MicroRNA-148a and -152 counteract RIPK1-mediated apoptosis and necroptosis to promote cancer cell proliferation and cisplatin resistance

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Abstract

Evasion of cell death is a hallmark of cancer cells. Receptor-interacting protein kinase 1 (RIPK1) is a common mediator in cancer cell death signaling pathways of apoptosis and necroptosis. MicroRNAs (miRNAs) are non-coding small RNAs that are involved in various biological processes such as cell proliferation and death by regulating target genes. Here, we identified miR-148a and miR-152 as suppressors of TNF-induced apoptosis based on the screening of apoptosis-regulating miRNAs. Elevated expression of miR-148a or miR-152 blocks cellular activation of caspase-8 and caspase-3 in multiple cancer cells. Moreover, overexpression of miR-148a or miR-152 inhibits TNF-induced necroptosis as well as cellular activation of RIPK1, RIPK3 and MLKL. We found that both miR-148a and miR-152 downregulate the expression of RIPK1, an essential regulator of both TNF-induced apoptosis and necroptosis. MiR-148a and miR-152 directly target the 3'UTR of RIPK1 to inhibit RIPK1 expression. Importantly, miR-148a or miR-152 overexpression promotes colony formation in multiple types of cancer cells. Of note, Kaplan-Meier Plotter analysis reveals that gastric carcinoma patients with high miR-152 expression are associated with lower overall survival. Overexpression of miR-148a or miR-152 significantly counteracts the chemotherapy drug cisplatin-induced RIPK1-mediated cell death and promotes gastric cancer cell survival and proliferation. These findings demonstrate the miR-148a and miR-152 as a class of oncogenic miRNAs capable of evading apoptosis and necroptosis via the suppression of RIPK1. Our study also suggests the miR-148a and miR-152 as a potential anti-cancer target for overcoming cell death resistance to chemotherapy drugs such as cisplatin.

Introduction

Cell death plays a critical role in the development and homeostasis maintenance in multicellular organisms. Apoptosis, a form of programmed cell death, is tightly controlled by a class of cysteine proteases called caspases [1]. Apoptotic cells display morphological alterations including cell shrinkage, DNA fragmentation and the formation of apoptotic bodies surrounded by membranes [2]. In mammalian cells, apoptosis can be induced through the mitochondrial pathway (the intrinsic pathway) or the death receptor pathway (the extrinsic pathway). Activation of mitochondria leads to cytochrome c release from mitochondrial into the cytosol. Cytochrome c further forms a protein complex (termed apoptosome) with pro-caspase-9 and apoptotic protease activating factor-1 (Apaf-1), leading to the activation of caspase-9 [3]. The pro-apoptotic protein Smac/Diablo is also released from mitochondria to the cytosol and subsequently binds to inhibitors of apoptosis proteins (IAPs) for relief of IAPs-mediated caspase inhibition. The extrinsic pathway is activated via the ligation of death ligands of the TNF receptor superfamily (e.g. TNF-α, FasL and TRAIL) to their respective death receptors (TNFR, Fas and TRAILR) [4, 5]. The binding of TNF to TNFR1 triggers the assembly of a membrane protein complex (Complex I) that contains TNFR1, receptor-interacting protein kinases 1 (RIPK1), TRAF2 and cIAP1/2 [6]. This process induces the ubiquitination of RIPK1, leading to NF-κB activation [7–9]. The small molecule (Smac mimetic) can mimic the function of Smac protein [10]. The addition of Smac mimetic activates the degradation of cIAP1/2, facilitating the deubiquitination of RIPK1 by cylindromatosis (CYLD) [11, 12].
This event promotes the formation of a cytosolic protein complex (Complex II) consisting of RIPK1, FADD and procaspase-8, leading to caspase-8 activation [6, 13]. Active caspase-9 and caspase-8 further cleave and activate the executor caspases such as caspase-3 for the execution of apoptosis.

