Overexpression of miR-382 sensitizes hepatocellular carcinoma cells to $\gamma\delta$ T cells through inhibiting the expression of c-FLIP

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ABSTRACT

Immunotherapy is an important strategy for cancer treatment. Recently, human $\gamma\delta$ T lymphocytes are reported to display anti-tumor effect against multiple cancers, including hepatocellular carcinoma (HCC). However, resistance to $\gamma\delta$ T cells is a major obstacle in immunotherapy of HCC. Studies have demonstrated that microRNAs (miRNAs) are usually dysregulated in cancer cells. Aberrant expression of miRNAs leads to low response to immunotherapy. In the present study, 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. Mechanically, we proved that mRNA of 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 cells treatment. These data strongly demonstrates that 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 due to the late diagnosis and poor prognosis [1]. Surgery and liver transplantation treatment are the most effective treatment for early stage HCC patients [2, 3]. However, many patients were diagnosed as advanced HCC at their first visit. For these patients, the cancer is unresectable, and the chemotherapy and immunotherapy are considered as the alternative option [4, 5]. However, HCC cells are usually resistant to these anti-tumor drugs [6, 7]. Drug-resistance has become a major obstacle for 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 MHC unrestricted manner [8-10].

Strategies using $\gamma\delta$ T cells have been evaluated in clinical trials for cancer treatment. However, as $\gamma\delta$ 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 nucleotides in length. MiRNAs functions as gene regulators, because they can partially pair with the complementary sequences of mRNA at the region of 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, epithelial-mesenchymal transition. In addition, studies 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 present 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 effect 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 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 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 cells 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 level of miR-382 compared to the paracancerous normal tissues (Figure 2A). In addition, we also observed that relative expression of miR-382 in HepG2 and PLC cells was significantly lower than that in the HCC patients' paracancerous normal 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

effect of miR-382 on $\gamma\delta$ T cells-induced cell death. As shown in Figure 2C, transfection with miR-382 caused 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 cells-induced cell lysis (Figure 2D). We demonstrate that overexpression of miR-382 sensitizes HCC cells to $\gamma\delta$ T cells treatment.

Specific killing of HCC cells co-treated with $\gamma\delta$ T cells and miR-382 is dependent on TRAIL and FasL signaling

TRAIL and 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 this, we observed significant reduction of cell lysis in $\gamma\delta$ T cells-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 cells-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 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 cells-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, TargetScan public database (www.targetscan.org) was used to search for the putative target of miR-382 in human cells. As shown in Figure 4A, c-FLIP was predicted as the potential target

Figure 1: Cytotoxicity of *ex vivo* expanded $\gamma\delta$ T cells against HCC cell lines. (A) *Ex vivo* expanded $\gamma\delta$ T cells from total 15 donors were identified by flow cytometry. (B) Specific killing of HepG2 and PLC induced by $\gamma\delta$ T cells was measured by using ^{51}Cr release assay. Data were represented as the mean $\pm$ SD from at least three independent experiments in each condition.

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 to the normal tissues. On the contrary, 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, 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 LV-miR-382 transfected xenografts expressed significantly lower level of c-FLIP compared

to 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 wildtype 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 cells-induced cytotoxicity against HCC, we knockdown c-FLIP by using its specific siRNA in HepG2 and PLC cells before they were co-cultured with $\gamma\delta$ T cells. Similarly with the miR-382, we showed that transfection with c-FLIP siRNA was able to enhance the $\gamma\delta$ T cells-induced cell death in these HCC cells (Figure 4F). We emphasized the importance of c-FLIP in resistance to $\gamma\delta$ T cells treatment.

