Knockdown of miR-25 increases the sensitivity of liver cancer stem cells to TRAIL-induced apoptosis via PTEN/PI3K/Akt/Bad signaling pathway

XIAONING FENG[1-3*], JINGJIN JIANG[4*], SHAOHUA SHI[1-3], HAIYANG XIE[1-3],
LIN ZHOU[1-3] and SHUSEN ZHENG[1-3]

[1]Key Laboratory of Combined Multi-Organ Transplantation, Ministry of Public Health and
Key Laboratory of Organ Transplantation of Zhejiang Province; [2]Collaborative Innovation Center for Diagnosis and
Treatment of Infectious Diseases; [3]Division of Hepatobiliary and Pancreatic Surgery, Department of Surgery,
The First Affiliated Hospital, School of Medicine, Zhejiang University; [4]Department of VIP,
The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang 310003, P.R. China

Abstract. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is considered as a promising anticancer agent, it induced apoptosis in cancer cells selectively without damaging the normal tissue cells. However, the resistance of cancer cells to TRAIL limits its application. Since the cancer stem cells (CSCs) are believed to be responsible for the treatment failure in multiple cancers including hepatocellular carcinoma (HCC), the aim of this study was to investigate the strategies to increase the sensitivity of liver cancer stem cells (LCSCs) to TRAIL. In the present study, we observed significant upregulation of miR-25 in LCSCs compared with the non-CSCs. Furthermore, we found that knockdown of miR-25 by its antisense oligonucleotide (anti-miR-25) significantly increased the sensitivity of LCSCs to TRAIL-induced apoptosis. The gene of phosphatase and tensin homologue (PTEN), which is a natural inhibitor of PI3K, was found to be directly regulated by miR-25 in HepG2-CSCs. We demonstrated that knockdown of miR-25 increased the expression of PTEN. Mechanistically, inhibition of Bad phosphorylation, which is regulated by the PTEN/PI3K/AKT pathway, is essential for the functional roles of anti-miR-25 in HepG2-CSCs. In conclusion, our findings indicate that overexpression of miR-25 is associated with the low-sensitivity to TRAIL in LCSCs. Knockdown of miR-25 may represent a potential strategy for increasing the efficacy of TRAIL by targeting the PTEN/PI3K/Akt/Bad signaling pathway.

Introduction

Hepatocellular carcinoma (HCC) represents the fifth most commonly diagnosed malignancy and the third leading cause of cancer-related death worldwide (1). Due to the extensive research efforts, understanding of the molecular mechanisms involved in the tumorigenic transformation of HCC has been clarified gradually. However, the molecular targets for HCC therapy remain insufficient (2). Recent studies suggest that human cancer can be considered as a stem cell disorder. According to these studies, tumors may be viewed as abnormal growth which is driven by a small population of cancer cells called cancer stem cells (CSCs) that are endowed with the ability of self-renewal (3,4).

Previous studies have identified the CD133+ phenotype as the molecular marker of liver cancer stem cells (LCSCs) (5). The LCSCs were found to show a greater colony-forming efficiency and greater ability to form a tumor *in vivo*. In addition, the LCSCs are believed to be responsible for the high resistance to chemotherapeutic drugs (6). Therefore, to treat the HCC more effectively, it is imperative that LCSCs are specifically targeted.

Tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) is a cytokine, which belongs to the TNF superfamily (7). As the TRAIL triggers apoptosis selectively in tumor cells without damaging the normal tissue cells, it has been tested in clinical trials for cancer therapy (8). However, unfortunately, some cancer cells (especially CSCs) are resistant to TRAIL and the mechanism of this resistance is not fully understood (9). Therefore, it is urgent to identify the mechanisms and take strategies to decrease the resistance of CSCs to TRAIL.