Necroptosis is a form of regulated necrosis that is controlled by the activation of RIPK1, RIPK3 and mixed lineage kinase domain-like protein (MLKL). Necroptotic cells have typical necrotic morphological features such as cell swelling and membrane breakdown. Necroptosis can be initiated by activation of death receptors [14, 15], Toll-like receptors [16, 17] and interferon receptors (IFNRs) [18], and by infection of pathogens [19] and endogenous retroviruses [20, 21]. Impaired caspase-8 activity leads to the switch from TNF-induced apoptosis to TNF-induced necroptosis [14, 22]. In TNF-induced necroptosis, RIPK1 interacts with RIPK3 to form a protein complex (necrosome) via their RIP homotypic interaction motif (RHIM) domains, leading to RIPK3 activation [23–25]. The activated RIPK3 recruits and phosphorylates the substrate MLKL, and thus results in MLKL oligomerization and translocation to the cell membrane, eventually leading to necroptosis [26]. Necroptosis causes the release of damage-associated molecular patterns (DAMPs) to trigger inflammatory responses.

Evasion of cell death is regarded as a hallmark of cancer cells. Cancer cells often display defects in the regulation of apoptosis, which is associated with the upregulation of anti-apoptotic genes and the downregulation of pro-apoptotic genes [27]. It has been reported that many cancer cells exhibit defective necroptosis arising from epigenetic silence of Ripk3 gene transcription. Resisting cell death programs such as apoptosis and necroptosis promote cancer cell survival and increased the resistance of cancer cells to chemotherapeutic drugs. Therefore, resistance to cell death programs is a major obstacle to successfully treating cancer.

MicroRNAs (miRNAs) are small endogenous noncoding RNAs that negatively regulate the expression of target genes by binding to their 3’-UTR region. MiRNAs have been reported to regulate various biological processes including cell proliferation, differentiation, survival and cell death [28]. MiR-148/152 family includes three members miR-148a, miR-148b and miR-152[29, 30]. MiR-148/152 family members have been implicated in inflammatory diseases and multiple types of cancer [29, 30]. Increasing evidence suggest that miR-148/152 family members have diverse effects on apoptosis-related genes. MiR-148a was shown to promote glioblastoma cell growth and survival by inhibiting MIG6 and BIM and indirectly upregulating EGFR protein expression [31]. MiR-148a inhibits B-cell receptor engagement-induced apoptosis in immature B cells by reducing the expression of pro-apoptotic genes such as Bim and thus impairs B-cell tolerance [32]. Overexpression of miR-152 inhibits hypoxia-induced apoptosis by inhibiting PTEN expression and this process was associated with the downregulation of pro-apoptotic protein Bax in endothelial cells [33]. On the contrary, miR-148a promotes apoptosis by reducing anti-proapoptotic gene Bcl-2 expression in colorectal cancer cells [34]. However, the precise roles of miR-148/152 family members in apoptosis remain incompletely understood. Moreover, the effects of miR-148/152 family members on necroptosis are largely unknown.
Here, we demonstrate that miR-148a/152 act as suppressors of both TNF-induced apoptosis and necroptosis in multiple human cancer cells. RIPK1, a common molecule involved in both TNF-induced apoptosis and necroptosis, was found to be the direct target of miR-148a and miR-152. MiR-148a and miR-152-mediated downregulation of RIPK1 leads to inhibition of downstream caspase-8 activation and RIPK3 activation upon apoptotic and necroptotic stimuli, respectively. Notably, elevated expression of miR-148a or miR-152 promoted cancer cell proliferation and colony formation in multiple types of cancer cells. Kaplan-Meier Plotter analysis has shown that gastric carcinoma patients with high miR-152 expression correlate with lower overall survival. Elevated expression of miR-148a and miR-152 increased the resistance of gastric cancer cells to the chemotherapeutic drug cisplatin by inhibiting RIPK1-mediated apoptosis and necroptosis.

