

Overexpression of miR-382 Sensitizes Hepatocellular Carcinoma Cells to $\gamma\delta$ T Cells by Inhibiting the Expression of c-FLIP

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Human $\gamma\delta$ T lymphocytes were reported to display anti-tumor effects against multiple cancers, including hepatocellular carcinoma (HCC). Aberrant expression of microRNAs (miRNAs) leads to a low response to immunotherapy. Thirty-five HCC tumor tissues and their adjacent healthy tissues were collected from patients with primary HCC who underwent tumor resection in the Third People's Hospital of Hainan Province, China. The purity of the resulting $\gamma\delta$ T cells was identified by anti- $\gamma\delta$ -T cell receptor-phycoerythrin (anti- $\gamma\delta$ -TCR-PE) and anti-CD3-fluorescein isothiocyanate (anti-CD3-FITC) antibodies on flow cytometry. Human HCC cell lines HepG2 and PLC were cultured. We observed that *ex vivo*, expanded human $\gamma\delta$ T cells were able to induce cell lysis of HCC. Furthermore, as miR-382 was observed to be downregulated in HCC tissues and cell lines, we found that overexpression of miR-382 increased the sensitivity of HCC cells to $\gamma\delta$ T cells. We proved that mRNA of cellular FADD-like interleukin-1 β -converting enzyme-inhibitory protein (c-FLIP) was the target of miR-382. Inhibition of c-FLIP by miR-382 significantly promotes the cell lysis of HCC through strengthening the activation caspase 8 induced by $\gamma\delta$ T cell treatment. In conclusion, overexpression of miR-382 promotes HCC cell lysis induced by $\gamma\delta$ T cells through inhibiting the expression of c-FLIP.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer deaths worldwide because of the late diagnosis and poor prognosis.¹ Surgery and liver transplantation treatment were the most effective treatment for patients with early-stage HCC.^{2,3} However, many patients were diagnosed with advanced HCC at their first visit. For those patients, the cancer is unresectable, and chemotherapy and immunotherapy are considered as the alternative option.^{4,5} However, HCC cells are usually resistant to those anti-tumor drugs.^{6,7} Drug resistance has become a major obstacle to the clinical application of chemotherapy or immunotherapy.

$\gamma\delta$ T cells belong to one subgroup of T lymphocytes. Although $\gamma\delta$ T cells account for only 5% of total T lymphocytes, they own potent anti-cancer activity because they exhibit direct cytotoxicity against

multiple malignancies with major histocompatibility complex (MHC) in an unrestricted manner.^{8–10} Strategies using $\gamma\delta$ T cells have been evaluated in clinical trials for cancer treatment. However, because $\gamma\delta$ T cell-based therapy exhibits limited success in cancer treatment,^{11,12} it suggests that adjuvant therapy is required to improve the efficiency of $\gamma\delta$ T cell-based immunotherapy.

MicroRNAs (miRNAs) are a group of non-coding RNAs. They are highly conserved sequences with 18–25 nt in length. miRNAs functions as gene regulators because they can partially pair with the complementary sequences of mRNA at the 3' untranslated region (3' UTR). As the results of miRNA-mRNA pairing, translation of targeted mRNA is inhibited.^{13,14} Reports have demonstrated that miRNAs are usually dysregulated in cancers. Dysregulation of miRNAs in cancers promote cell proliferation and epithelial-mesenchymal transition. In addition, studies have indicated that expression profile of miRNAs determines the sensitivity of tumor cells to cancer therapy.^{15–17} However, the role of miRNAs in cancer immunotherapy is still unclear. In the current study, we observed significantly decreased expression of miR-382 in HCC cells. Overexpression of miR-382 in HCC may represent a novel strategy to increase the sensitivity of HCC cells to $\gamma\delta$ T cell-based immunotherapy.

RESULTS

Cytotoxicity of *Ex Vivo* Expanded $\gamma\delta$ T Cells against HCC

To investigate the anti-tumor effects of $\gamma\delta$ T cells, we first expanded the human $\gamma\delta$ T cells by using peripheral blood mononuclear cells (PBMCs) in blood samples of healthy donors. As shown in **Figure 1A**, purified $\gamma\delta$ T cells, which were *ex vivo*-expanded, highly expressed CD3 and $\gamma\delta$ TCR. Subsequently, we co-cultured the $\gamma\delta$ T cells with the HCC cell lines in different E:T ($\gamma\delta$ T effector/targeted HCC cells) ratios. We observed that $\gamma\delta$ T cells displayed lytic activity against both

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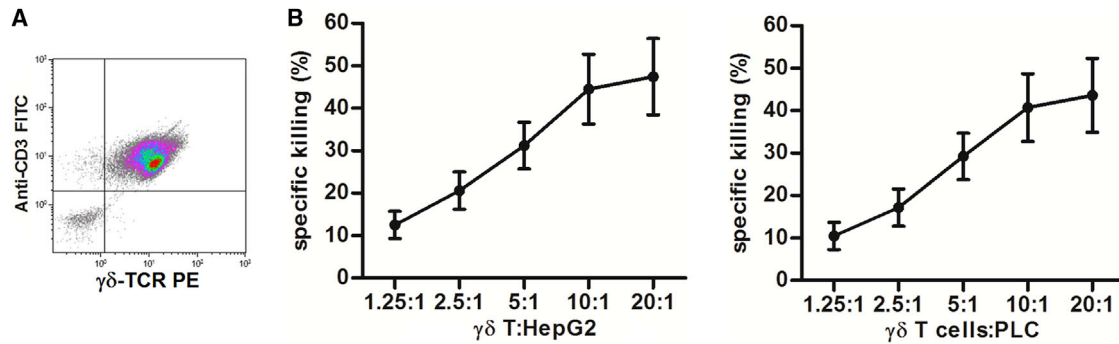


Figure 1. Cytotoxicity of Ex Vivo Expanded $\gamma\delta$ T Cells against HCC Cell Lines

(A) *Ex vivo*, expanded $\gamma\delta$ T cells from 15 donors were identified by flow cytometry. (B) Specific killing of HepG2 and PLC, induced by $\gamma\delta$ T cells, was measured with a ^{51}Cr release assay. Data are represented as means \pm SD from at least three independent experiments in each condition.

