cells induced by radiation is significantly different from the conventional type such as induced by LPS+Nigericin, which is characterized as delayed occurrence and with concomitant up-regulation of GSDMD expression.

The radiation-induced GSDMD cleavage is dependent on the activation of Caspase-1 and Caspase-11

Former result had demonstrated concurrent activation in Caspase-1 and Caspase-11 (Figure 3A). In order to investigate the effect of Caspase-1 and Caspase-11 on GSDMD cleavage, 293T cell lines was then used, because 293T cell lines poorly express Caspase-1, Caspase-11 (Caspase-4 for human derived cells) and GSDMD as compared to Mode-k cell line (Figure 4A). Next, GSDMD was over-expressed in 293T cells, followed by radiation. After 8 h, no remarkable increase of GSDMD cleavage was observed (Figure 4B), which suggested that lack of Caspase-1 and Caspase-11 cannot led to radiation-induced GSDMD cleavage. Moreover, Caspase-1 or Caspase-11 expression in Mode-k cells was silenced by using targeted siRNA (Figure 4C). Compared to radiation alone group, Caspase-1 siRNA, and Caspase-11 siRNA both decreased absorption of SYTOX Green (Figure 4D-E) and reduced release of LDH (Figure 4F-G), of which Caspase-11 knock-down had the most significant effect (Figure 4H). A conclusion could be obtained that, the radiation-induced GSDMD cleavage is dependent on both activation of Caspase-1 and Caspase-11, in which Caspase-11 is predominate.

Radiation up-regulates the expression of GSDMD by P53 transcription

As aforementioned, radiation could not only induce GSDMD cleavage but also up-regulate GSDMD expression especially after 12 h of radiation exposure. This may also a vital cause of the delayed

pyroptosis. In order to clarify the upstream regulatory molecules of GSDMD, we performed a predictive analysis of transcription factors combining JASPAR and PROMO database. Simultaneously, the RNA-seq of radiated and no-radiated Mode-k cells were also conducted to screen the differentially expressed genes (DEGs). Next, the intersection between predictive results of transcription factors and DEGs was made. As a result, five molecules including *AR, RELA, SP1, TP53* and *TWIST1* were identified as common transcription factors predicted from both two database (Figure 5A-B), among which *AR, TP53* and *TWIST1* were common genes from DEGs (Figure 5B). Gene ontology (GO) analysis and KEGG pathway analysis showed that the DEGs are mostly involved in the cellular processes of programmed cell death (Figure 5C left) and KEGG pathway of NOD-like receptor signaling pathway (related to pyroptosis) and apoptosis (Figure 5C right).

Subsequently, their expressions were detected by qPCR and results validated that A*R, RELA, TP53* and *TWIST1* were up-regulated upon radiation, within them *TP53* had the most significant fold of change (Figure 5D). *TP53* is the gene of P53, which is known for its anti-tumor activity. It is also one of the most common molecules which show aberrant expression after radiation. Upon radiation, P53 can be phosphorylated in early DNA injury, which is conducive to recruiting proteins including ART to promote DNA damage repair and concurrently making cell cycle arrest to provide enough time for DNA damage repair. Following that, P53 expression increases with concomitant increasing apoptotic proteins such as Bax, eventually inducing cell apoptosis [15]. Combing the previous findings, we reasoned that the up-regulation of P53 might be the culprit for increasing GSDMD expression and incidence of delayed pyroptosis after radiation exposure.

In order to investigate the relationship between P53 and GSDMD, PTF-α, an inhibitor to P53 transcription was used. As compared with no IR group, radiation up-regulated the expression of GSDMD and P53 in a time-dependent manner. However, PTF-α eliminated this up-regulated trend in GSDMD, indicating that P53 can regulate GSDMD expression after radiation (Figure 5E). Further, the transcriptional effect of P53 on GSDMD was validated by dual-luciferase reporter gene assay. Results demonstrated profound activation of GSDMD promotor in presence of high P53 expression (Figure 5F), which was absent upon mutations in promotor (MUT1+MUT2, H20316) (Figure 5G). Chip-qPCR assay also confirmed that P53 indeed binds GSDMD promotor (Figure 5H).

Thereafter, the role of P53 in radiation-induced pyroptosis was also investigated. With PTF-α pretreatment, the release of LDH (Figure 5I) and absorption of SYTOX Green (Figure 5J-K) were decreased obviously after radiation. However, the application of PTF-α exhibited not remarkable effect on the absorption of SYTOX Green (Figure S3A-B) and release of LDH (Figure S3C) after LPS+Nigericin treatment.

Considering that DNA damage is the main biological effect upon radiation, we then investigated if DNA damage was related to transcription of P53 on GSDMD. Accordingly, the Etoposide, an inducer of DNA damage was applied in Mode-k cells.

Comet assay indicated that Etoposide induced comet tails comparable to radiation (Figure S4A), and simultaneously up-regulated the expression of γ-H2AX proteins, marker of DNA damage (Figure S4B), indicating an evident DNA damage upon Etoposide treatment. Next, using PTF-α, the expression of γ-H2AX had not obvious change, however GSDMD up-regulation induced by Etoposide was significantly suppressed (Figure S4B-C), meanwhile the release of LDH was also reduced (Figure S4D).

Above results demonstrated that radiation-induced DNA damage up-regulates P53 which subsequently transcribes GSDMD to finally bring about pyroptosis.

Radiation-induced pyroptosis promotes apoptosis in intestinal epithelial cells

P53 is a well-known pro-apoptotic molecule that can directly transcribe apoptosis-related genes, such as Bax, as a transcription factor. On the other hand, we had proved that P53 could transcribe GSDMD and thereby induce the occurrence of pyroptosis after radiation exposure. In the following part, the association between P53-induced apoptosis and pyroptosis after radiation was investigated. At first, GSDMD was knocked out in Mode-k cells (Figure 6A-B) and was confirmed by decreasing pyroptosis as demonstrated by reductions in release of LDH (Figure 6C) and absorption of SYTOX Green (Figure 6D, F). After knocking out GSDMD, the apoptosis induced by radiation was further detected. As a result, the expression of Bax was decreased remarkably (Figure 6A-B), meanwhile flow cytometry assay also demonstrated a reduced apoptosis in Mode-k cells with GSDMD knock-out (Figure 6E-G). Both two findings indicated that the GSDMD-mediated pyroptosis facilitated cell apoptosis. Reversely, the effect of apoptosis on pyroptosis was studied. By using siRNA-Bax, the expression of Bax after radiation was decreased, while there was no notable change in GSDMD expression and cleavage (Figure 6H-J). In the flow cytometry, application of Peptide V5, a Bax inhibitor, did not show significant effect on the release of LDH induced by radiation (Figure 6K-L), suggesting that the radiation-induced apoptosis exhibited less effect on pyroptosis.

Previous research revealed that GSDMD expression on cell membrane could increase Ca^{2+} influx, and the Ca^{2+} overload was regarded as an important promoter for cell apoptosis. It triggered speculation that the GSDMD-mediated pyroptosis might promote cell apoptosis by increasing Ca^{2+} influx. Here, Ca^{2+} probe was used to evaluate intracellular Ca^{2+} content at different time points after radiation. At 4 h after radiation, the fluorescence intensity of Ca^{2+} in Mode-k cells gradually increased, which was higher and more sharply than that in cells with GSDMD knock-out (Figure 7M-O). This demonstrated that, the up-regulation and activation of GSDMD-mediated pyroptosis after radiation could accelerate cell apoptosis mainly via increasing Ca^{2+} influx in late stage.