MicroRNAs (miRNAs), small functional RNAs of 19-25 nt, are a class of short non-coding RNA molecules and play

Correspondence to: Dr Shusen Zheng, Division of Hepatobiliary and Pancreatic Surgery, Department of Surgery, The First Affiliated Hospital, School of Medicine, Zhejiang University, 79 Qingchun Road, Hangzhou, Zhejiang 310003, P.R. China
E-mail: hangzhoussz@163.com

*Contributed equally

important regulatory roles by binding to the target mRNAs at the 3'-untranslated region (3'-UTR) (10,11). Increasing evidence shows that miRNAs have significant roles in a number of biological processes such as proliferation, differentiation, metabolism and apoptosis. Aberrant expression of miRNAs is associated with multiple human diseases including cancer (12,13). In the present study, we found the miR-25 was dysregulated in LCSCs. We therefore investigated the relationship between the miR-25 and the treatment of TRAIL in LCSCs, which may serve as a potential target for HCC therapy.

Materials and methods

Animals. Nude mice (BALB/c, nu/nu; 4-5 week old; 18-23 g in weight) were obtained from the Shanghai Laboratory Animal Center (Shanghai, China). The animals were kept in ventilated, pathogen-free racks under a 12 h light-dark photoperiodicity with controlled humidity (50-60%) and temperature (22-24°C). The animals were kept with free access to food and water. Animal care and the experimental procedures in this study were approved by the Animal Care Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University and complied with the recommendations of the Chinese guidelines for the care and use of laboratory animals.

Cell lines. The HCC cells lines of HepG2, Huh7, PLC and the normal liver cell line of L-O2 were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM basic medium containing 10% fetal bovine serum (FBS) (both from Gibco, Grand Island, NY, USA) at 37°C in a humidified 5% CO_2 incubator. To evaluate the role of anti-miR-25 in HCC *in vivo*, the stable HepG2 cell line with miR-25 knockdown was generated. Briefly, we purchased the recombinant lentivirus contained miR-25 antisense nucleotide sequence from the Shanghai Genechem Co., Ltd. (Shanghai, China). The routine HepG2 cells were then transfected with $5x10^5$ transducing units of lentivirus, and the cells were selected with 1 μg/ml puromycin for 2 weeks.

Measurement of miR-25 expression level. The expression level of miR-25 was measured by reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR). Briefly, total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The reverse transcription reaction for miR-25 was performed by using the One Step PrimeScript miRNA cDNA synthesis kit (Takara, Dalian, China). Subsequently, the qRT-PCR reaction was performed by SYBR® Premix Ex Taq™ II (Takara). The expression of miR-25 was determined using the $2^{-\Delta\Delta CT}$ analysis method (14) taking the U6 snRNA as the internal control.

Identification and sorting of LCSCs and non-CSCs. HCC cells were collected and washed twice. Cell samples were then stained with anti-CD133-FITC (Miltenyi Biotec, Gladbach, Germany) for 20 min at room temperature. The percentages of CD133+ cells were analyzed and sorted as the LCSCs using the fluorescent-activated cell sorting equipment (Beckman Coulter, Brea, CA, USA).

Transfection. Cell-based experiments were carried out by transfection of 50 pmol/ml RNA oligonucleotides including miR-25 mimics, miR-25 antisense-oligonucleotides (anti-miR-25), control RNA oligonucleotides (miR-NC) and small interfering RNA of phosphatase and tensin homologue (PTEN) (PTEN siRNA). All of these RNAs were purchased from RiboBio Co., Ltd. (Guangzhou, China) and transfected into the cells using Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's instructions.

Cell viability assay. The sorted LCSCs and non-CSCs were seeded in 96-well plates at $5x10^3$ cells/well and transfected with 50 pmol/ml RNA oligonucleotides. Twenty-four hours post-transfection, cells were incubated in different concentrations of TRAIL for 48 h. Subsequently, 20 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to the medium and incubated at 37°C for 4 h followed by lysing in dimethyl sulfoxide at room temperature for 30 min. The absorbance in each well was measured at 570 nm using a microplate reader (Sunrise Microplate Reader; Tecan, Männedorf, Switzerland).

Luciferase reporter assay. Fragment of PTEN 3'-UTR containing the seed region of miR-25 was cloned into the pGL3 Luciferase Reporter Vectors (Promega, Madison, WI, USA) named pGL3-PTEN. The mutant PTEN reporter was created by mutating the seed regions of the miR-25 binding sites (GUGCAAU) by using the site-directed mutagenesis kit (Takara, Shiga, Japan) named pGL3-mutant PTEN. For luciferase reporter assay, LCSCs were seeded in 48-well plates followed by co-transfection with 50 pmol/ml miR-25 mimics or inhibitors together with 2 μg/ml Firefly luciferase reporters and 100 ng/ml *Renilla* luciferase pRL-TK vector (Promega). Luciferase activity was measured 48 h after transfection by using the Dual-Luciferase Reporter assay system (Promega) according to the manufacturer's instructions.