HepG2 and PLC cell lines (Figure 1B). We demonstrated that $\gamma\delta$ T cells were able to kill the HCC cells in a dose-dependent manner.

Overexpression of miR-382 Sensitizes HCC Cells to $\gamma\delta$ T Cell Treatment

To explore the potential role of miR-382 in HCC, we detected the expression of miR-382 in HCC patients' tumor tissues. We found that the HCC tumor tissues expressed significantly lower levels of miR-382 compared with the precancerous healthy tissues (Figure 2A). In addition, we also observed that the relative expression of miR-382 in HepG2 and PLC cells was significantly lower than that in the HCC patients' precancerous healthy tissues (Figure 2B). These results suggested that miR-382 was a potential tumor suppressor in HCC. Subsequently, we introduced HCC cells with miR-382 to investigate the effects of miR-382 on $\gamma\delta$ T cell-induced cell death. As shown in Figure 2C, transfection with miR-382 caused an overexpression of miR-382 in HepG2 and PLC cells. Furthermore, the results of cytotoxicity assays showed that introduction with miR-382 was able to increase the sensitivity of HCC cells to $\gamma\delta$ T cell-induced cell lysis (Figure 2D). We demonstrate that overexpression of miR-382 sensitizes HCC cells to $\gamma\delta$ T cell treatment.

Specific Killing of HCC Cells Co-treated with $\gamma\delta$ T Cells and miR-382 Is Dependent on TRAIL and FasL Signaling

Tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL), which are secreted by $\gamma\delta$ T cells, have been reported to participate in the specific killing of tumor cells.^{18,19} In accordance with that, we observed a significant reduction of cell lysis in $\gamma\delta$ T cell-treated HepG2 and PLC cells after they were incubated with TRAIL- and FasL-neutralizing antibodies (Figure 3A). Furthermore, we found that the promotion of miR-382 on $\gamma\delta$ T cell-induced cell lysis can be also inhibited by TRAIL- and FasL-neutralizing antibodies (Figure 3B). However, our results showed that miR-382 transfection in HCC cells didn't change the levels of TRAIL and FasL secreted by $\gamma\delta$ T cells (Figure 3C). Therefore, our results suggested that the cytotoxicity of $\gamma\delta$ T cells to HCC is dependent on TRAIL and FasL signaling. Transfection with miR-382 is able to sensitize the HCC cells to $\gamma\delta$ T cell-secreted TRAIL and FasL.

MiR-382 Targets c-FLIP in HCC

To explore the mechanism by which miR-382 sensitizes HCC cells to $\gamma\delta$ T cells, the TargetScan public database (<http://www.targetscan.org>) was used to search for the putative target of miR-382 in human cells. As shown in Figure 4A, cellular FADD-like interleukin-1 β -converting enzyme-inhibitory protein (c-FLIP) was predicted as the potential target of miR-382 because c-FLIP is the cellular inhibitor of caspase 8.²⁰ We have demonstrated that expression of miR-382 was decreased in HCC cells compared with that in healthy tissues. In contrast, we found that the expression of c-FLIP was increased in HCC cells (Figure 4B). These results showed a negative correlation between miR-382 and c-FLIP. To determine whether the expression of c-FLIP was regulated by miR-382, the protein level of c-FLIP in HepG2 and PLC was detected after they were transfected with miR-382. As shown in Figure 4C, overexpression of miR-382 was able to suppress the protein level of c-FLIP in both HepG2 and PLC cell lines. In addition, the lentivirus-mediated miR-382 (LV-miR-382)-transfected xenografts expressed significantly lower levels of c-FLIP compared with the LV-control-transfected xenografts *in vivo* (Figure 4D). Furthermore, after performing the luciferase reporter assays, we found that transfection with miR-382 decreased the luciferase activity of the wild-type c-FLIP 3' UTR reporter, but not the mutant and empty reporters (Figure 4E). Taken together, we demonstrate that miR-382 targets c-FLIP in HCC. To test the role of c-FLIP in $\gamma\delta$ T cell-induced cytotoxicity against HCC, we knocked down c-FLIP with its specific small interfering RNA (siRNA) in HepG2 and PLC cells before they were co-cultured with $\gamma\delta$ T cells. Similarly, with miR-382, we showed that transfection with c-FLIP siRNA was able to enhance the $\gamma\delta$ T cell-induced cell death in those HCC cells (Figure 4F). We emphasized the importance of c-FLIP in resistance to $\gamma\delta$ T cell treatment.