Materials And Methods

Cell Culture

Panc-1, MKN45, T98G, HT-29, SGC-7901 and HEK-293T cells were obtained from ATCC. T98G-hRIPK3 cell line was constructed in our lab. MKN45 and SGC-7901 cells were cultured in the RPMI-1640 (Hyclone) medium, HT-29 cells were cultured in McCoy’s 5A (Gibco) medium, and the rest of the cell lines were cultured in the DMEM (Hyclone) media containing 10% fetal bovine serum (Gibco) and 100 units/mL Penicillin-Streptomycin-Glutamine (Gibco) in a humidified environment at 37°C and 5% CO₂ in an incubator.

Cell Viability Assay

Cells were seeded in 96-well plates and then treated as indicated. The cell viability was measured by using the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega, United States) according to the manufacturer’s instruction.

Reagents and Antibodies

TNF-α recombinant protein was generated as previously described [13]. z-VAD was purchased from Bachem. Cisplatin (CDDP) was purchased from Selleck. The following antibodies were used: caspase-8 (CST, 9746), cleaved caspase-3 (CST, 9661), caspase-3 (CST, 9662), FADD (CST, 2782), p-RIPK1 (CST, 65746), RIPK1 (BD Biosciences, 610458), p-RIPK3 (Abcam, 209384), p-MLKL (Abcam, 187091), MLKL (Abcam, 184718), β-actin (Sigma, A2066). The Smac mimetic compound and anti-human RIPK3 antibody were provided by Dr. Xiaodong Wang (National Institute of Biological Sciences, Beijing).

MiRNA and siRNA Transfection

Lipofectamine 2000 (Invitrogen, United States) was used to transfect siRNA oligos into cells according to the manufacturer’s instruction. The siRNA oligos were bought from GenePharma Co., Ltd. (Shanghai, China). The following siRNA oligos were used: miR-NC: 5’-AACGUACGCGGAUACUUCGA-3’; miR-148a: 5’-
Western Blot Analysis

The cell pellet was harvested by centrifugation at 13000 × g for 1 min and then resuspended in a lysis buffer containing 20 mM Tris–HCl pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM Na$_3$VO$_4$, 1% Triton X-100, 25 mM β-glycerol phosphate, 0.1 mM PMSF, a complete protease inhibitor set (Roche). The cell lysate was incubated on ice for around 20 min and then centrifuged at 13000 × g for 20 min at 4°C. The supernatants were collected for further western blot analysis.

Flow Cytometry Analysis

The Annexin V-FITC/PI apoptosis detection kit (BD Biosciences, United States) was used to detect apoptotic cells according to the manufacturer's instructions. In brief, cells were transfected with miR-NC, miR-148a or miR-152. After around 60 hours (h), cells were treated with TNF-α and Smac mimetic. 16 hours later, cells were harvested and washed with cold PBS. Then these cells were resuspended in a staining buffer containing Annexin V- FITC and incubated in dark at room temperature. After 20 minutes, these cells were stained with PI. Flow cytometry analysis was obtained on a Gallios Flow Cytometer (Beckman Coulter, United States). The data were analyzed by the software Kaluza Analysis.

Dual-Luciferase Reporter Assay

3’-UTR of human RIPK1 was cloned into the pmirGLO vector (Promega, United States). The recombinant reporter plasmids were validated by DNA sequencing. RIPK1-3’UTR reporter plasmid was co-transfected with miR-NC, miR-148a, miR-152 and miR-152 mutant into HEK-293T cells. The luciferase activity was measured 48 h after transfection by using the Dual-Luciferase Reporter Assay System (Promega, United States). Luciferase activity was normalized to Renilla luciferase activity.

Colony Formation Assay

Panc-1, T98G and MKN45 cells were transfected with miR-NC, miR-148a, miR-152 or siRIPK1. After 48 hours, these cells (1 × 10$^3$/well) were seeded in 6-well plates. The medium was changed every two days. After 7 days, cells were fixed with 10% formalin and then stained with Giemsa (Nanjing Jiancheng Chemical Industrial Co., Ltd. (Nanjing, China)) for counting. Colonies were counted and analyzed using Image J software.