Mitochondria isolation. For detecting the release of cytochrome *c*, second mitochondria derived activator of caspase/direct IAP binding protein with low pI (Smac/DIABLO) and apoptosis-inducing factor (AIF) in cytoplasm, mitochondria of LCSCs were isolated using the Mitochondria Isolation kit for Mammalian Cells (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's guidance.

Immunoprecipitation. Cells were lysed in RIPA buffer and centrifuged at 12,000 x g for 10 min. The resulting supernatants were collected and incubated with primary antibody of Bad (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4°C. Subsequently, the protein A agarose beads were added and incubated for 2 h. After washing the beads with cold RIPA buffer, the co-precipitated proteins were removed from the beads by boiling in sodium dodecyl sulfate (SDS) sample buffer.

Western blot analysis. Fifty micrograms of the extracted and the co-precipitated proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The membrane was then blocked with 5% non-fat milk
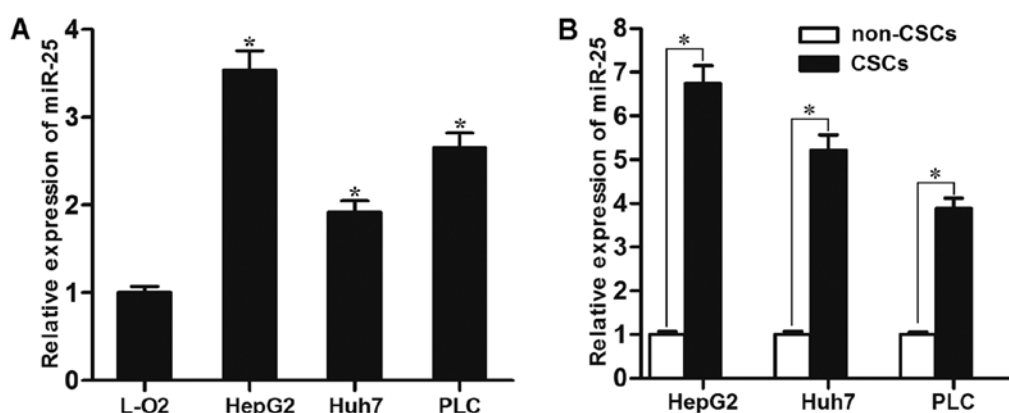
Figure 1. miR-25 is overexpressed in liver cancer stem cells (LCSCs). (A) miR-25 expression levels were examined by quantitative real-time polymerase chain reaction (qRT-PCR) in normal liver cells (L-O2, as control group) and three hepatocellular carcinoma (HCC) cell lines (HepG2, Huh7 and PLC). *P<0.05 vs. control group. (B) The LCSC population was sorted as CD133+ cells, and the CD133-/low cells were sorted as the non-cancer stem cells (non-CSCs) in HCC cell lines. *P<0.05.

and incubated with various antibodies followed by incubation of horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Inc.). Signals were detected using enhanced chemiluminescence (ECL) reagents (Thermo Fisher Scientific) according to the manufacturer's guidance.

Detection of apoptosis and reactive oxygen species (ROS). LCSCs were treated with RNAs and TRAIL. Then, the cells were stained with PI and Annexin V (Sigma-Aldrich) for apoptosis detection and dihydroethidium (DHE; Molecular Probes, Camarillo, CA, USA) for measurement of ROS. Both apoptosis and ROS were detected on the flow cytometry according to the manufacturer's guidance, respectively.