MiR-382 Promotes $\gamma\delta$ T Cell-Induced Activation of Caspases through Decreasing the Expression of c-FLIP

To investigate whether a decrease in c-FLIP was essential in miR-382-promoted cell death, we introduced HepG2 and PLC cells with a c-FLIP plasmid to oppose the miR-382-induced knockdown of c-FLIP. We found that the effect of miR-382 on $\gamma\delta$ T cell-

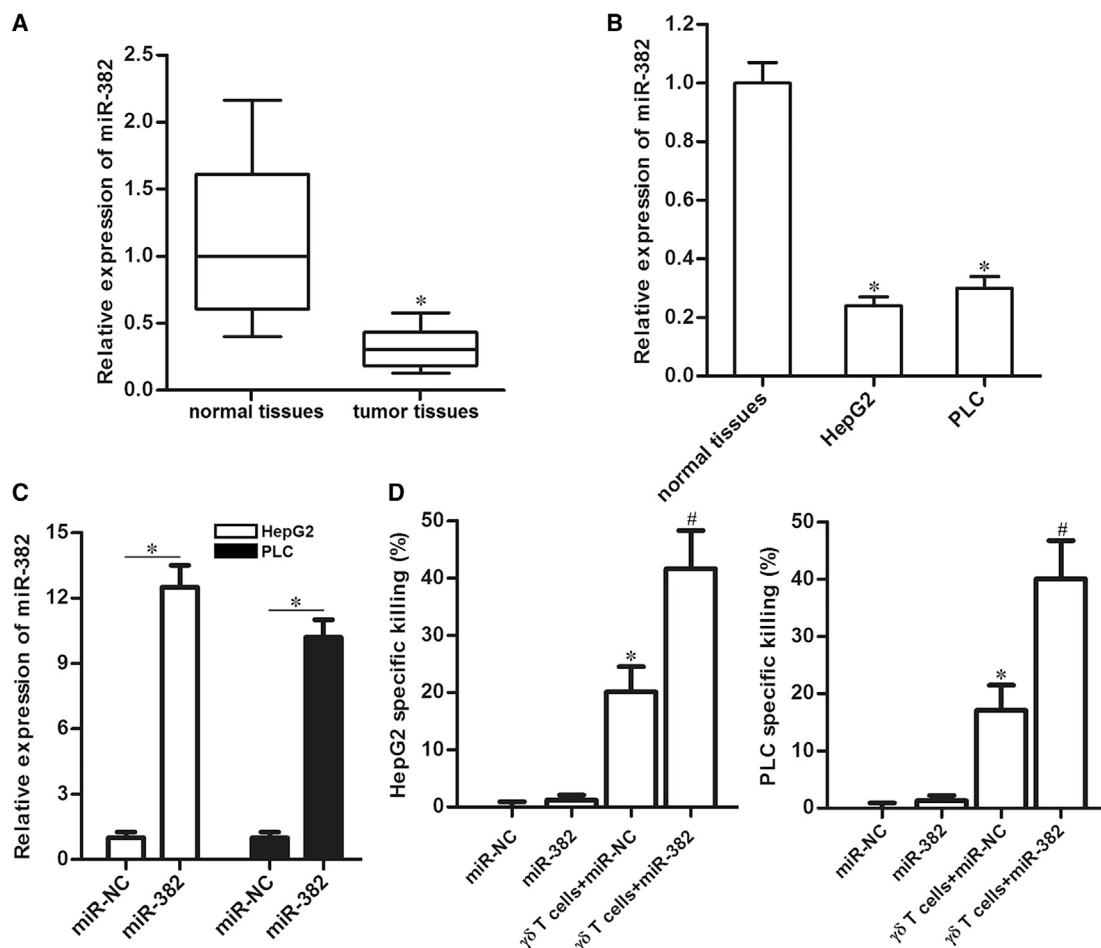


Figure 2. Transfection with miR-382 Increased the Sensitivity of HCC Cells to $\gamma\delta$ T Cell-Induced Cell Lysis

(A) Quantitative real-time PCR analysis was performed to detect expression of miR-382 in the tumors and precancerous tissues of patients with HCC. * $p < 0.05$. (B) Relative expression of miR-382 in precancerous tissues. HepG2 and PLC cell lines were measured by quantitative real-time PCR analysis. * $p < 0.05$ versus healthy tissues. (C) Transfection with miR-382 mimics increased expression of miR-382 in HepG2 and PLC cells. * $p < 0.05$. (D) After treatment with miR-382 and $\gamma\delta$ T cells (E:T = 2.5:1) in HepG2 and PLC cell lines, specific killing was measured with a ^{51}Cr release assay. * $p < 0.05$ versus miR-negative control (miR-NC) group. # $p < 0.05$ versus $\gamma\delta$ T cells + miR-NC group. All data are represented as means \pm SD from at least three independent experiments in each condition.

induced cell lysis was obviously abolished by overexpression of c-FLIP (Figure 5A). Those results indicated that a decrease of c-FLIP was essential in miR-382-promoted cell death. Because c-FLIP is the cellular inhibitor of caspase 8,²⁰ we next performed western blot analysis to evaluate the activation of caspase 8. As would be expected, miR-382 enhanced $\gamma\delta$ T cell-induced activation of caspase 8 in both HepG2 and PLC cells through decreasing the expression of c-FLIP (Figure 5B). Bid is the substrate of caspase 8. It can be cleaved by caspase 8 and then translocated from the cytosolic fraction to mitochondria as truncated Bid (tBid).²¹ Therefore, we separated the mitochondria from the HepG2 and PLC cells to detect the level of tBid. We found that co-treatment with miR-382 and $\gamma\delta$ T cells induced significant translocation of tBid to the mitochondria through the c-FLIP/caspase 8 pathway (Figure 5C). Because the translocation of tBid induced an opening in the mitochondrial permeability transition pore (mPTP),²¹ we found that a

combination of miR-382 and $\gamma\delta$ T cells induced release of cytochrome *c* and second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pl (Smac/DIABLO), which were the apoptotic inducers,²² from mitochondria into the cytoplasm. Therefore, caspase 9 and its substrate caspase 3 were significantly activated (Figure 5D). Taken together, we demonstrate that miR-382 promotes $\gamma\delta$ T cell-induced activation of caspases through decreasing the expression of c-FLIP.