Disulfiram, a GSDMD inhibitor alleviates radiation-induced intestinal injury

Since the GSDMD-mediated pyroptosis could promote the occurrence and development of radiation-induced intestinal injury, suppressing GSDMD, therefore, might be potentially protection for radiation-induced intestinal injury. Here we applied a new GSDMD inhibitor called disulfiram. It is a classical

alcohol deterrent. In 2021, Jun Jacob Hu et al [16] firstly reported the suppressive action of disulfiram for pyroptosis, which was achieved by inhibiting membrane pore formation activated by N-GSDMD. Our study found that disulfiram could significantly block the aggregation of GSDMD on cell membrane under radiation (Figure 3F) and decrease the absorption of SYTOX Green (Figure 7A-B) as well as the release of LDH (Figure 7C), indicative of decreasing pyroptosis. In the meantime, the cell apoptosis was concurrently inhibited remarkably (Figure 7D-E), which further demonstrated the relationship between pyroptosis and apoptosis. In vivo experiment was performed to identify the role of disulfiram in radiation-induced intestinal injury. It was found that application of disulfiram prolonged the survival of mice exposed to radiation (Figure 7F), remitted intestinal injury (Figure 7G-I), and simultaneously decreasing intestinal epithelial death (Figure 7J-K). In all, disulfiram could suppress the GSDMD-mediated pyroptosis thereby decreasing radiation-induced intestinal injury. Disulfiram, therefore, is a potential new preventive agent for radiation-induced intestinal injury.

Discussion

The propose of the concept of pyroptosis not only provides a new view of cell death but also helps clarify the mechanism underlying inflammation. Since the discovery of GSDMD as the key executor of pyroptosis in 2015 by Shao et al [14], pyroptosis has been greatly attached to disease studies. Current studies believed that the GSDMD-mediated pyroptosis is important in the occurrence and development of a variety of diseases, such as neurodegenerative disease [17] and pneumonia [18]. However, there is a lack of study on its role in radiation-induced intestinal injury. Although some studies had suggested the involvement of pyroptosis in radiation damage of cells [10, 11], the properties specific to radiation is not highlighted and the role of GSDMD is not clear. To fill this gap, we respectively explored the in vivo effect of GSDMD knock-out on the survival and intestinal function in mice, and its in vitro effect on radiation sensitivity of intestinal epithelial cells. The results indicated that mice exposed to radiation had significantly up-regulated GSDMD expression in intestinal tissue cells and epithelial cells. Knock-out of GSDMD could led to prolonged survival after radiation, improved intestinal function, less injury and decreasing radiation sensitivity. It fully indicated that, the GSDMD-mediated pyroptosis, expectedly, could induce or even advance the occurrence and development of radiation-induced intestinal injury. It is noting that the pyroptosis induced by ionizing radiation had a late onset, which is beyond our expectation. It is different with the routine pyroptosis, which is mediated by canonical inflammasome or non- canonical inflammasome activation pathways, with a later-onset and concomitant remarkably increasing GSDMD transcription. It is generally believed that pyroptosis is a result of a series of reactions by enzymes, which is acute and highly responsive. In this setting, pyroptosis is considered to play a key part in the rapid progression of infectious diseases, such as the LPS-induced sepsis. To the contrary, the radiation-induced pyroptosis appears to occur mildly, despite the similar presentations with the routine cases, such as GSDMD cleavage and membrane pore formation, increasing permeability. It is noting that this type of pyroptosis comes with remarkable increasing expression of GSDMD in late stage of radiation. It is tempting to speculate whether the radiation induces a specific type of pyroptosis.

For the above speculation, there are two issues that need to be clarified: 1) whether the activation of this type of pyroptosis is dependent on the canonical inflammasome or non-canonical inflammasome activation pathways as the routine pyroptosis; 2) how the GSDMD expression is regulated in late stage of radiation and what's the biological meaning.

To address the two questions, Caspase-1, and Caspase-11, respectively representative of canonical inflammasome and non-canonical inflammasome activation pathways, were examined. We found that both Caspase-1 and Caspase-11 experienced cleavage after radiation and suppressing Caspase-1 or Caspase-11 all decreased the pyroptosis induced by radiation. This indirectly suggested that radiation exposure could activate both canonical inflammasome and non-canonical inflammasome activation pathways, which was once reported by Daniel AR et al [19]. Radiation is a special pathogenic factor, which is massively different with the normal "ligand-to-receptor" binding, generalized biomacromolecules are involved in that process. It suggests that the effect of radiation on canonical inflammasome and non-canonical inflammasome activation might be non-specific or indirectly. However, the final outcome comes out the same, with GSDMD cleavage and incidence of pyroptosis. Additionally, this also indicates that the canonical inflammasome and non-canonical inflammasome activation remain the main approaches involved in GSDMD cleavage and related pyroptosis, as the cleavage of GSDMD is dependent on the activation of Caspase-1 or Caspase-11 (Caspase-4,5 in human), despite different stimuli, such as radiation and LPS.

We further studied the mechanism underlying the up-regulation of GSDMD after radiation and corresponding biological significance. Radiation is a strong stimulus affecting large numbers of molecules, the majority of which is non-specific. Here, bioinformatics analyses were conducted to study the upstream transcription factors of GSDMD upon radiation. *AR, RELA, SP1, TWIST1* and *TP53* were identified to show significant research value. Further, the RNA-seq of no-radiated and radiated Mode-k cells were also conducted to screen DEGs. By making intersection between the predicted results of transcription factors and DEGs, the *TP53, AR* and *TWIST1* were identified. After verifying their expression by q-PCR, *TP53*, which exhibited the most remarkable changes after radiation, was particularly concerned. P53 is a type of transcription factor with powerful functions, and it is a well-known tumor suppressor [20]. It has been established that P53 plays a key part in radiation-induced injury. The current view is that, in early DNA damage, P53 advances DNA damage repair mainly by phosphorylation and aggregation in DNA damage regions [21]. In that way, P53 content gradually increases with time, which makes cell cycle arrest to create more time for DNA damage repair on the one hand and increases cell apoptosis by augmenting the transcription of apoptosis-related proteins, such as Bax, on the other hand. Biologically, such process helps remove cells which fail to repair DNA damage and in turn decreases the incidence of mutations [21]. In this study, unexpectedly we found another role of radiation-induced P53 in cell death which is pyroptosis. By the dual-luciferase reporter gene assay and Chip assay, we further proved that P53 could directly transcribe GSDMD. This well explains the incidence of delayed pyroptosis after radiation. It is a fact that P53 in early radiation damage does not change significantly but increases in late stages with difficulty in repair, which is relatively consistent with the trend of GSDMD expression. In this study, we also proved that suppressing P53 inhibited the delayed pyroptosis induced by ionizing

radiation. However, based on our current findings, the decrease in GSDMD expression appears to be the main cause of decreasing pyroptosis, rather than the GSDMD cleavage and the formation of membrane pores.

Since P53 could mediate the occurrence of both pyroptosis and apoptosis, we further studied their role in radiation damage. Bax and GSDMD were executor for apoptosis and pyroptosis respectively, and them all had been verified to be target of P53 transcription, thereby the relationship between P53-mediated apoptosis and pyroptosis was investigated by regulating Bax or GSDMD. The results revealed that GSDMD silence decreased Bax expression and increased cell apoptosis, while inhibiting Bax did not show significant effect on GSDMD expression, suggesting that pyroptosis could promote apoptosis, in turn apoptosis exerts no considerable influence on pyroptosis. Actually, the relationship between pyroptosis and apoptosis is an important scientific question and had been discussed in some studies. Some studies suggest that pyroptosis or apoptosis is an alternative selection when cells have to die, during which the Caspase-1 initiates apoptosis in the absence of gasdermin D may be critical mechanism [22]. However, there are still some views that apoptosis and pyroptosis are mutually reinforcing processes, which was supported by a finding that Caspase-3 could cleave GSDME, another important executor to pyroptosis [23, 24]. Despite above contradictory views, this research firstly investigated mutual regulation between apoptosis and pyroptosis under radiation condition and demonstrated accelerative role of pyroptosis in apoptosis. Furthermore, the mechanism was revealed that it is Ca^{2+} influx and Ca^{2+} overload due to cleaved GSDMD aggregation in cytomembrane that accelerates apoptosis. This supports the study of Zhong Q et al [25], and more importantly unifies the P53-mediated pyroptosis and apoptosis.