Tumor growth in nude mice. An equal number (5×10^6) of HepG2 cells transfected with lentivirus anti-miR-25 or lentivirus control were harvested and washed. The experimental animals were divided into four groups (8 mice/group). For xenografts, two groups of mice were subcutaneously injected with lentivirus control HepG2 cells (for lenti control group and TRAIL group), and two groups of mice were subcutaneously injected with lentivirus anti-miR-25 HepG2 cells (for anti-miR-25 group and TRAIL plus anti-miR-25 group). The tumor volume (V) was calculated based on the following equation: $1/2 \times length \times width^2$. The mice in TRAIL group and TRAIL plus anti-miR-25 group received intraperitoneal (i.p.) injections of 40 µg/kg TRAIL per two days when the tumors reached a mean volume of 100 mm³ (15). The tumor formation was monitored every three days using calipers. Nude mice were euthanized at the experimental end-point (31 days post-injection).

To measure the population of CSCs in tumor tissues, we purified the cells from the tumor. Briefly, the tumor xenografts were cut and digested with 1 mg/ml of collagenase type III (5 ml/g tissue; Worthington Biochemical, Lakewood, NJ, USA) in 10% FBS containing DMEM medium at 37˚C for 2 h. The resulting cells were then stained with anti-CD133-FITC for 20 min before analyzing on the FACS vantage.

Statistical analysis. The data are presented as the mean ± standard deviation and carried out by at least three independent experiments. Statistical analysis was performed by Student's t-test using SPSS 15.0 software. Values of P<0.05 were considered statistically significant.

Results

miR-25 is overexpressed in LCSCs. To investigate the role of miR-25 in HCC, its expression levels between normal liver cell line and HCC cell lines were compared by qRT-PCR. The expression of miR-25 was significantly higher in HCC cell lines than that in the L-O2 cells (Fig. 1A). We further evaluated the expression of miR-25 in the CSCs of HepG2, Huh7 and PLC, as well as in the non-CSCs of these cell lines. As shown in Fig. 1B, miR-25 was upregulated overall in all tested HCC cell lines. Taken together, these results suggested that the upregulation of miR-25 may be involved in LCSCs.