MiR-382-Overexpressed HCC Model Is Sensitive to $\gamma\delta$ T Cell Treatment *In Vivo*

Because the introduction of miR-382 increased the cytotoxicity of $\gamma\delta$ T cells to HCC *in vitro*, we next investigated whether there was a similar effect of miR-382 seen for *in vivo* HCC models. After separating the xenografts from the tumor-bearing mice, we performed quantitative real-time PCR assays to detect the expression of miR-

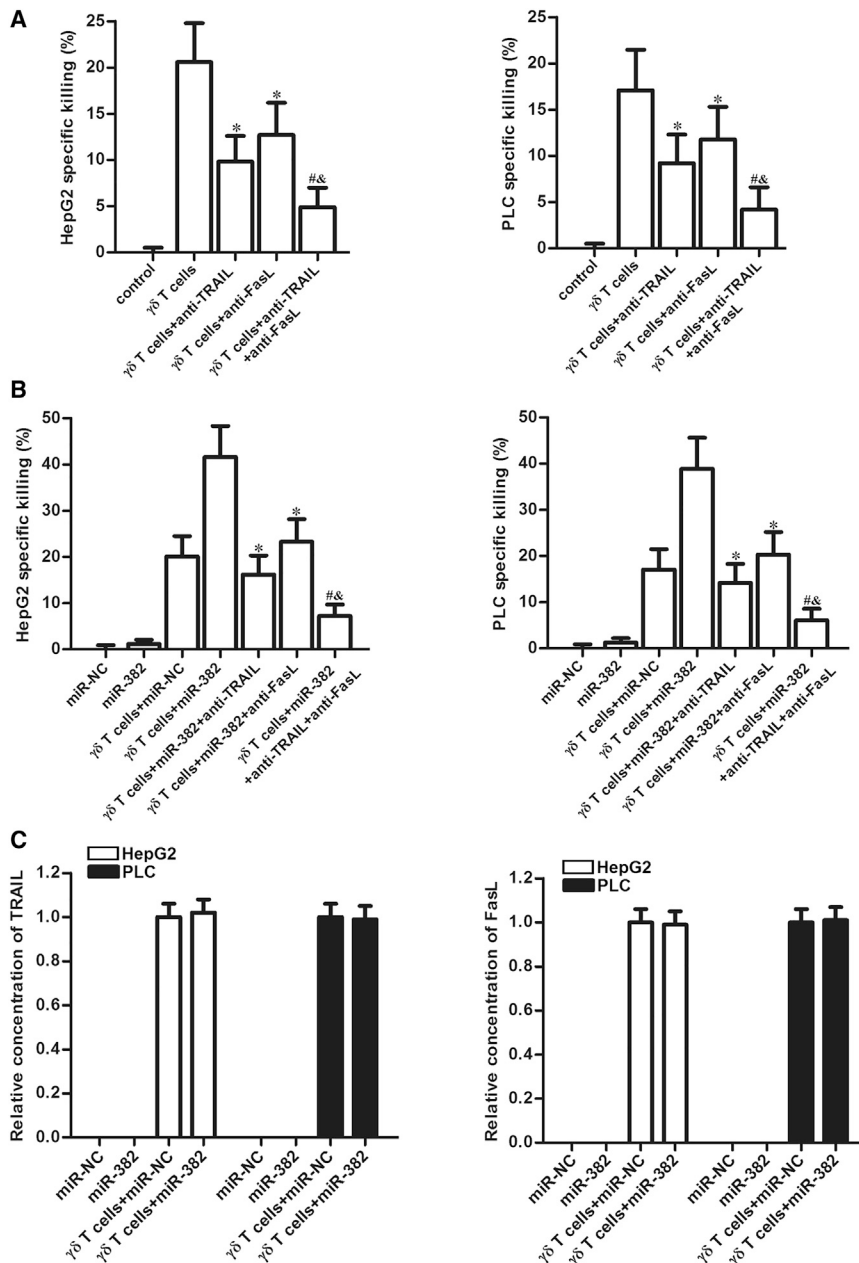


Figure 3. Role of TRAIL and FasL in Cell Lysis of HCC Cells Co-treated with miR-382 and $\gamma\delta$ T Cells

(A) TRAIL- and FasL-neutralizing antibodies inhibited the cytotoxicity of $\gamma\delta$ T cells (E:T = 2.5:1) to HepG2 and PLC cells. * $p < 0.05$ versus $\gamma\delta$ T cells group. # $p < 0.05$ versus $\gamma\delta$ T cells + anti-TRAIL group. ## $p < 0.05$ versus $\gamma\delta$ T cells + anti-FasL group. (B) Cell lysis of HCC cells co-treated with $\gamma\delta$ T cells (E:T = 2.5:1) and miR-382 was inhibited by TRAIL- and FasL-neutralizing antibodies. * $p < 0.05$ versus $\gamma\delta$ T cells + miR-382 group. # $p < 0.05$ versus $\gamma\delta$ T cells + miR-382 + anti-TRAIL group. ## $p < 0.05$ versus $\gamma\delta$ T cells + miR-382 + anti-FasL group. (C) miR-382 transfection in HCC cells didn't change the levels of TRAIL and FasL secreted by $\gamma\delta$ T cells (E:T = 2.5:1). All data are represented as means \pm SD from at least three independent experiments in each condition.