To conclude, this study identified the association between the GSDMD-mediated pyroptosis and radiation-induced intestinal injury. This type of pyroptosis is quite different with the conventional type (such as the LPS-induced pyroptosis), with a later onset and concomitant up-regulation of GSDMD, the key executor of pyroptosis. We further proved that P53 plays a vital part in the occurrence of this type of pyroptosis. Biologically, P53 increases cell pyroptosis in late stage of radiation, and the mediated up-regulation of GSDMD enhances the Ca^{2+} influx, which accelerates the apoptosis (Fig. 8). The findings of our study not only enhance our understanding on P53 but also lay a basis for the relationship between apoptosis and pyroptosis. Based on above findings, the disulfiram, a GSDMD inhibitor, was firstly used in the prevention of radiation-induced intestinal injury, and results also supported disulfiram as a potential preventive agent. However, the current study inevitably has some limitations, including further validation on the relationship between P53 and the whole pyroptosis-related pathway is needed to perform, and the specific mechanism between radiation-induced pyroptosis and apoptosis is still clarified. In the future, more in-depth studies along this direction will be devised, in an attempt to provide new strategies for the prevention and treatment of radiation-induced intestinal injury.

Declarations

Ethics approval and consent to participate

All animal experiments were undertaken in accordance with the National Institute of Health “Guide for the Care and Use of Laboratory Animals” (NIH Publication No.85-23, National Academy Press, Washington, DC, revised 1996), with the approval of the Laboratory Animal Center of Second Military Medical University.

Consent for publication

All authors reached an agreement to publish the study in this journal.

Availability of data and material

All data generated or analyzed during this study were included in this published article and its supplemental material.

Competing interests

The authors have no potential conflict of interests

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Authors' contributions

Hu Liu and Hao Hu designed the study. Tingting Liu, Suhe Dong, Ruling Liu, Yuhan Lin and Liuhuan Yao performed the experiments. Ruling Liu and Jicong Du analyzed the data. Tingting Liu wrote the paper, Fu Gao and Cong Liu supported fund assistance. All authors read and approved the final manuscript.

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Tables

Table 1. Primer information of detected genes

Gene Symbol	RefSeq accession numbers	Forward sequence	Reverse sequence
AR	NM_013476.4	AAAGAGCCGCTGAAGGGAAA	GGAGACGACAAGATGGGCAA
RELA	NM_001365067.1	CCTCGGGACAAACAGCCTC	CACGGCGCGCTAAAGTAAAG
SP1	NM_013672.2	GCCACCATGAGCGACCAAGA	CATTGCCGCTACCCCCATTA
TP53	NM_001127233.1	ATTCAGGCCCTCATCCTCCT	CCATGGCAGTCATCCAGTCT
TWIST1	NM_011658.2	GGCCGGAGACCTAGATGTCATT	CTGGGAATCTCTGTCCACGG
β-actin	NM_007393.5	CACTGTCGAGTCGCGTCC	TCATCCATGGCGAACTGGTG

Figures

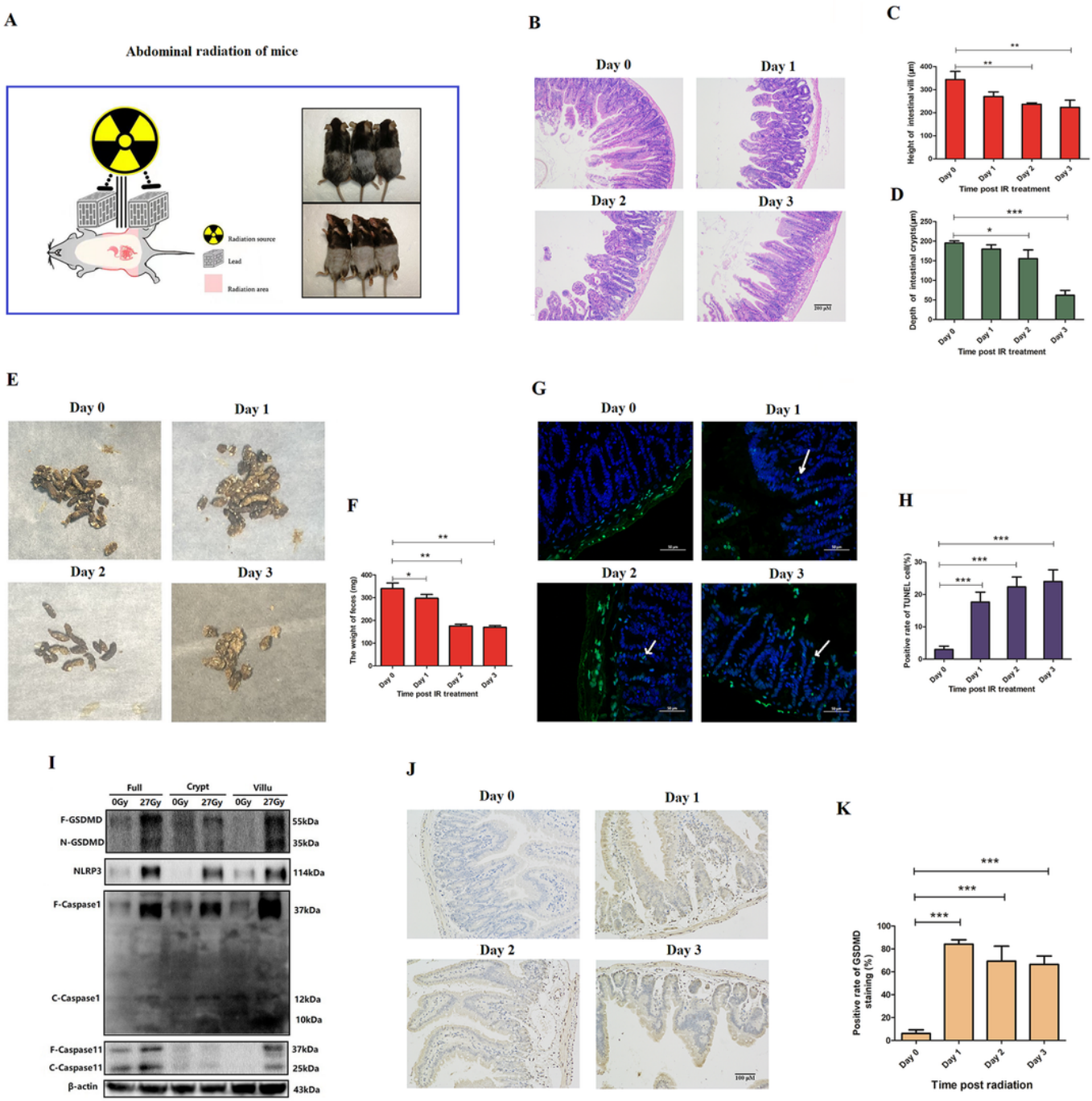

Figure 1

Pyroptosis is induced in radiation-induced intestinal injury. (A) Schematic diagram of local abdominal radiation. Briefly, after anesthesia, mice were fixed in a plastic box for radiation, by which the abdomen was exposed to ray of 27Gy (dose rate was 1Gy/min) and others were shielded by lead bricks. (B-D) On the 1st, 2nd and 3rd day after abdominal radiation, the small intestines of mice (N=6) were taken to perform H&E staining (B), and height of villi (C) and depth of crypts (D) were measured to quantify

intestinal damage. (E-F) Faeces of mice were collected on the on the 1st, 2nd and 3rd day after abdominal radiation. The appearance of feces was showed (E) and weights were measured (F). (G-H) By TUNEL fluorescent staining, the cell death was labeled (G) and positive rate of TUNEL cells was calculated (H). (I) 24 hours after radiation, small intestines of mice were taken, then crypt and villi were separated respectively to detect expression of GSDMD, Caspase-1, Caspase-11 and NLRP3 by western blotting. (J-K) On the 1st, 2nd and 3rd day after abdominal radiation, small intestines of mice were subject to immunohistochemical staining of GSDMD (J) and positive area was quantified (K). Image analysis was conducted using Image J software. The error value was expressed as mean±SD. *, ** and *** represented P<0.05, 0.01 and 0.001 between the corresponding groups, respectively.