miR-25 is associated with the sensitivity of LCSCs to TRAIL in vitro. In order to evaluate the sensitivity of LCSCs and non-CSCs to TRAIL, we sorted them in HepG2 cell line using the anti-CD133 antibody. Subsequently, the HepG2-CSCs and non-CSCs were treated with different concentrations of TRAIL. The sensitivity to TRAIL was significantly lower in HepG2-CSCs than that in the non-CSCs (Fig. 2A). The results suggest the high resistance of LCSCs to TRAIL. To evaluate the role of miR-25 in LCSCs, we transfected the HepG2-CSCs and non-CSCs with the miR-25 mimic or inhibitors. Transfection of miR-25 mimics significantly upregulated the expression level of miR-25 in both the HepG2-CSCs and non-CSCs, whereas the expression of miR-25 was significantly downregulated due to the transfection of anti-miR-25 (Fig. 2B). Interestingly, the flow cytometric analysis showed that the percentage of CD133+ HepG2-CSCs population was increased after the single treatment of TRAIL. On the other hand, although changing the expression of miR-25 did not influence the percentage of HepG2-CSCs population significantly, the anti-miR-25 significantly inhibited the effect of TRAIL on enriching the HepG2-CSCs population, whereas the miR-25 mimics even increased the enrichment of HepG2-CSCs population induced by TRAIL (Fig. 2C and D). We consider that the treatment of TRAIL alone probably killed the TRAIL-sensitive non-CSCs,
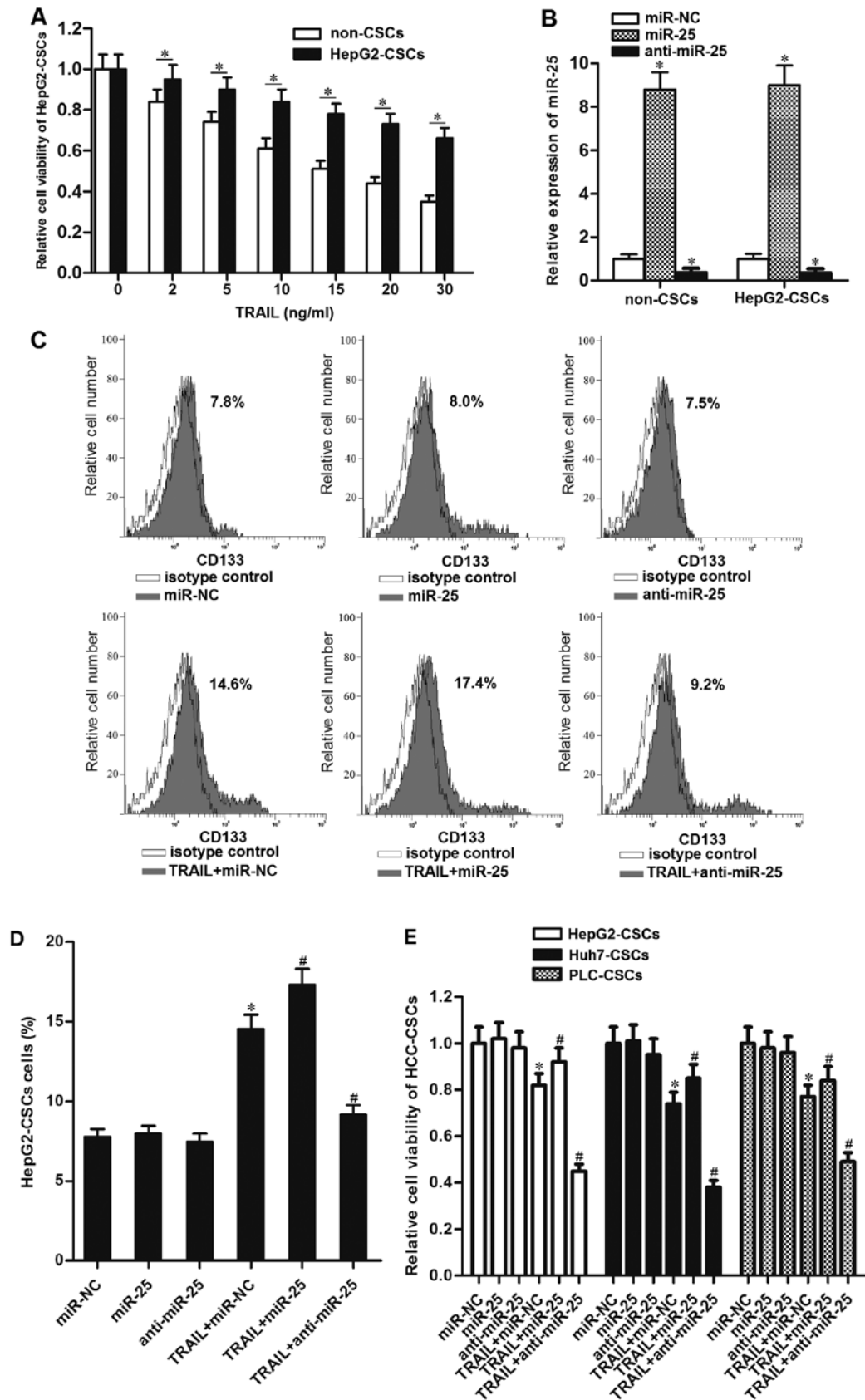