Overall Survival Analysis

The dataset of a total of 431 patients with gastric carcinoma was obtained from the online database Kaplan-Meier Plotter. The overall survival was analyzed by using Kaplan-Meier Plotter and GraphPad Prism.

Statistical Analysis
Data are expressed as mean ± SD. Unpaired Student’s t-test or an analysis of variance (ANOVA) were used to compare two or different groups, respectively. A P-value of < 0.05 was considered statistically significant.

Results

MiR-148a and miR-152 inhibit TNF-induced apoptosis

To identify miRNAs that may regulate apoptosis, we tested a set of miRNAs by assessing their effects on TNF-induced apoptosis in human pancreatic cancer Panc-1 cells, which are known to undergo caspase-8-mediated apoptosis upon treatment with TNF-α plus Smac mimetic [13]. Panc-1 cells were transfected with miRNAs for around 60 hours (h), followed by the treatment of TNF-α plus Smac mimetic (T + S). After 24 h, cell viability was measured based on the ATP levels. The RIPK1 siRNA oligo was used as the positive control (Fig. 1A). We found that both miR-148a and miR-152 efficiently blocked TNF-induced apoptosis in Panc-1 cells (Fig. 1A). Furthermore, we confirmed that miR-148a and miR-152 significantly inhibited TNF-induced apoptosis in human glioblastoma T98G, human gastric adenocarcinoma SGC-7901 and human gastric carcinoma MKN45 cells (Fig. 1B-D).

Apoptotic cells expose phosphatidylserine at the outer surface of the cell membrane, which can be detected by Annexin V staining. We evaluated the effects of miR-148a and miR-152 on the Annexin V-positive and propidium iodide (PI) positive cells. Transfection of miR-148a or miR-152 in Panc-1 and T98G cells significantly reduced the percentage of Annexin V and PI positive cells upon stimulation of TNF-α plus Smac mimetic (Fig. 1E-F). Collectively, these results demonstrate that miR-148a and miR-152 act as suppressors of TNF-induced apoptosis in multiple cancer cells.

MiR-148a and miR-152 negatively regulate TNF-induced apoptosis by interfering with caspase-8 activation or its upstream signals

Having shown that miR-148a and miR-152 are capable of inhibiting TNF-induced apoptosis, we sought to evaluate the effects of miR-148a and miR-152 on caspase activation. TNF-induced apoptosis requires the activation of the initiator caspase-8 and the executioner caspase-3. Both caspase-8 and caspase-3 are activated by proteolytic cleavage during apoptosis. Therefore, we examined the impacts of miR-148a and miR-152 on the activation of caspase-8 and caspase-3 by assessing their proteolytic cleavage products. We found that either miR-148a or miR-152 transfection in Panc-1, inhibited the cleavage of both caspase-8 and caspase-3 (Fig. 2A). Further, we confirmed that miR-148a or miR-152 transfection led to the suppression of caspase-8 and caspase-3 activation in T98G and SGC-7901 cells treated with TNF-α/Smac mimetic (Fig. 2B-C). Of note, miR-148a or miR-152 transfection did not affect the expression levels of caspase-8 and caspase-3 in cells under normal culture conditions (Fig. 2D-F). Taken together, these results indicate that miR-148a and miR-152 negatively regulate TNF-induced apoptosis by interfering with caspase-8 activation or its upstream signals.
MiR-148a and miR-152 block TNF-induced necroptosis

TNF-α is a pleiotropic cytokine that can trigger necroptosis in addition to apoptosis. It is known that TNF-induced apoptosis switches into TNF-induced necroptosis in the presence of pan-caspase inhibitor z-VAD. Three reagents TNF-α, Smac mimetic and z-VAD are commonly used as necroptotic stimuli to induce necroptosis in RIPK3-expressing cells such as human colon cancer HT-29 cells and human gastric adenocarcinoma SGC-7901 cells [23]. We found that both miR-148a and miR-152 efficiently blocked TNF-induced necroptosis in HT-29 and SGC-7901 cells (Fig. 3A-B), suggesting that miR-148a and miR-152 are suppressors of TNF-induced necroptosis. Unlike HT-29 cells, T98G cells were insensitive to the treatment of TNF-α, Smac mimetic and z-VAD, due to the lack of RIPK3 expression (Fig. S1A-B). Next, we generated a stable T98G cell line expressing human RIPK3 (T98G-hRIPK3), which was committed to TNF-induced necroptosis upon the treatment of TNF-α, Smac mimetic and z-VAD (Fig. S1C). Transfection of miR-148a or miR-152 also reduced TNF-induced necroptosis in T98G-hRIPK3 cells (Fig. S1C).