DISCUSSION

miRNAs have been reported to participate in carcinogenesis and cancer progression. Furthermore, dysregulation of miRNAs leads to drug resistance in cancers.^{23–25} Among these miRNAs, miR-382, which acts as a tumor suppressor, is reported to inhibit tumor growth and to sensitize cancer cells to anti-tumor drugs. For instance, overexpression of miR-382 inhibits cell proliferation and metastasis in prostate cancer.²⁶ In osteosarcoma, increased expression of miR-382 reduces the resistance against cisplatin, doxorubicin, and methotrexate.²⁷ However, the relationship between miR-382 and immunotherapy in cancers is still unclear.

c-FLIP belongs to the anti-apoptotic members of the Bcl-2 protein family. Studies demonstrate that c-FLIP has homology to caspase-8 but lacks its protease activity. Therefore, c-FLIP, a competitive inhibitor of caspase-8, suppresses the apoptotic pathway, which is dependent on caspase-8 signaling.^{20,28} In human cancer cells, c-FLIP is usually overexpressed. High levels of c-FLIP may protect cancer cells from apoptotic signaling. Therefore, reducing the expression of

c-FLIP in tumor cells is able to improve the sensitivity of those cells to drugs.^{29,30} In this study, we demonstrated that overexpression of c-FLIP in HCC can be abolished by miR-382 introduction. Furthermore, sensitivity of HCC cells to $\gamma\delta$ T cells was found to be significantly increased because of overexpression of miR-382. We proved that the miR-382/c-FLIP axis was associated with the efficiency of immunotherapy in HCC.

In the $\gamma\delta$ T cell-mediated cell lysis pathway, TRAIL and FasL, which are secreted by them, are essential because TRAIL and FasL directly trigger caspase-8 and thereby cause the translocation of tBid to the

382 in the tumors. As shown in Figure 6A, tumors transduced with LV-miR-382 expressed significantly higher levels of miR-382 compared with the LV control groups. In contrast, the miR-382-overexpressed tumors expressed lower protein level of c-FLIP compared with the control groups (Figure 6B). There exists a negative correlation between miR-382 and c-FLIP *in vivo*. In accordance with the *in vitro* results, we observed that miR-382-overexpressed HepG2 models were more sensitive to $\gamma\delta$ T cell treatment compared with the control HepG2 models (Figure 6C). These results indicate that overexpression of miR-382 was able to increase the anti-tumor effect of $\gamma\delta$ T cells in HCC *in vivo*.

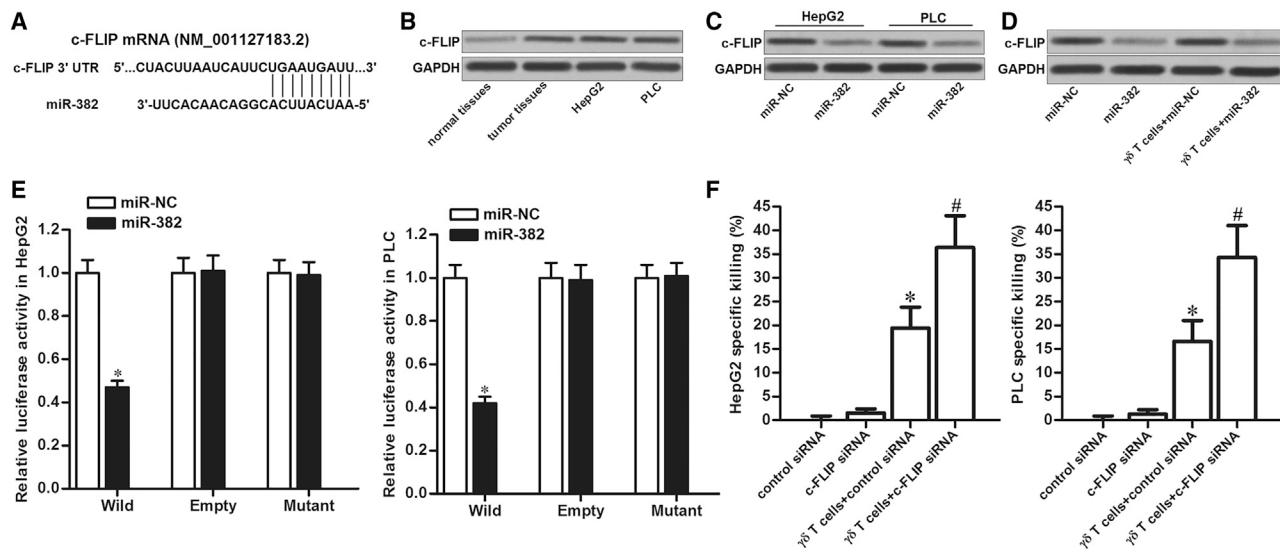


Figure 4. c-FLIP Is the Target of miR-382 in HCC

(A) Putative binding site of miR-382 in c-FLIP 3' UTR predicted by TargetScan database. (B) Western blot analysis was performed to detect the expression of c-FLIP in the tumors and precancerous tissues of patients HCC and HepG2 and PLC cell lines. (C) Overexpression of miR-382 suppressed the protein level of c-FLIP in HepG2 and PLC cells. (D) Expression levels of c-FLIP in LV-miR-382 and LV-control-transfected xenografts. (E) Relative LV activities of wild-type and mutant c-FLIP 3' UTR reporters in the presence of miR-382. * $p < 0.05$ versus miR-NC group. (F) After treatment with c-FLIP siRNA and $\gamma\delta$ T cells (E:T = 2.5:1) in HepG2 and PLC cell lines, specific killing was measured with a ^{51}Cr release assay. * $p < 0.05$ versus control siRNA group. # $p < 0.05$ versus $\gamma\delta$ T cells + control siRNA group. All data are represented as means \pm SD from at least three independent experiments in each condition.

mitochondria. Subsequently, the mitochondrial permeability transition pore is open, and then, cytochrome *c* and Smac/DIABLO, which are apoptotic inducers derived from the mitochondria, are released into the cytoplasm. Finally, caspase-9 and caspase-3 are cleaved, and apoptosis occurs in cancer cells.^{19,21,31,32} Consistent with these reports, our results emphasize the importance of TRAIL and FasL in $\gamma\delta$ T cell-mediated cell lysis in HCC. Moreover, we demonstrate that overexpression of miR-382 decreases the expression of c-FLIP and thereby promotes the activation of caspase-8 mediated by $\gamma\delta$ T cells. Downstream of caspase-8 activation, we observed significant mitochondrial apoptosis in HCC cells caused by co-treatment with $\gamma\delta$ T cells and miR-382 (Figure 7).