Figure 2. miR-25 is associated with the sensitivity of liver cancer stem cells (LCSCs) to tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) *in vitro*. (A) HepG2-CSCs and non-CSCs were sorted and subsequently treated with different concentrations of TRAIL. The sensitivity of these cells was evaluated by MTT assay. $^{*}P<0.05$. (B) After transfection with miR-NC, miR-25 and anti-miR-25, the expression of miR-25 in the HepG2-CSCs and non-CSCs were monitored using qRT-PCR. $^{*}P<0.05$ vs. miR-NC group. (C) The effect of miR-25, anti-miR-25 and TRAIL (10 ng/ml) on changing the percentage of the HepG2-CSCs population was evaluated by flow cytometry using CD133 antibody. (D) miR-25 inhibitors inhibited the effect of TRAIL on enriching the HepG2-CSCs population in HepG2 cell line. $^{*}P<0.05$ vs. miR-NC group; $^{#}P<0.05$ vs. TRAIL+miR-NC group. (E) After treatment with miR-25, anti-miR-25 and TRAIL (10 ng/ml), the cell viability of HepG2-CSCs, Huh7-CSCs and PLC-CSCs were measured by MTT assay. $^{*}P<0.05$ vs. miR-NC group; $^{#}P<0.05$ vs. TRAIL+miR-NC group.
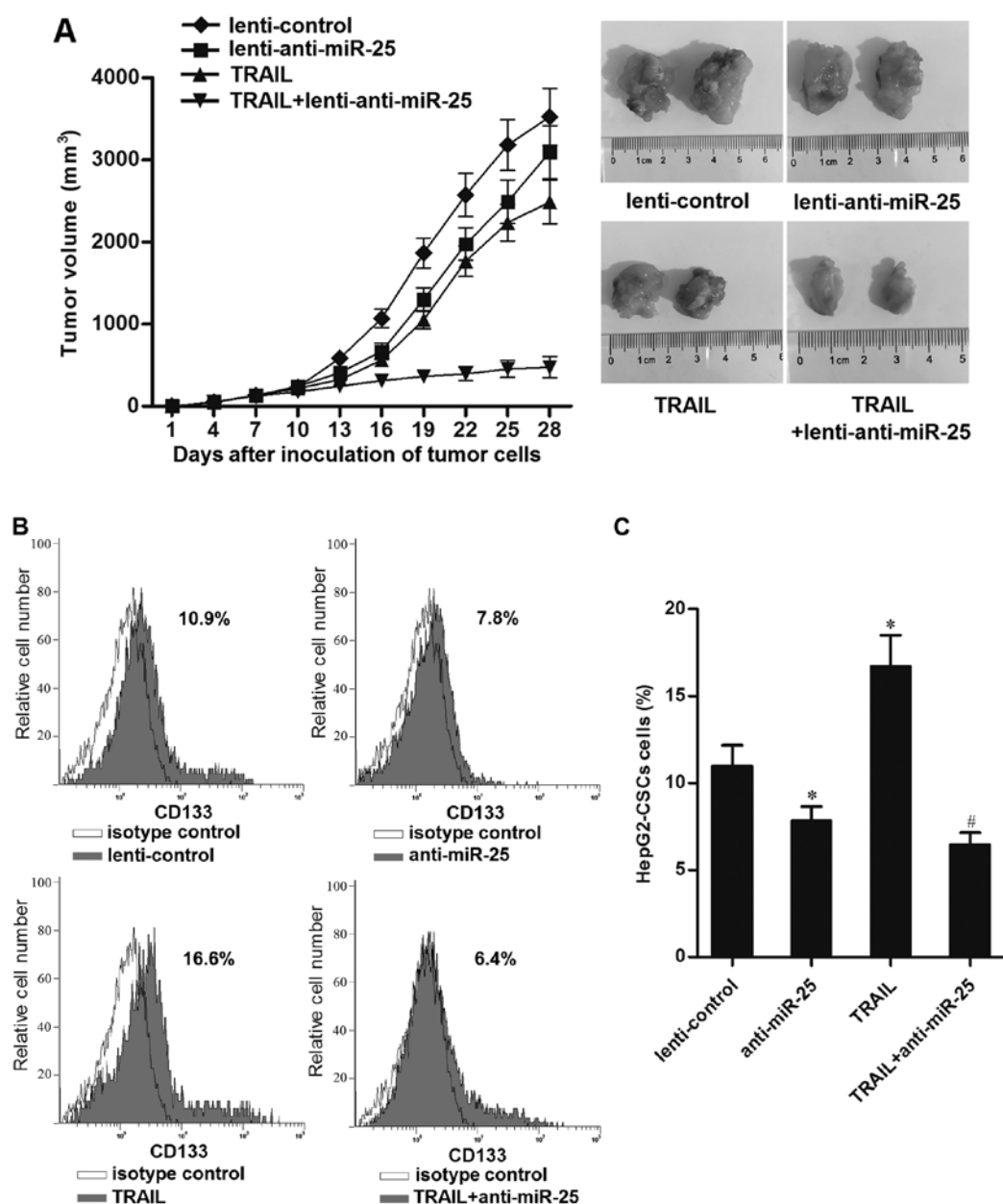
Figure 3. Knockdown of miR-25 increases the antitumor effect of tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) on hepatocellular carcinoma (HCC) *in vivo*. (A) Volumes of HepG2 tumors *in vivo* were detected every three days with or without anti-miR-25 transfection and TRAIL treatment. (B) The population of CSCs in tumor cells *in vivo* was detected by flow cytometry. (C) Knockdown of miR-25 inhibited the effect of TRAIL on enriching the HepG2-CSCs population *in vivo*. *P<0.05 vs. lenti-control group; #P<0.05 vs. TRAIL group.