In TNF-induced necroptosis, RIPK1 and RIPK3 are activated, leading to the phosphorylation of RIPK1 and RIPK3. Activated RIPK3 further phosphorylates the substrate MLKL. We further examined the effects of miR-148a and miR-152 on the activation of RIPK1, RIPK3 and MLKL upon necroptotic stimuli. MiR-148a or miR-152 transfection resulted in decreased phosphorylation levels of RIPK1, RIPK3 and MLKL in HT-29 and SGC-7901 cells treated with TNF-α/Smac mimetic/z-VAD (Fig. 3C-D). We also observed a decreased level of RIPK1 in cells transfected with miR-148a or miR-152 (Fig. 3C-D). Moreover, we found that miR-148a or miR-152 transfection reduced the levels of RIPK1 phosphorylation and RIPK1 expression in T98G-hRIPK3 cells(Fig. S1D). These results demonstrate that miR-148a and miR-152 negatively regulate TNF-induced necroptosis as well as cellular activation of RIPK1, RIPK3 and MLKL.

MiR-148a and miR-152 downregulate RIPK1 expression by directly targeting its 3’UTR

Since miR-148a and miR-152 function as suppressors of both TNF-induced apoptosis and necroptosis, miR-148a and miR-152 may directly target the common molecule RIPK1. We searched the miRWalk database and found a putative binding site at the 3’UTR of RIPK1 for miR-148a and miR-152 (Fig. 4A). We evaluated the effects of miR-148a and miR-152 on the protein level of RIPK1 under normal culture condition. Transfection of either miR-148a or miR-152 led to a significant reduction in RIPK1 expression in Panc-1, HT-29, SGC-7901 and T98G cells (Fig. 4B-E).

To further evaluate whether RIPK1 is a direct target for miR-148a and miR-152, we generated a luciferase reporter construct (pmirGLO-RIPK1-3’UTR) containing the 3’UTR of RIPK1. We found that transfection of miR-152 significantly decreased the luciferase signal of 3’-UTR of RIPK1 (Fig. 4F). Of note, miR-152 failed to affect a mutant form of RIPK1-3’UTR in which the predicted miR-148a/152 binding sequences “GCACUGA” was replaced by “CGUGACU” (Fig. 4F), supporting that the predicted sequences of “GCACUGA” at RIPK1-3’UTR are critical recognition sites for miR-148a and miR-152. Moreover, we generated a mutant form of miR-152 (miR-152-mut) in which the predicted binding sites were mutated
This mutant miR-152 did not affect the luciferase signal of 3'-UTR of RIPK1 (Fig. 4G). Moreover, we found that the mutant miR-152 transfection failed to inhibit TNF-induced apoptosis and had no effect on the protein level of RIPK1 expression in T98G cells (Fig. 4H-I). Consistently, the mutant miR-152 failed to inhibit TNF-induced necroptosis in both HT-29 and T98G-hRIPK3 cells (Fig. 4J-K; Fig. S2A-B). Collectively, these results demonstrate that miR-148a and miR-152 block TNF-induced apoptosis and necroptosis by directly downregulating RIPK1 expression.