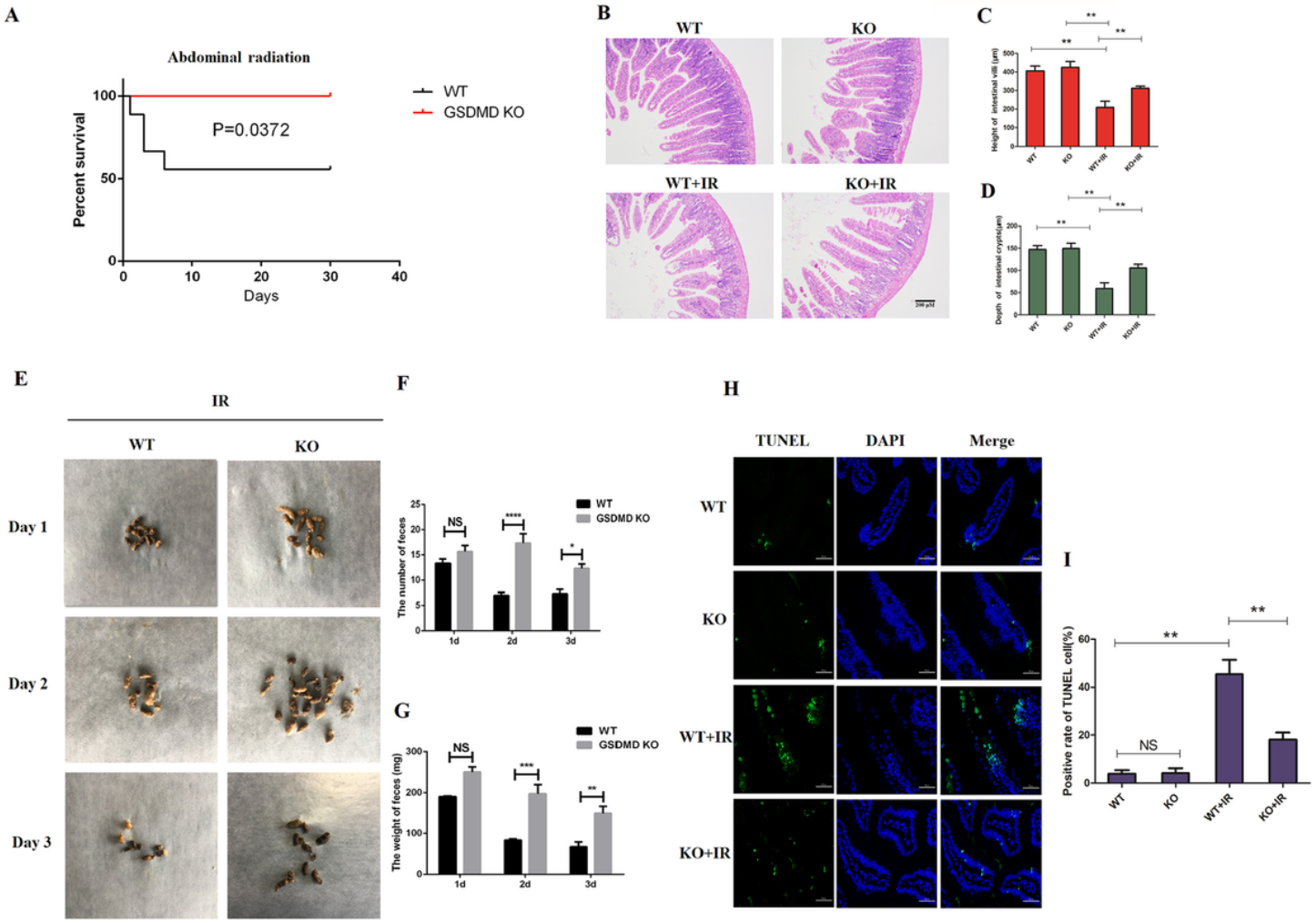

Figure 2

GSDMD knock-out can alleviate the radiation-induced intestinal injury.

(A) Mice of GSDMD knock-out (N=10) were subject to abdominal radiation of 27Gy (dose rate was 1Gy/min), and same age wild type of C57BL/6 mice (N=10) also received radiation of 27Gy as control

group. After radiation, their survival were observed for 30 consecutive days. (B-D) On the 3rd day after radiation, the small intestines of wild type mice (N=5) and GSDMD knock-out mice (N=5) were taken to perform H&E staining (B), and height of villi (C) and depth of crypts (D) were measured. (E-G) Feces of mice were collected on the on the 1st, 2nd and 3rd day after abdominal radiation. The appearance of feces was showed (E), and numbers (F) and weights (G) were measured. (H-I) On the 3rd day after radiation, the death of small intestines of mice were labeled by TUNEL fluorescent staining (H) and positive rate of TUNEL cells were quantified (I). Image analysis was conducted using Image J software. The error value was expressed as mean±SD. Every experiment was repeated tree times. NS represented no significant difference. *, ** and *** represented $P<0.05$, 0.01 and 0.001 between the corresponding groups, respectively.