whereas the TRAIL-resistant HepG2-CSCs survived under the TRAIL treatment. To demonstrate the effect of miR-25 on the sensitivity of LCSCs to TRAIL, we next performed a cell viability assay. The HCC-CSCs (HepG2-CSCs, Huh7-CSCs and PLC-CSCs) transfected with anti-miR-25 showed significantly higher sensitivity to TRAIL than those in the miR-NC group (Fig. 2E). However, the cells transfected with miR-25 mimics showed the opposite effect on the sensitivity of TRAIL compared with those in the miR-NC groups. Taken together, these results indicated that knockdown of miR-25 could increase the sensitivity of LCSCs to TRAIL-induced cell death *in vitro*.

Knockdown of miR-25 increases the antitumor effect of TRAIL on HCC in vivo. To investigate the effect of miR-25 inhibitors

on TRAIL treatment *in vivo*, a total of 5x10^{6} of HepG2 cells transfected with lentivirus anti-miR-25 or lentivirus control were inoculated to the nude mice. Then, the tumor volumes were calculated every three days. The results showed that the growth of HepG2 cells transfected with lentivirus anti-miR-25 was significantly slower than the cells transfected with lentivirus control when they were treated with equal dose of TRAIL *in vivo* (Fig. 3A). Furthermore, the results of flow cytometry indicated that the percentage of CSC population in lentivirus control tumor tissues was significantly higher than the lentivirus anti-miR-25 tumor tissues when the mice were treated with equal dose of TRAIL *in vivo* (Fig. 3B and C). Taken together, these results indicated that knockdown of miR-25 could enhance the curative effect of TRIAL and increase the sensitivity of LCSCs to TRAIL *in vivo*.
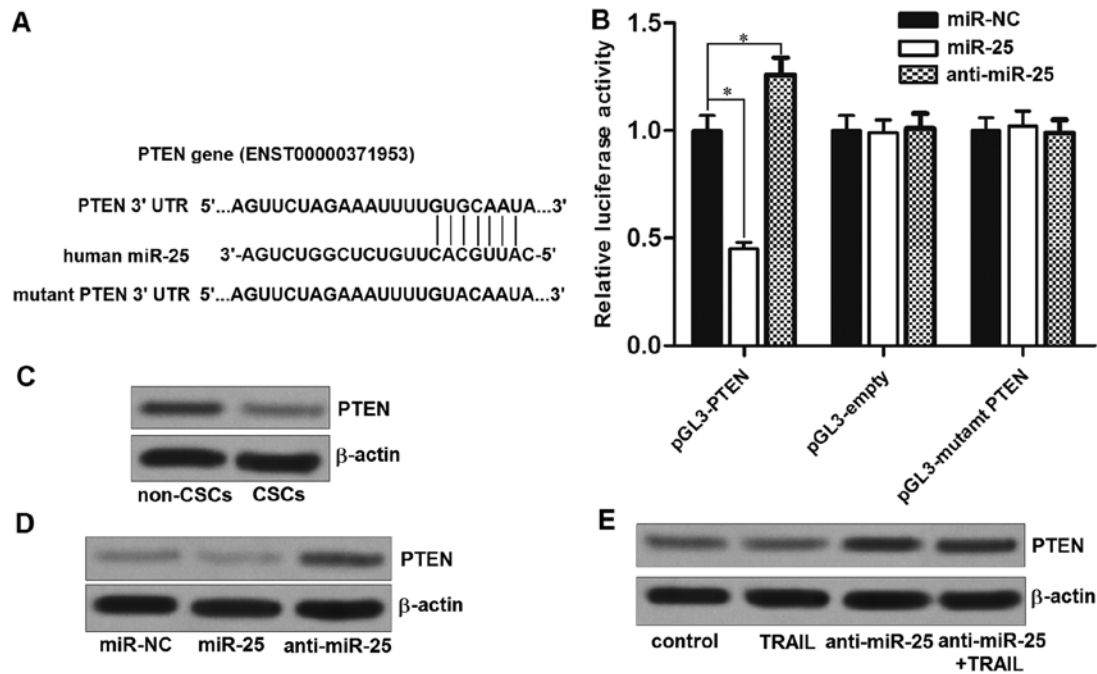
Figure 4. Phosphatase and tensin homologue (PTEN) is the target of miR-25 in HepG2-CSCs. (A) The putative miR-25 binding sites on 3'-untranslated region (3'-UTR) of PTEN mRNA was predicted by the TargetScan, miRanda and PicTar databases. (B) After transfection, the luciferase activity was detected using dual-luciferase reporter assay system according to the manufacturer's instruction. Each value is evaluated by the relative luciferase activity of Firefly to *Renilla*. *P<0.05. (C) The expression of PTEN in HepG2-CSCs and non-CSCs was evaluated by western blot analysis. (D) The effect of miR-25 and anti-miR-25 on regulating the expression of PTEN in HepG2-CSCs. (E) Knockdown of miR-25 increased the expression of PTEN HepG2 cells *in vivo*.