There were some limitations with this study. We used an immune-suppressed mice model. However, the immune microenvironment in HCC is highly complex. Although we observed that co-injection of $\gamma\delta$ T cells and the human HCC cell line at the time of xenograft implantation is efficient in the nude mice model, it is still unknown whether that strategy is efficient in humans. The mechanisms of co-injection of therapy and xenografts at the same time are also not fully understood. Further study with xenografts is needed before intratumoral or systemic injection of $\gamma\delta$ T cells can be used. Alternatively, an immune-competent model could be evaluated.

In summary, we provide strong evidence that miR-382 increases the sensitivity of HCC cells to $\gamma\delta$ T cell-mediated cytotoxicity. Moreover, we explored whether miR-382-promoted cell lysis is dependent on the c-FLIP/caspase-8 pathway. Further studies are, however, required to

explore whether this strategy can also be applied to other cancers and immunotherapy.

MATERIALS AND METHODS

$\gamma\delta$ T Cell Amplification

$\gamma\delta$ T cells were expanded *ex vivo* as described.³³ In brief, PBMCs were collected from blood samples of healthy donors ($n = 15$) by the density-gradient-separation method. Together, these PBMCs were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS) in the presence of 3 μM BrHPP (Innate Pharma, Marseille, France) and 400 IU interleukin-2 (IL-2)/mL (R&D Systems, Minneapolis, MN, USA) for 2 weeks. Subsequently, $\gamma\delta$ T cells were purified by negatively selecting with anti- $\alpha\beta$ -TCR antibody (BioLegend, San Diego, CA, USA) and MACS LD depletion columns (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the resulting $\gamma\delta$ T cells was identified by anti- $\gamma\delta$ -TCR-PE and anti-CD3-FITC antibodies on flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

Tumor Cell Lines

Human HCC cell lines HepG2 and PLC were purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 supplemented with 10% FCS. To generate stable miR-382 overexpression in the HCC cell model, we transfected the HepG2 cells with 5×10^5 units of recombinant LV that contained miR-382 precursor sequences (LV-miR-382) (GeneChem, Shanghai, China). Puromycin (1 $\mu\text{g}/\text{mL}$) was used for selecting culture of LV-transfected HepG2 cells.

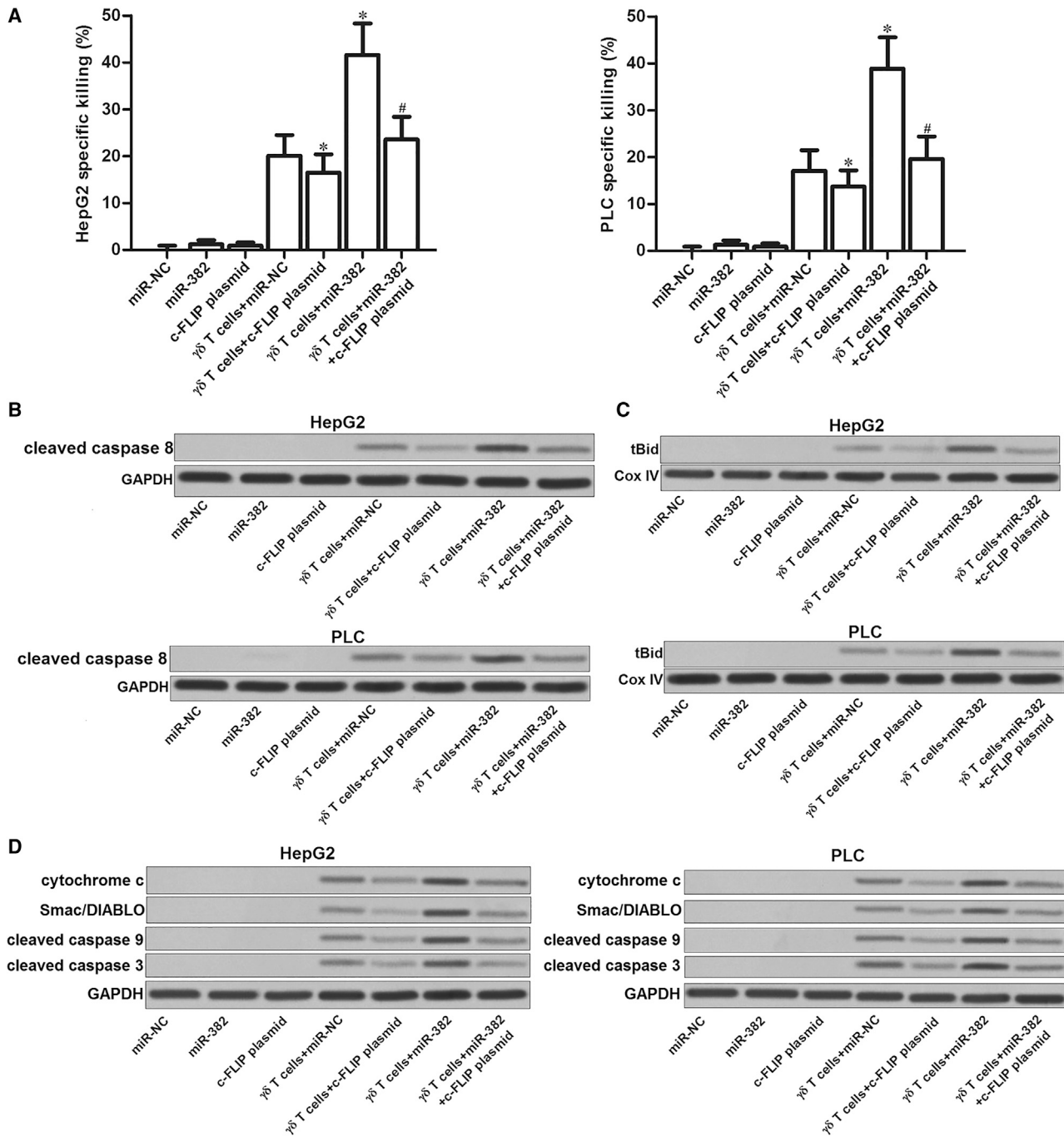


Figure 5. MiR-382 Promotes $\gamma\delta$ T Cell-Induced Activation of Caspases through the c-FLIP/Caspase 8 Pathway