Figure 2: Transfection with miR-382 increased the sensitivity of HCC cells to $\gamma\delta$ T cells-induced cell lysis. (A) QRT-PCR analysis was performed to detect the expression of miR-382 in HCC patients' tumor and paracancerous tissues. * $p < 0.05$. (B) Relative expression of miR-382 in paracancerous tissues, HepG2 and PLC cell lines was measured by using qRT-PCR analysis. * $p < 0.05$ vs. normal tissues. (C) Transfection with miR-382 mimics increased the 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, specific killing of them was measured by using ^{51}Cr release assay. * $p < 0.05$ vs. miR-NC group. # $p < 0.05$ vs. $\gamma\delta$ T cells + miR-NC group. All data were represented as the mean $\pm$ SD from at least three independent experiments in each condition.

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 vs. $\gamma\delta$ T cells group. # p <0.05 vs. $\gamma\delta$ T cells + anti-TRAIL group. & p <0.05 vs. $\gamma\delta$ 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 vs. $\gamma\delta$ T cells + miR-382 group. # p <0.05 vs. $\gamma\delta$ T cells + miR-382 + anti-TRAIL group. & p <0.05 vs. $\gamma\delta$ 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 were represented as the mean $\pm$ SD from at least three independent experiments in each condition.

MiR-382 promotes $\gamma\delta$ T cells-induced activation of caspases through decreasing the expression of c-FLIP

To investigate whether decrease of c-FLIP was essential in miR-382-promoted cell death, we introduced the HepG2 and PLC cells with 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 cells-induced cell lysis was obviously abolished by overexpression of c-FLIP (Figure 5A). It indicated that decrease of c-FLIP was essential in miR-382-promoted cell death. As 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 cells-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 the form of truncated Bid (tBid) [21]. We therefore 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 mitochondria through the c-FLIP/caspase 8 pathway (Figure 5C). Since the translocation of tBid induced opening of mitochondrial permeability transition pore (mPTP) [21], we found that combination with miR-382 and $\gamma\delta$ T cells induced release of cytochrome c and Smac/DIABLO, which

were the apoptotic inducer [22], from mitochondria into the cytoplasm. As the results, caspase 9 and its substrate caspase 3 were significantly activated (Figure 5D). Taken together, we demonstrate that miR-382 promotes $\gamma\delta$ T cells-induced activation of caspases through decreasing the expression of c-FLIP.

MiR-382-overexpressed HCC model is sensitive to $\gamma\delta$ T cells treatment *in vivo*

As introduction with miR-382 increased the cytotoxicity of $\gamma\delta$ T cells to HCC *in vitro*, we next investigated whether similar effect of miR-382 can be seen *in vivo* HCC models. After separating the xenografts from the tumor-bearing mice, we performed qRT-PCR assays to detect the expression of miR-382 in them. As shown in Figure 6A, tumors transduced with lentivirus-miR-382 (LV-miR-382) expressed significantly higher levels of miR-382 compared to the LV-control groups. In contrast, the miR-382-overexpressed tumors expressed lower protein level of c-FLIP compared to the control groups (Figure 6B). There exists 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 cells treatment compared to the control HepG2 models (Figure 6C). These results indicated that overexpression of miR-382 was able to increase the anti-tumor effect of $\gamma\delta$ T cells on 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 HCC patients' tumor and paracancerous tissues, 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 luciferase activities of wildtype and mutant c-FLIP 3' UTR reporters in the presence of miR-382. * $p < 0.05$ vs. miR-NC group. (F) After treatment with c-FLIP siRNA and $\gamma\delta$ T cells (E:T=2.5:1) in HepG2 and PLC, specific killing of them was measured by using ^{51}Cr release assay. * $p < 0.05$ vs. control siRNA group. # $p < 0.05$ vs. $\gamma\delta$ T cells + control siRNA group. All data were represented as the mean $\pm$ 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 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