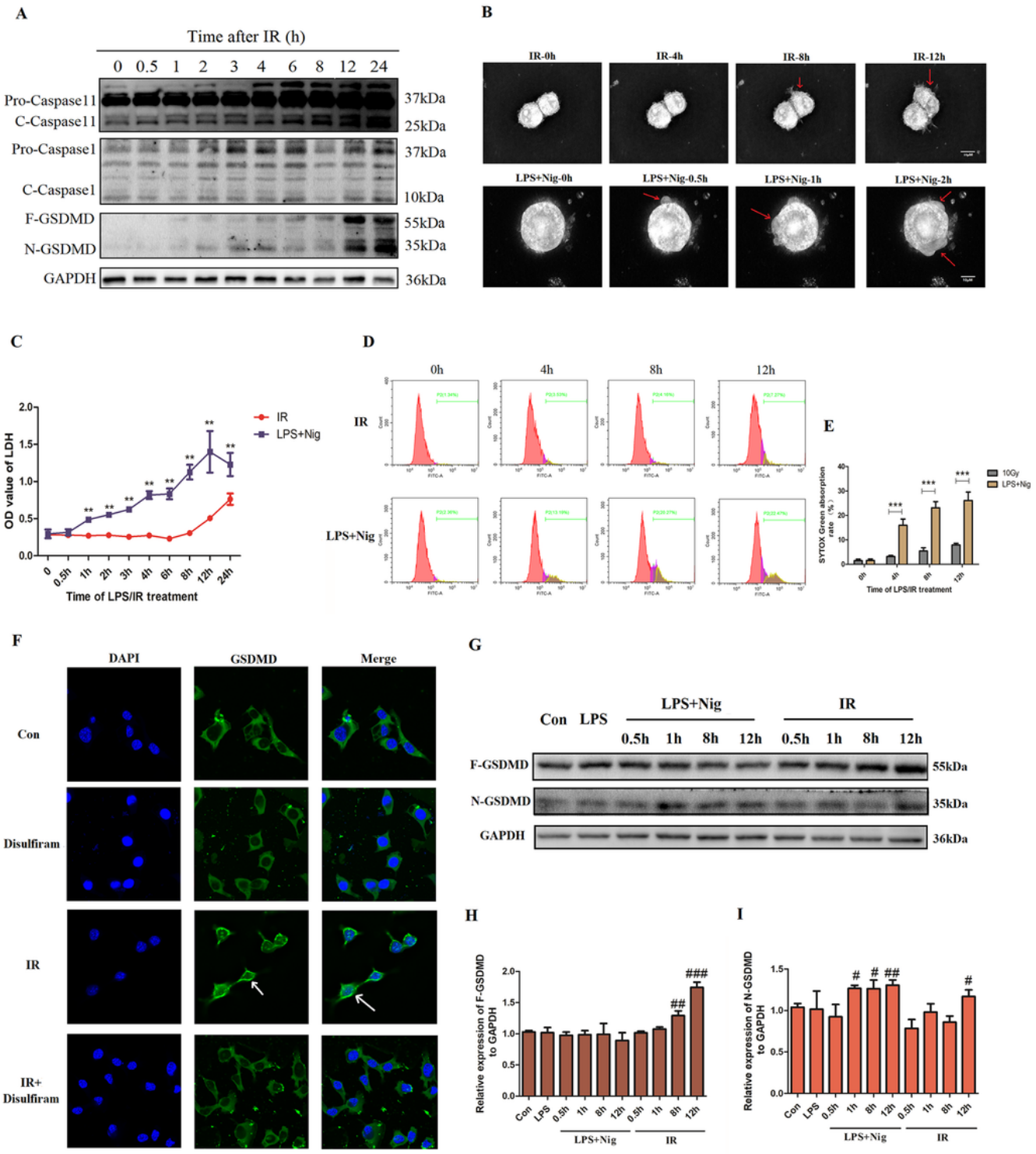

Figure 3

Radiation exposure induces the incidence of delayed pyroptosis and up-regulates GSDMD expression. (A) Mode-k cells received radiation of 10Gy (dose rate was 1Gy/min), and at designed time point (0.5, 1, 2, 3, 4, 6, 8, 12, 24 hours) the proteins of Mode-k were extracted to detect the expression of Caspase-1/cleaved Caspase-1, Caspase-11/cleaved Caspase-11, GSDMD/N-GSDMD. (B) Mode-k cells were radiated or treated with 1μg/ml LPS and 20μM Nigericin. At 4, 8, 12 hours after radiation and 0.5, 1, 2 hours after

LPS+Nigericin treatment, the morphology were observed and recorded by 3D Live Cell Imaging System. The red arrows indicated swell vesicle following pyroptosis. (C-E) With radiation and LPS+Nigericin treatment, the release of LDH were detected at 0.5-24 hours (C) and the absorption of SYTOX Green were tested at 4, 8 ,12 hours by Flow Cytometer (D-E). (F) Mode-k cells were treated with or without disulfiram (30μM) for 2h, and then cells were radiated at dose of 10Gy. 12 hours later, cells were stained with GSDMD immunofluorescent antibody (Green). The white arrows indicated GSDMD aggregation in cytomembrane. (G-I) After radiation and LPS+Nigericin treatment, at 1, 8, 12 hours the expression of F-GSDMD (full-length GSDMD) and N-GSDMD were detected by western blotting (G), and expression level were quantified respectively (H-I). Image analysis was conducted using Image J software. The error value was expressed as mean±SD. Every experiment was repeated tree times. *** represented P<0.001 between the two groups, respectively. $^{\#}$, $^{\#\#}$ and $^{\#\#\#}$ represented P<0.05, 0.01 and 0.001 between the corresponding group *vs* control group.