**MiR-148a and miR-152 promote cancer cell growth and colony formation**

Since overexpression of miR-148a or miR-152 increased the resistance of cancer cells to apoptosis and necroptosis, we speculated that miR-148a and miR-152 might contribute to cell proliferation and clonogenicity in glioblastoma cells. We further examined the effects of miR-148a and miR-152 on the colony formation of Panc-1, T98G, MKN45 and SGC-7901 cells. As shown in Fig. 5A-B, both miR-148a and miR-152 overexpression significantly enhanced the colony formation of Panc-1 cells. MiR-148a and miR-152 induced colony formation ability of tumor cells were further confirmed in T98G, MKN45 and SGC-7901 cells (Fig. 5C-H). These results suggest that miR-148a and miR-152 promote the survival and proliferation of cancer cells.

**High expression of miR-152 is correlated with poor prognosis of gastric carcinoma patients and resistance of cancer cells to cisplatin**

Evasion from cell death such as apoptosis is considered a hallmark of cancer cells. We sought to clarify whether cellular expression levels of miR-148 and miR-152 correlate with the resistance of cancer cells to TNF-induced apoptosis. Therefore, we examined the prognostic value of miR-148/152 expression in human cancer patients using the online database Kaplan-Meier Plotter (http://kmplot.com/analysis/). Kaplan–Meier overall survival analysis showed that gastric carcinoma patients with the high expression level of miR-152 had shorter overall survival time compared to those patients with low miR-152 expression (Fig. 6A). Cisplatin is a first-line chemotherapy drug for gastric cancer. Cisplatin was shown to induce apoptosis and necroptosis in multiple types of cancer cells including gastric cancer cells[35–37]. To further explore the effects of miR-152 on cisplatin induced apoptosis and necroptosis. We transfected miR-152 and siRIPK1 oligo into human gastric cancer SGC-7901 cells, followed by the treatment of cisplatin. MiR-152 overexpression could inhibit cisplatin-induced cell death (Fig. 6B), associated with significant reduction of RIPK1 phosphorylation, MLKL phosphorylation and caspase-3 cleavage (Fig. 6C). These results indicate that miR-152 decreases the sensitivity of gastric cancer cells to cisplatin by inhibiting RIPK1-mediated apoptosis and necroptosis. We further examined the effect of miR-152 on the colony formation of SGC-7901 cells. Overexpression of miR-152 significantly enhanced the colony formation of SGC-7901 cells upon cisplatin treatment (Fig. 6D-E). Collectively, these results suggest that high expression of miR-152 increases the resistance of cancer cells to cisplatin by counteracting RIPK1-mediated cell death.
Discussion

Evading cell death programs such as apoptosis and necroptosis are crucial mechanisms exploited by cancer cells to escape from immunosurveillance and increase their oncogenic potential. MiRNAs play important roles in tumor development as oncogenes or tumor suppressors by regulating the expression of target genes. In this study, we demonstrate that miR-148a and miR-152 negatively regulate apoptosis and necroptosis by directly inhibiting RIPK1 expression. Upregulation of miR-148a or miR-152 is associated with their oncogenic activity and chemotherapy drug resistance.

MiR-148a and miR-152 have been reported to regulate cell proliferation and cell death in diverse cancer cells depending on the target mRNAs [29, 30]. Based on a cell-based screening for miRNAs regulating apoptosis, we identified miR-148a and miR-152 as suppressors of TNF-induced apoptosis in multiple cancer cell lines. Overexpression of either miR-148a or miR-152 inhibited the activation of caspase-8 and caspase-3 upon apoptotic stimuli (Fig. 2). Furthermore, we found that miR-148a and miR-152 significantly blocked TNF-induced necroptosis, which was associated with reduced activation of RIPK1, RIPK3 and MLKL (Fig. 3). Taken together, our study reveals that miR-148a and miR-152 can evade both apoptosis and necroptosis in cancer cells.