(A) After treatment with miR-382, c-FLIP plasmid and $\gamma\delta$ T cells (E:T = 2.5:1) in HepG2 and PLC cell lines, their specific killing was measured with a ^{51}Cr release assay. * $p < 0.05$ versus $\gamma\delta$ T cells + miR-NC group. # $p < 0.05$ versus $\gamma\delta$ T cells + miR-382 group. (B) After treatment with miR-382, c-FLIP plasmid, and $\gamma\delta$ T cells (E:T = 2.5:1) in HepG2 and PLC cell lines, cleaved caspase 8 was detected by western blot analysis. (C) After separating the mitochondria from the HepG2 and PLC cells, the level of tBid in the mitochondria was detected by western blot analysis. (D) Levels of cytochrome c, Smac/DIABLO, cleaved caspase 9, and cleaved caspase 3 in the cytosolic fraction of HepG2 and PLC cells were detected by western blot analysis. All data are represented as means \pm SD from at least three independent experiments in each condition.

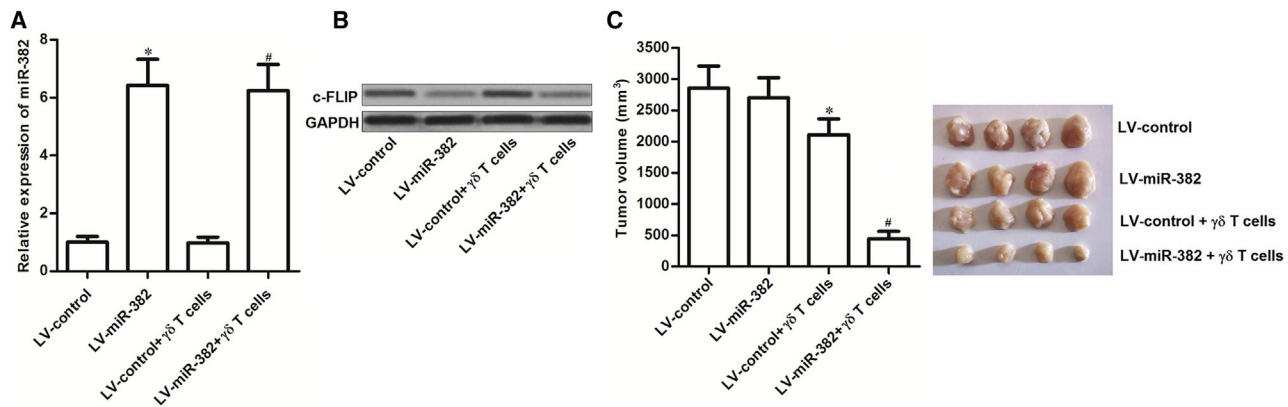


Figure 6. MiR-382-Overexpressed HCC Model Is Sensitive to $\gamma\delta$ T Cells Treatment *In Vivo*

(A) Relative expression of miR-382 in tumors transduced with LV-miR-382 or LV-control was measured by using quantitative real-time PCR analysis. * $p < 0.05$ versus LV-control group. # $p < 0.05$ versus LV-control + the $\gamma\delta$ T cell group. (B) Protein level of c-FLIP in mice tumor tissues was measured by western blot analysis. (C) After separating the xenografts from the tumor-bearing mice, tumor volume was measured. * $p < 0.05$ versus LV-control group. # $p < 0.05$ versus LV-control + the $\gamma\delta$ T cell group; 28 mice were used (seven mice were included in each group), and data are represented as means \pm SD.

Tissue Samples

Thirty-five HCC tumor tissues and their adjacent healthy tissues were collected from patients with primary HCC who underwent tumor resection in The Third People's Hospital of Hainan Province from December 2013 to May 2016. All HCC patients provided their informed consents for use of the clinical specimens for medical research. We obtained the approval of the ethics committee of The Third People's Hospital of Hainan Province for the use of the clinical samples.

Quantitative Real-Time PCR

Total RNAs were extracted from tissues samples and HCC cell lines (HepG and PLC) with Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Specific stem-loop RT primer and PrimeScript RT reagent Kit (Takara Bio, Kusatsu, Japan) were used for reverse transcription of miR-382. The sequence of the mentioned RT primer was as follows: 5'-CTCAACTGGTGTCTGGAGT CGGCAATT CAGTTGAGAAGTGTTG-3'. After reverse transcription, real-time PCR was performed to detect the expression of miR-382 with SYBR Premix Ex Taq (Takara) on an ABI PRISM 7900 Sequence Detection System (Thermo Fisher Scientific). Expression of U6 small nuclear RNA (snRNA) was used as internal control to normalize the expression level of miR-382.

Plasmid and Transfection

The open reading frame of c-FLIP was amplified and ligated into pcDNA3.1 plasmid (Thermo Fisher Scientific). c-FLIP and control siRNA were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). For transfection, c-FLIP plasmid (2 μ g/mL), miR-382 (5'-AAUCAUUCACGGACAACACUU-3', 50 pmol/mL, GeneChem), negative control oligonucleotide (miR-NC, 5'-CGACAACAUG AUAUAACUCUC-3', 50 pmol/mL, GeneChem), c-FLIP, and control siRNA (50 pmol/mL) were transfected into the HCC cell lines by using lipofectamine 2000 (Thermo Fisher Scientific), according to the manufacturer's protocol.