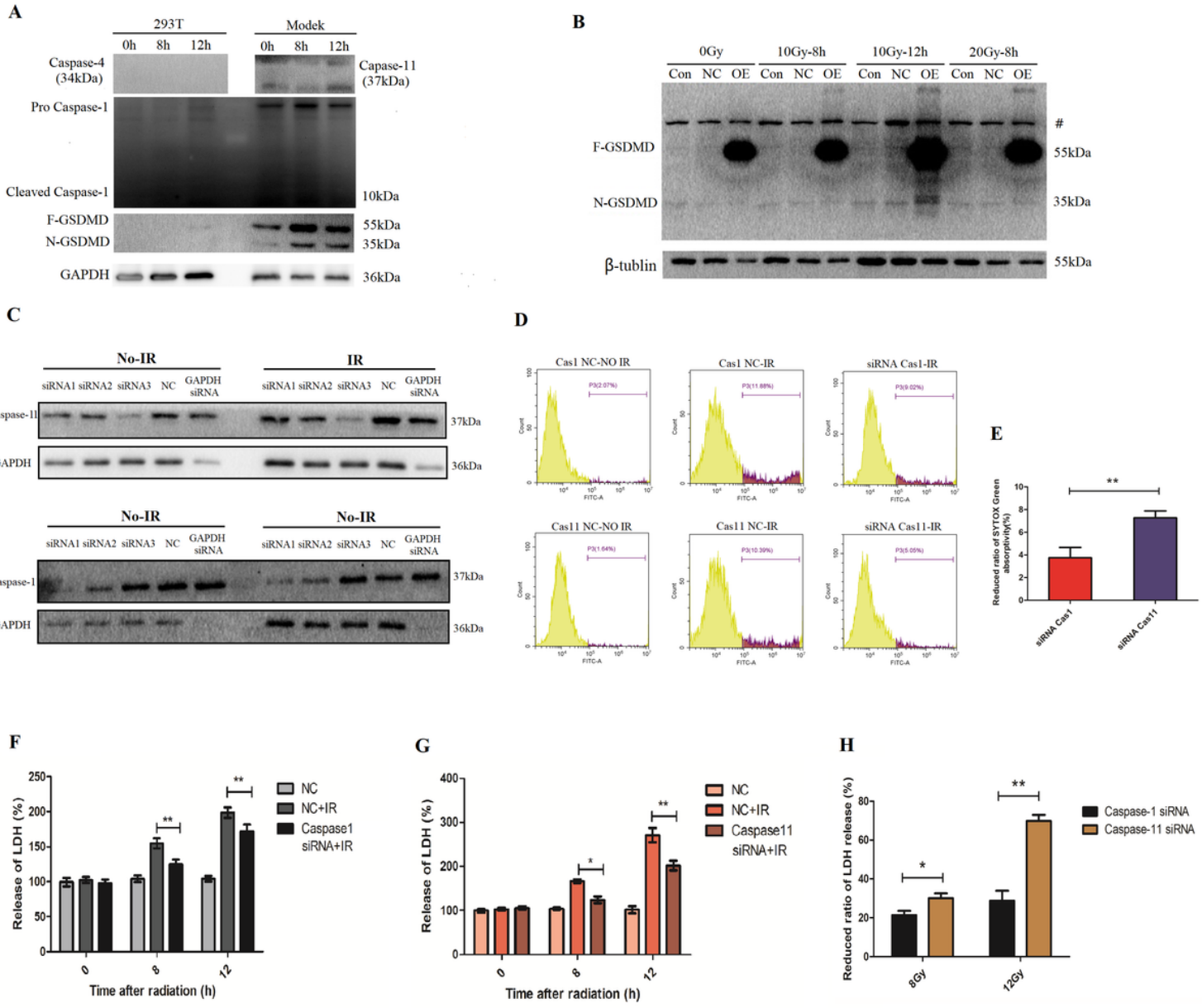

Figure 4

The radiation-induced GSDMD cleavage is dependent on the activation of Caspase-1 and Caspase-11. (A) 293T cells and Mode-k cells were radiated at dose of 10Gy, and at 8, 12 hours after radiation the proteins from both cells were extracted. Next, the expressions of GSDMD, Caspase-1 and Caspase-11 (for Mode-k), Caspase-4 (for 293T) were detected by western blotting. (B) GSDMD was over-expressed in 293T cells, and cells were radiated at dose of 10Gy and 20Gy, respectively. At 8 and 12 hours after radiation, proteins of 293T cells were extracted to detect the expression of F-GSDMD/N-GSDMD. # indicated, nonspecific binding. (C) Caspase-1 and Caspase-11 siRNA (including 3 siRNAs respectively) were transfected into Mode-k cells. Then cells received 10Gy radiation, and the expression of Caspase-1 and Caspase-11 were detected, respectively. Simultaneously, GAPDH siRNA was also used to verify transfection efficiency. (D-E) Having verified and been transfected with the most efficient siRNA for Caspase-1 and Caspase-11, Mode-k cells were radiated at dose of 10Gy, and the absorption of SYTOX Green were tested at 12 hours by Flow Cytometer. (F-H) Simultaneously, the release of LDH after Caspase-1 (F) and Caspase-11 siRNA (G) transfection were also detected, and reduced ratio was calculated (H). The error value was expressed as mean±SD. Every experiment was repeated tree times. ** represented $P<0.01$ between the two groups.

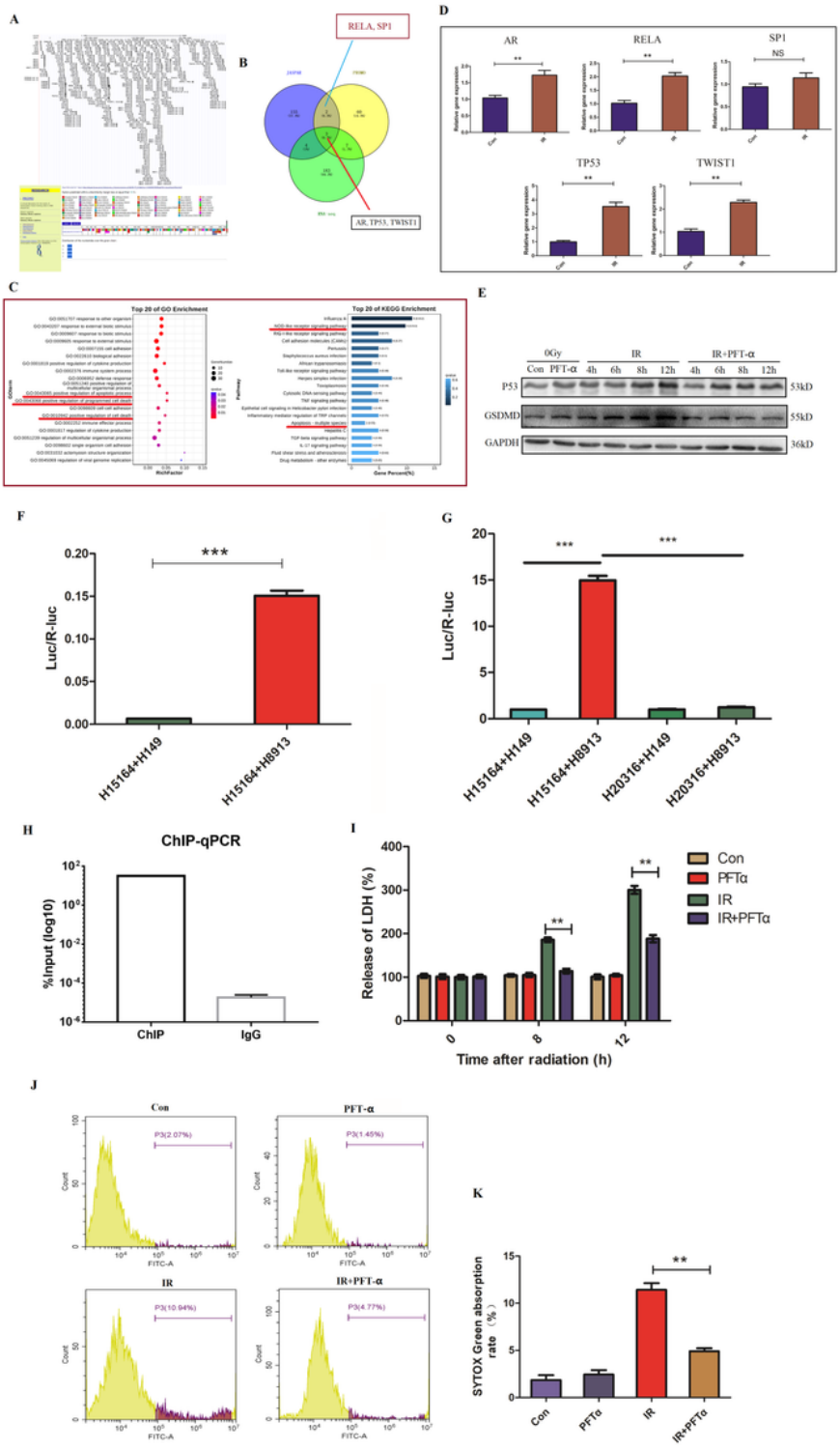

Figure 5

Radiation up-regulates the expression of GSDMD by P53 transcription.

(A-B) Prediction of upstream transcription factors for GSDMD. Firstly, the promoter sequence of GSDMD was obtained by retrieving NCBI database. Next, the promoter sequence of GSDMD was put into JASPAR and PROMO database respectively, and then predicted transcription factors were listed (A).

Simultaneously, the RNA-seq of no-radiated and radiated Mode-k cells were performed, and differentially expressed genes (DEGs) between the two groups were screened. Using Venn diagram, the shared transcription factors between databases of predicted transcription factors and DEGs were displayed (B). (C) The Gene ontology (GO) analysis and KEGG pathway analysis from DEGs were conducted. (D) Further the expression of shared transcription factors (including AR, RELA, SP1, TP53 and TWIST1) after radiation were validated by qPCR. (E) Mode-k cells were treated with PFT-α (10μM) for 2h, then cells received 10Gy radiation. At 4, 6, 8, 12 hours after radiation, the expressions of P53 and GSDMD were detected. (F) Double luciferase reporter gene system was used to verify the transcription of P53 on GSDMD. 48 hours after co-transfection with P53 plasmid and GSDMD promotor plasmid into 293T cells, the intensity of luciferase was detected. H15164, H149, H8913 and H321 indicated pGL4.10-Gsdmd Promoter, pLenti-EF1a-EGFP-P2A-Puro-CMV-MCS-3Flag, pLenti-EF1a-EGFP-P2A-Puro-CMV-Trp53-3Flag and pRL-CMV, respectively. (G) The GSDMD promotor was mutated to further detect the combination between P53 and GSDMD promotor. H20316 indicated pGL4.10-Gsdmd promotor (MUT1+MUT2). (H) In order to further confirm the combination between P53 and GSDMD promotor, the Chip-qPCR assay was conducted. (I-K) Mode-k cells were treated with PFT-α (10μM) for 2h, then cells received 10Gy radiation. At 8, 12 hours after radiation, the release of LDH was detected by ELISA assay (I), and the absorption of SYTOX Green was tested at 12 hours by Flow Cytometer (J-K). The error value was expressed as mean±SD. Every experiment was repeated tree times. ** and *** represented $P<0.01$ and 0.001 between the two groups, respectively.