RIPK1 is an essential regulator of both TNF-induced apoptosis and necroptosis. We found that upregulation of miR-148a or miR-152 led to a significant reduction of RIPK1 expression in multiple cancer cells. Moreover, both miR-148a and miR-152 directly inhibited the luciferase activity of 3’-UTR of RIPK1. The “GCACUGA” region at the RIPK1-3’UTR is crucial for the recognition of RIPK1 by miR-148a and miR-152a. Thus, RIPK1 is a direct target of miR-148a and miR-152. In addition to regulating apoptosis and necroptosis, RIPK1 is required for NF-κB activation. NF-κB signaling pathway plays important roles in inflammatory and immune responses by regulating the transcription of various target genes [38]. MiR-148a was previously found to inhibit the NF-κB signaling pathway and inflammatory gene expression [39, 40]. Therefore, miR-148/152-mediated suppression of RIPK1 expression is likely to be one of the mechanisms leading to the inhibited NF-κB activation.

Considering the ability of miR-148a and miR-152 to counteract apoptosis and necroptosis signaling pathways, it is attempting to speculate that the miR-148a and miR-152 may have oncogenic activity. Indeed, miR-148a or miR-152 overexpression enhances the clonogenicity of human cancer cells (Fig. 5). Moreover, gastric carcinoma patients with the high expression level of miR-152 are correlated with a poor prognosis (Fig. 6). Therefore, miR-148a/152 could be a biomarker for predicting the sensitivity of cancer cells to apoptosis and necroptosis. Cisplatin has been widely used for the treatment of gastric carcinoma, associated with RIPK1-mediated cell death [35–37]. We found that overexpression of miR-152 significantly inhibits cisplatin induced apoptosis and necroptosis in gastric cancer cells, suggesting that miR-152 is an important mediator for cisplatin resistance in gastric cancer cells. Our findings make miR-152 a potential target for overcoming chemotherapy drug resistance. Given the important role of the miR-148a and miR-152 in inflammatory diseases and cancers, future studies will be required to explore the
precise role of miR-148a and miR-152-mediated RIPK1 silence in inflammatory diseases and cancer in vivo.

Declarations

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Author contributions

All authors contributed to the study’s conception and design. XY and SH designed the study. Experiment operation, data collection and analysis were performed by JL, YJ, CZ, YD, XD, XW, HZ, XX, SD, HL, TY, WZ, CY and XY. XY, SH, JL, YJ, CZ wrote the manuscript. All authors reviewed the manuscript and consented for publication.

Data Availability

The data underlying this article will be shared on reasonable request to the corresponding author.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

References


Figures

Fig. 1

A  

B

C  

D

E  

F

Figure 1
MiR-148a and miR-152 inhibit TNF-induced apoptosis.

A-D. Panc-1, T98G, SGC-7901 and MKN45 cells were transfected with miR-NC, miR-148a, miR-152 or RIPK1 siRNA (siRIPK1) oligo for 60 h prior to the treatment with PBS (control) or 100ng/ml TNF-α plus 100nM Smac mimetic (T+S) for 24 h, and then cell viability was determined by measuring ATP levels. E-F. Panc-1 and T98G cells were transfected with miR-NC, miR-148a, miR-152 or siRIPK1 for 60 h, and then cells were treated with control or T+S, subsequently subjected to Annexin V/PI staining, followed by flow cytometric analysis of dead cells. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Figure 2

MiR-148a and miR-152 negatively regulate TNF-induced apoptosis by interfering with caspase-8 activation or its upstream signals.

A-C. Panc-1, T98G and SGC-7901 cells were transfected with miR-NC, miR-148a, miR-152 or siRIPK1 for 60 h and then treated with control PBS or 100ng/ml TNF-α plus 100nM Smac mimetic for 8h. Cell lysates
were collected for western blot analysis of full-caspase-3, cleaved-caspase-3, full-caspase-8, cleaved-caspase-8 and β-actin. D-F. Panc-1, T98G and SGC-7901 cells were transfected with miR-NC, miR-148a, miR-152 or siRIPK1 for 60 h, and then cell lysates were collected for western blot analysis of caspase-3, caspase-8, FADD and β-actin.

Fig. 3

Figure 3
MiR-148a and miR-152 block TNF-induced necroptosis.