Luciferase Reporter Assay

Fragments of c-FLIP 3' UTR containing the binding sites of miR-382 were amplified and ligated into pMIR-REPORT Luciferase vector (Thermo Fisher Scientific). For the luciferase reporter assay, HepG2 and PLC cells were co-transfected with miR-382, pMIR-REPORT Luciferase vector and Renilla luciferase-thymidine kinase (pRL-TK) vector (Promega, Madison, WI, USA). After 48 h of incubation, cells were lysed, and the firefly and Renilla luciferase activities were measured with the Dual-Luciferase Reporter System Kit (Promega), according to the manufacturer's protocol.

Cytotoxicity Assay

$\gamma\delta$ T cells expanded *ex vivo* were tested for cytotoxicity against HepG2 and PLC cells. Their cell lysis was measured with a ⁵¹Cr release assay. In brief, HepG2 or PLC cells were labeled with ⁵¹Cr sodium chromate. Subsequently, the labeled cells were seeded into 96-well plates (2 \times 10³ cells per well) and co-cultured with $\gamma\delta$ T cells for 4 h. Released ⁵¹Cr in culture supernatants was measured with a Top-Count gamma counter (Packard Instrument, Meriden, CT, USA). Specific killing of target cells was calculated with the standard formula: $\{[\text{counts per minute (cpm)}]_{\text{experimental}} - \text{cpm}_{\text{spontaneous}}\} / (\text{cpm}_{\text{maximum}} - \text{cpm}_{\text{spontaneous}}) \times 100\%$. For blocking assays, targeted HepG2 or PLC cells were co-cultured with $\gamma\delta$ T cells in the presence of TRAIL- and FasL-neutralizing antibodies (R&D Systems).

TRAIL and FasL ELISA

HepG2 or PLC cells were co-cultured with $\gamma\delta$ T cells at an E:T ratio of 2.5:1. After 4 h co-culture, supernatants were collected, and the production of TRAIL and FasL was determined with TRAIL and FasL ELISA kits (R&D Systems) according to the manufacturer's protocol.

Mitochondria Isolation

To evaluate the release of cytochrome *c* and Smac/DIABLO from mitochondria into the cytoplasm of targeted HepG2 and PLC cells,

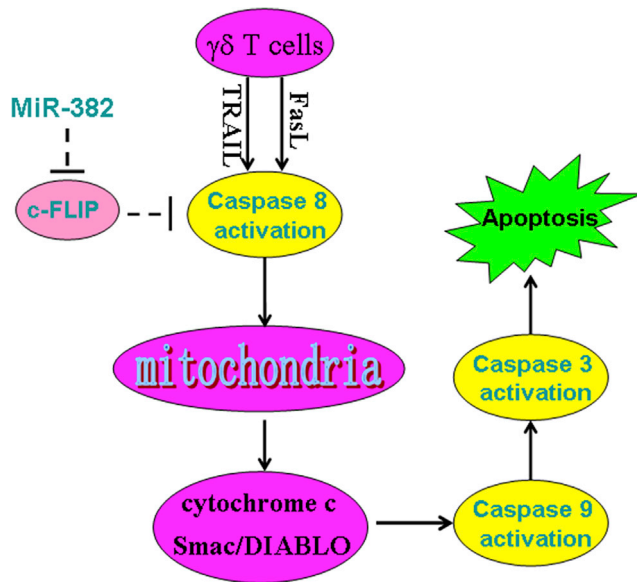


Figure 7. Overexpression of miR-382 Inhibited Expression of c-FLIP, thus Enhancing the $\gamma\delta$ T Cell-Induced Apoptosis through the Mitochondrial Pathway

we isolated the mitochondria using Mitochondria/Cytosol Fraction Kit (BioVision Technologies, Exton, PA, USA), according to the manufacturer's protocol.

Western Blot Analysis

Proteins extracted from the mitochondria or cytoplasm were analyzed by western blot assays. c-FLIP, cleaved caspase-8, Bid, Cox IV, cytochrome c, Smac/DIABLO, cleaved caspase-9, cleaved caspase-3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were used to detect their expression levels.

Xenografts of the Tumor Cells

Nude mice (4 weeks old) were purchased from Shanghai Super-B&K Laboratory Animal Corporation (Shanghai, China), and 2×10^6 LV-miR-382-transfected HepG2 (or LV-control-transfected HepG2) and 6×10^7 $\gamma\delta$ T cells were subcutaneously co-injected. After 28 days, tumor tissues were harvested to measure their size. Tumor volumes (V) were calculated based on the formula $V = [(length \times width^2)/2]$. The animal care and experimental protocols were approved by the Animal Care Committee of The Third People's Hospital of Hainan Province.

Statistical Analysis

Values were expressed as means \pm SE and were analyzed by one-way analysis of variance (ANOVA). $p < 0.05$ was considered statistically significant.

Ethical Approval

We obtained the approval of the ethics committee of The Third People's Hospital of Hainan Province for the use of clinical samples.

Consent for Publication

All authors read, approved the content, and agreed to submit this material for publication in the journal.

Availability of Data and Material

The datasets used or analyzed during the current study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

B.H., Z.C., Z.Z., and L.F. contributed to the study design; Z.H., L.H., M.F., Q.C., Y.K., and J.Y. contributed to data and statistical analyses; B.H. contributed to manuscript preparation and manuscript editing.

CONFLICTS OF INTEREST

The authors declare no competing interests

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