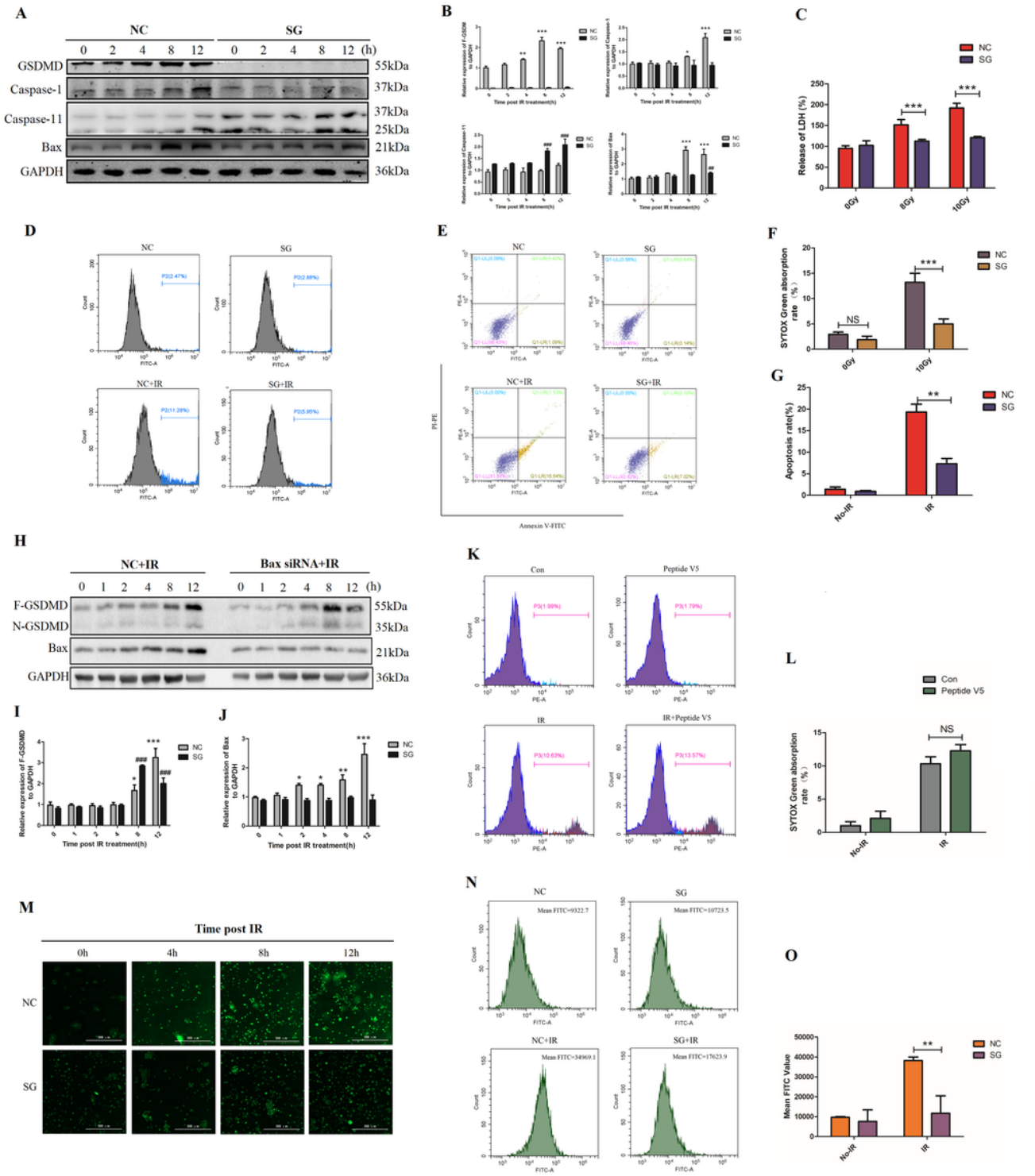

Figure 6

Radiation-induced pyroptosis promotes apoptosis in intestinal epithelial cells. (A-B) The GSDMD of Mode-k cells was knock-out by CRISPR-CAS9 technique, then GSDMD knock-out (SG) cells and control (NC) were radiated at dose of 10Gy. At 2, 4, 8, 12 hours after radiation, the expressions of GSDMD, Caspase-1, Caspase-11 were detected by western blotting (A) and were subsequently quantified (B). (C) SG and NC Mode-k cells were radiated at dose of 8 and 10Gy respectively, and 12 hours after radiation

the LDH release was detected. (D-G) SG and NC Mode-k cells received 10Gy radiation, then at 12 hours after radiation the absorption of SYTOX Green was determined (D, F) and at 24 hours the apoptosis was tested by Flow Cytometer (E, G).

(H-J) Bax siRNA was used and the expressions of Bax and GSDMD were detected (H) and were quantified (I-J) at designed time points (1, 2, 4, 8, 12 hours) after 10Gy radiation. (K-L) Peptide V5, an inhibitor of Bax was used to verify the influence of apoptosis on pyroptosis. Pretreated with Peptide V5 (50μM) for 2h, Mode-k cells received 10Gy radiation, then absorption of SYTOX Green was determined by Flow Cytometer at 12 hours later. (M-O) The intracellular calcium concentration was detected. SG and NC Mode-k cells were radiated at dose of 10Gy, and at 4, 8, 12 hours the intracellular calcium concentration was detected by calcium probe-Fluo 4-AM, and fluorescence intensity was subsequently observed by fluorescence microscope (M) and was measure by Flow Cytometer (N-O). Image analysis was conducted using Image J software. The error value was expressed as mean±SD. Every experiment was repeated tree times. NS represented no significant difference. *, ** and *** represented $P<0.05$, 0.01 and 0.001 between the corresponding groups *vs* control groups (0h) in NC Mode-k cells, and $^{\#\#}$, $^{\#\#\#}$ represented $P<0.01$ and 0.001 in SG Mode-k cells.