A-B. HT-29 and SGC-7901 cells were treated with 100ng/ml TNF-α, 100nM Smac mimetic plus 20μM z-VAD (T+S+Z) for 24h, then cell viability was measured by ATP levels. C-D. HT-29 and SGC-7901 cells were transfected with miR-NC, miR-148a, miR-152 or siRIPK1 for 60 h, cell lysates were harvested for western blot analysis of p-RIPK1, RIPK1, p-RIPK3, RIPK3, p-MLKL, MLKL and β-actin. ***P < 0.001, ****P < 0.0001.
Figure 4

MiR-148a and miR-152 downregulate RIPK1 expression by directly targeting its 3’UTR.

A The putative binding sequences of miR-148a and miR-152 in the 3’-UTR of RIPK1. The binding prediction was analyzed with the miRWalk database. B-E. Panc-1, HT-29, SGC-7901 and T98G cells were transfected with miR-NC, miR-148a, miR-152 or siRIPK1 for 60 h, cell lysates were then collected for western blot analysis of RIPK1 and β-actin. F MiR-NC or miR-152 was co-transfected with pmir-GLO-RIPK1-3’UTR or a mutant form of pmir-GLO-RIPK1-3’UTR (pmir-GLO-mutant-RIPK1-3’UTR) into HEK-293T cells for 60 h, and then luciferase activity was measured. G MiR-NC, miR-152 or miR-152 mutant was co-transfected with pmir-GLO-RIPK1-3’UTR into HEK-293T cells for 60 h, and then luciferase activity was measured. H T89G cells were transfected with miR-NC, miR-152, miR-152 mutant or siRIPK1 for 60h, and treated with TNF plus Smac mimetic for 24 h, then cell viability was determined. I T98G cells were transfected as indicated for 60 h, and cell lysates were collected for western blot analysis of RIPK1 and β-actin. J HT-29 cells were transfected with indicated for 60h and then treated with TNF, Smac mimetic plus z-VAD for 24 h, then cell viability was determined. K HT-29 cells were transfected as indicated for 60 h and treated with TNF, Smac mimetic plus z-VAD for 6 h, then cell lysates were collected for western blot analysis of p-RIPK1, RIPK1 and β-actin. *P < 0.05, **P < 0.01, ****P < 0.001.
MiR-148a and miR-152 promote cancer cell growth and colony formation.

**A-H.** Panc-1, T98G, MKN45 and SGC-7901 cells were transfected with miR-NC, miR-148a or miR-152 for 48h and then seeded in 6-well plates. After 7 days, a colony formation assay was performed (**A, C, E, G**).
The number of colonies was quantified using the software Image J (B, D, F, H). **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 6

High expression of miR-152 is correlated with poor prognosis of gastric carcinoma patients and resistance of cancer cells to cisplatin.
A. The overall survival of gastric carcinoma patients was compared between individuals with high or low expression of miR-152. B. SGC-7901 cells were transfected as indicated for 60 h and treated with the control PBS or cisplatin for 24 h. Cell viability was determined by measuring ATP levels. C. SGC-7901 cells were transfected as indicated for 60 h and treated with cisplatin for 12 h, then cell lysates were collected for western blot analysis of p-RIPK1, RIPK1, p-MLKL, MLKL, caspase-3, cleaved caspase-3 and β-actin. D-E. SGC-7901 cells were transfected with miR-NC or miR-152 for 48 h and then seeded in 6-well plates. These cells were treated with cisplatin, and after 7 days, a colony formation assay was performed (D). The number of colonies was quantified using the software Image J (E). **P < 0.01, ***P < 0.001

**Figure 7**

The schematic model of miR-148a/152-mediated inhibition of apoptosis and necroptosis via suppression of RIPK1.

In TNF or cisplatin-induced apoptosis and necroptosis, miR-148a and miR-152 negatively regulate RIPK1 by directly targeting its “GCACUGA” at the 3′UTR region. MiR-148a/152-mediated downregulation of
RIPK1 results in suppressed downstream activation of caspase-8 and RIPK3, resulting in resistance to apoptosis or necroptosis.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supportingmaterial1.pdf
- supportingmaterial2.pdf