Figure 5: MiR-382 promotes $\gamma\delta$ T cells-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, specific killing of them was measured by using ^{51}Cr release assay. * $p<0.05$ vs. $\gamma\delta$ T cells + miR-NC group. # $p<0.05$ vs. $\gamma\delta$ 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, cleaved caspase 8 was detected by using western blot analysis. (C) After separating the mitochondria from the HepG2 and PLC cells, the level of tBid on mitochondria was detected by western blot analysis. (D) Levels of cytochrome c, Smac/DIABLO, cleaved caspase 9 and cleaved caspase 3 in cytosolic fraction of HepG2 and PLC cells were detected by using western blot analysis. All data were represented as the mean $\pm$ 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 qRT-PCR analysis. * $p < 0.05$ vs. LV-control group. # $p < 0.05$ vs. LV-control + $\gamma\delta$ T cells 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. * $p < 0.05$ vs. LV-control group. # $p < 0.05$ vs. LV-control + $\gamma\delta$ T cells group. 28 mice were used (7 mice were included in each group) and data were represented as the mean $\pm$ SD.

Figure 7: Overexpression of miR-382 inhibited expression of c-FLIP, and thus enhancing $\gamma\delta$ T cells-induced apoptosis through the mitochondrial pathway.

methotrexate [27]. However, relationship between miR-382 and immunotherapy in cancers is still unclear.

C-FLIP belongs to the anti-apoptotic members in Bcl-2 protein family. Studies demonstrate that c-FLIP has homology to caspase-8 but lacks of its protease activity. Therefore, c-FLIP, a competitive inhibitor of caspase-8, suppresses the apoptotic pathway which is dependent on the caspase-8 signaling [20, 28]. In human cancer cells, c-FLIP is usually overexpressed. High level of c-FLIP may protect cancer cells from the apoptotic signaling exists in circumstance. Therefore, reducing the expression of c-FLIP in tumor cells is able to improve the sensitivity of them 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 the overexpression of miR-382. We proved that miR-382/c-FLIP axis was associated with efficiency of immunotherapy on HCC.

In pathway of $\gamma\delta$ T cells-mediated cell lysis, TRAIL and FasL secreted by them are essential. It's because TRAIL and FasL directly trigger caspase-8 and thereby causing translocation of tBid to mitochondria. Subsequently, the mitochondrial permeability transition pore is open and then cytochrome c and Smac/DIABLO which are apoptotic inducers derived from mitochondria will be released into 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 emphasized the importance of TRAIL and FasL in $\gamma\delta$ T cells-mediated cell lysis in HCC. Moreover, we demonstrated that overexpression of miR-382 decreased the expression of c-FLIP and thereby promoting activation of caspase-8 mediated by $\gamma\delta$ T cells. As the downstream of caspase-8 activation, we observed significant mitochondrial apoptosis in HCC cells caused by the co-treatment with $\gamma\delta$ T cells and miR-382 (Figure 7).

In summary, we provide strong evidence that miR-382 increases sensitivity of HCC cells to $\gamma\delta$ T cells-mediated cytotoxicity. Moreover, we explore that miR-382-promoted cell lysis is dependent on c-FLIP/caspase-8 pathway. Nevertheless, further studies are required to explore whether this strategy can also apply to other cancers and other immunotherapy.

MATERIALS AND METHODS

$\gamma\delta$ T cells amplification

$\gamma\delta$ T cells were expanded *ex vivo* as described [33]. In brief, peripheral blood mononuclear cells (PBMCs) were collected from blood samples of healthy donors (n=15) by using density gradient separation method. Together, these PBMC were cultured in RPMI 1640 supplemented with 10% FCS in presence of 3 μ M BrHPP (Innate Pharma, France) and 400 IU IL-2/mL (R&D SYSTEM,

USA) for 2 weeks. Subsequently, $\gamma\delta$ T cells were purified by negatively selecting with anti- $\alpha\beta$ -TCR antibody (BioLegend, USA) and MACS LD depletion columns (Miltenyi Biotec, Germany). The purity of resulting $\gamma\delta$ T cells was identified by using anti- $\gamma\delta$ -TCR-PE and anti-CD3-FITC antibodies on flow cytometry (Becton Dickinson, 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 HCC cell model, we transfected the HepG2 cells with 5 $\times$ 10⁵ units of recombinant lentivirus contained miR-382 precursor sequences (LV-miR-382) (Genechem Corporation, Shanghai, China). Puromycin (1 μ g/ml) was used for selecting culture of lentivirus transfected HepG2 cells.