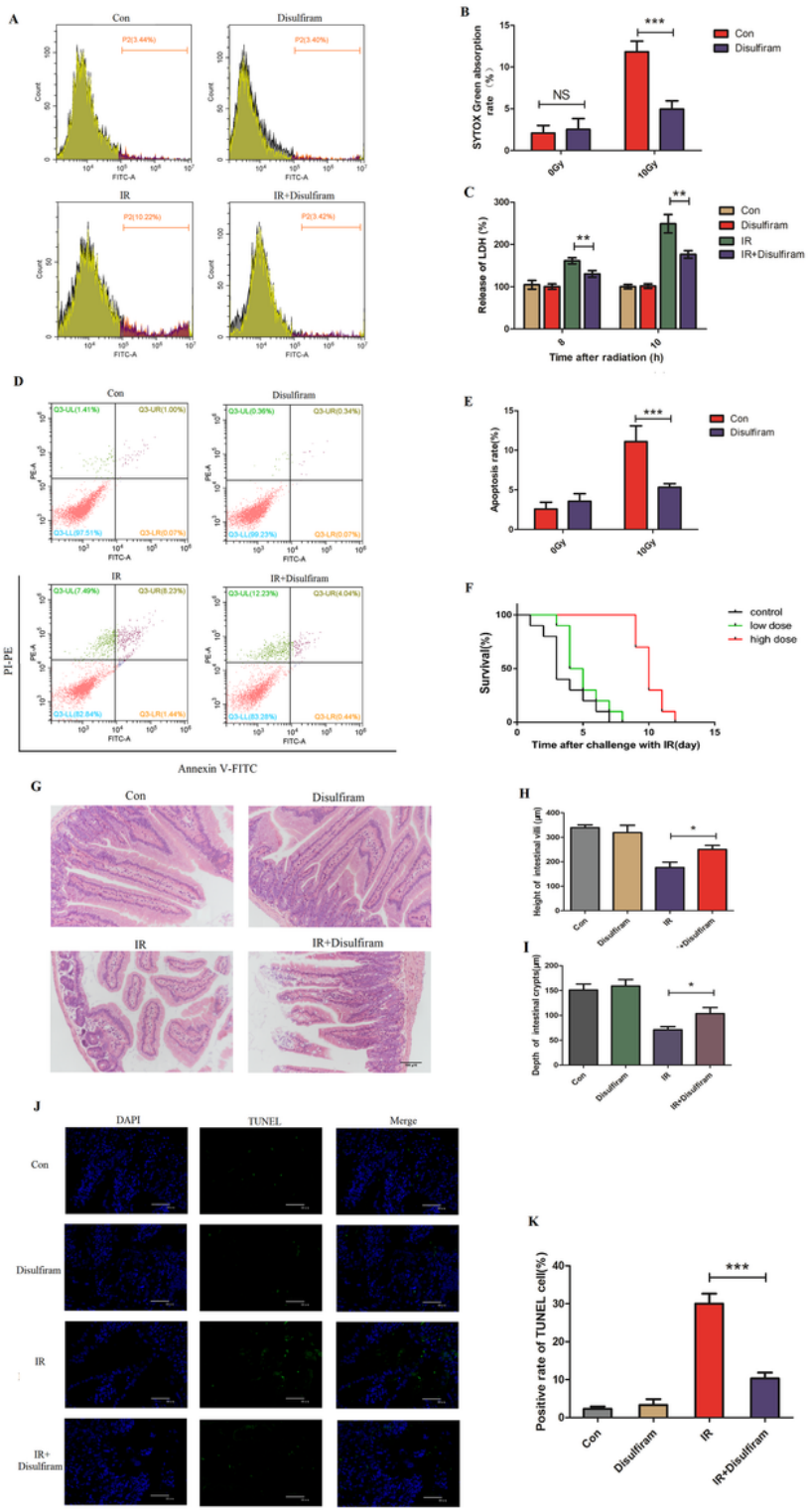

Figure 7

Disulfiram alleviates radiation-induced intestinal injury. (A-C) Mode-k cells were treated with disulfiram (5μM) for 2h, then received 10Gy radiation. At 12 hours after radiation the absorption of SYTOX Green was tested (A-B), and at 8, 12 hours the release of LDH was detected (C). (D-E) 24 hours after radiation the apoptosis of Mode-k cells was detected by PI/Annexin V staining, and the fluorescence intensity was tested by Flow Cytometer subsequently. (F) Mice (N=10) were in advance intraperitoneal injected with

disulfiram at 5mg/kg (low dose) and 25mg/kg (high dose), then receive 27Gy abdominal radiation and their survival were observed for 30 consecutive days. (G-I) Having intraperitoneal injection of disulfiram at 25mg/kg, the small intestines of mice (N=5) were taken to make H&E staining (G) and TUNEL fluorescence staining (J) on the 3rd day after radiation. The height of villi (H) and depth of crypts (I) were measured to quantify H&E staining, and positive rate of TUNEL cells (K) was calculated. The error value was expressed as mean±SD. Every experiment was repeated tree times. *, ** and *** represented P<0.05, 0.01 and 0.001 between the corresponding groups.

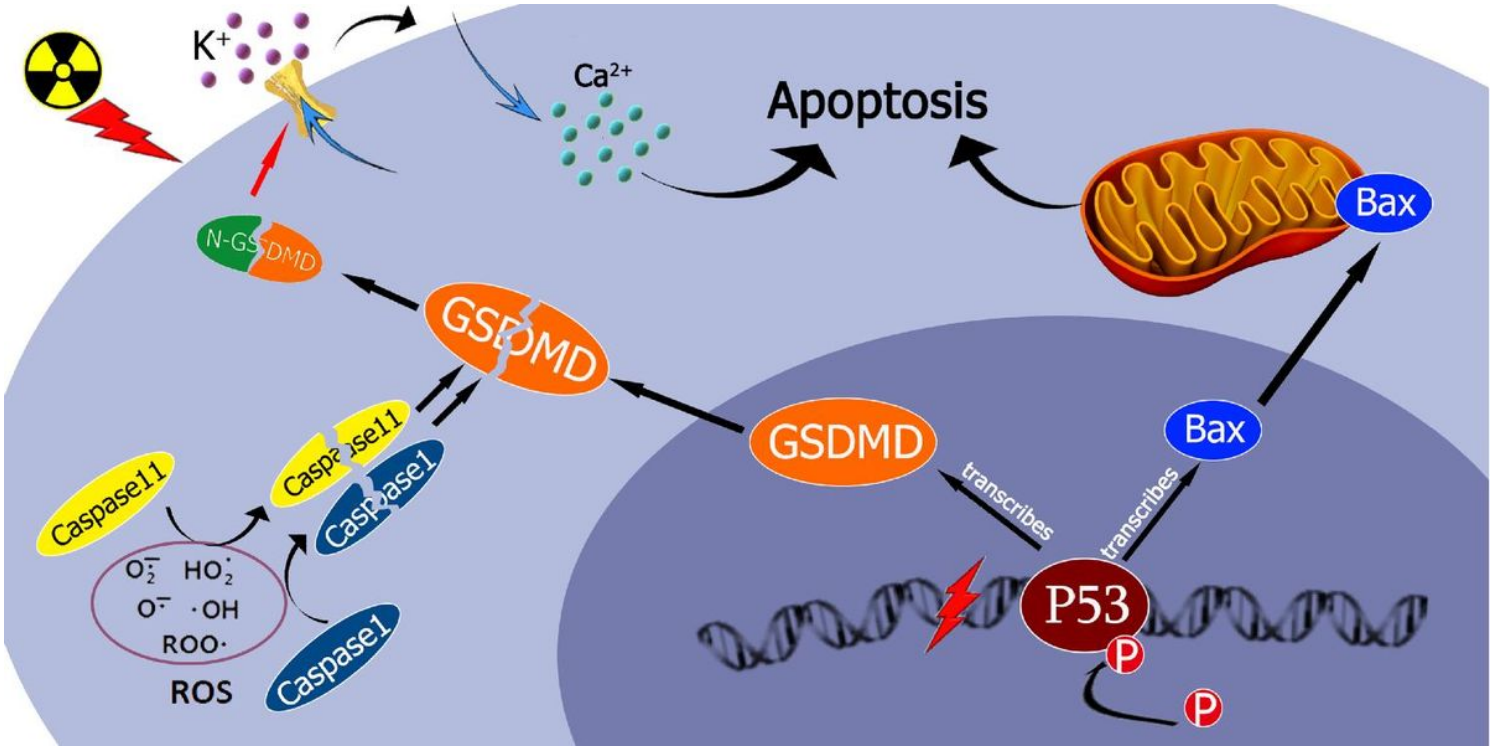

Figure 8

Summary of study. Radiation could induce delayed pyroptosis in the intestinal epithelial cell. During that process, GSDMD is cleaved by Caspase-1 and Caspase-11 cleavage and has inducible high expression by P53 transcription which simultaneously transcribes Bax to induce apoptosis. Subsequently, N-GSDMD aggregates on the cytomembrane, which then enhanced Ca2+ influx and further accelerated apoptosis.

Supplementary Files

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