Tissue samples

A total of 35 HCC tumor tissues and their adjacent normal tissues were collected from primary HCC patients who underwent tumor resection in The third people's Hospital of Hainan Province from 12/2013 to 5/2016. All HCC patients have given their informed consent on the use of 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 clinical samples.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs were extracted from tissues samples and HCC cell lines (HepG and PLC) by using Trizol reagent (Invitrogen, USA). Specific stem-loop RT primer and PrimeScript RT reagent Kit (TaKaRa, Japan) were used for reverse transcription of miR-382. The sequence of the mentioned RT primer was as follows: 5'-CTCAACTGGTGTCTGGAGT CGGCAATTCAAGTTGAGAAGTGTTG-3'. After reverse transcription, real-time PCR was performed to detect the expression of miR-382 by using the SYBR Premix Ex Taq (TaKaRa) on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, USA). Expression of U6 snRNA was used as internal control to normalize the expression level of miR-382.

Plasmid and transfection

Open reading frame of c-FLIP was amplified and ligated into pcDNA3.1 plasmid (Invitrogen). c-FLIP and control siRNA were purchased

from Santa Cruze Biotechnology (USA). For transfection, c-FLIP plasmid (2 µg/ml), miR-382 (5'-AAUCAUUCACGGACAACACUU-3', 50 pmol/ml, Genechem Corporation), negative control oligonucleotide (miR-NC, 5'-CGACAACAUGAUUAACUCUC-3', 50 pmol/ml, Genechem Corporation), c-FLIP and control siRNA (50 pmol/ml) were transfected into HCC cell lines by using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Luciferase reporter assay

Fragment of c-FLIP 3' UTR contained the binding sites of miR-382 was amplified and ligated into pMIR-REPORT Luciferase vector (Applied Biosystems). For luciferase reporter assay, HepG2 and PLC cells were co-transfected with miR-382, pMIR-REPORT Luciferase vector and Renilla luciferase pRL-TK vector (Promega, USA). After 48 h incubation, cells were lysed and the Firefly and Renilla luciferase activities were measured by using Dual-Luciferase Reporter System Kit (Promega) according to the manufacturer's protocol.

Cytotoxicity assay

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

TRAIL and FasL ELISA

HepG2 or PLC cells were co-cultured with γδT cells at an E:T (γδT effector : targeted HCC cells) ratios of 2.5:1. After 4 h co-culture, supernatants were collected, and the production of TRAIL and FasL was determined by using TRAIL and FasL ELISA kits (R&D SYSTEM) according to the manufacturer's protocol.

Mitochondria isolation

To evaluate the release of cytochrome c and Smac/DIABLO from mitochondria into cytoplasm of targeted HepG2 and PLC cells, we isolated the mitochondria by using Mitochondria/Cytosol Fraction Kit (BioVision, USA) according to the manufacturer's protocol.

Western blot analysis. Proteins extracted from mitochondria or cytoplasm were analyzed by using western blot assays. C-FLIP, cleaved caspase-8, Bid, Cox IV, cytochrome c, Smac/DIABLO, cleaved caspase-9, cleaved caspase-3 and 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). 2×10⁶ LV-miR-382 transfected HepG2 (or LV-control transfected HepG2) and 6×10⁷ γδT cells were subcutaneously co-injected. 28 days later, tumor tissues were harvested to measure the size of them. Tumor volumes (V) were calculated based on the formula $V = \text{length} \times (\text{width}^2) / 2$. 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 mean ± SE and analyzed by using One-way analysis of variance (ANOVA). $P < 0.05$ was considered to be statistically significant.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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