PTEN is the target of miR-25 in HepG2-CSCs. To understand how miR-25 regulated the sensitivity of TRAIL to LCSCs, three different public databases (TargetScan, miRanda and PicTar) were used. All of these databases showed a prediction that a highly conserved sequence in the 3'-UTR of the PTEN mRNA was targeted by miR-25 (Fig. 4A). To investigate whether miR-25 directly targets the PTEN mRNA, we cloned the PTEN 3'-UTR sequence into the pGL3 luciferase reporter vectors. The results of dual luciferase reporter assays showed that co-transfection of miR-25 mimics and luciferase reporter with wild-type of PTEN 3'-UTR significantly decreased the luciferase activities, whereas the anti-miR-25 increased the luciferase activities. Moreover, the mutant or empty plasmids exhibited no response to miR-25 or anti-miR-25 (Fig. 4B). These results suggest that miR-25 directly regulates the PTEN in HepG2-CSCs. Consistent with this, we found the expression level of PTEN was significantly lower in HepG2-CSCs than that in the corresponding non-CSCs (Fig. 4C), because the expression of miR-25 is overexpressed in HepG2-CSCs. To confirm that miR-25 regulates the expression of PTEN, the PTEN protein was measured after the miR-25 or anti-miR-25 was transfected into HepG2-CSCs. Transfection of miR-25 decreased the expression of PTEN (Fig. 4D). Whereas, the transfection of anti-miR-25 increased the expression of PTEN. In addition, we detected the expression of PTEN in the lenti-control tumor tissues (mice in control group and TRAIL group) and lenti anti-miR-25 tumor tissues (mice in anti-miR-25 group and anti-miR-25 plus TRAIL group) *in vivo*. We found that the protein levels of PTEN in miR-25 knockdown tumor cells were significantly increased compared with the control tumor cells (Fig. 4E). Taken together, we demonstrated that the PTEN gene is a functional target of miR-25 in HCC.

Knockdown of miR-25 increases the sensitivity of HepG2-CSCs to TRAIL through PTEN pathway. To investigate the role of PTEN in the anti-miR-25 promoted cell death in HepG2-CSCs, we knocked down the expression of PTEN by its specific siRNA, and the transfection efficiency of PTEN siRNA is shown in Fig. 5A. We observed that although the anti-miR-25 significantly enhanced the cytotoxicity of TRAIL to HepG2-CSCs, the promotion of anti-miR-25 was significantly inhibited when the PTEN siRNA was introduced (Fig. 5B). Furthermore, as the anti-miR-25 inhibited the enrichment of HepG2-CSC population induced by TRAIL, transfection of PTEN siRNA abolished the effect of anti-miR-25 in HepG2 cells (Fig. 5C and D). Taken together, these results indicate that the anti-miR-25 increases the sensitivity of HepG2-CSCs to TRAIL by upregulating the expression of PTEN.

Knockdown of miR-25 increases the sensitivity of HepG2-CSCs to TRAIL through apoptosis pathway. We observed that although the anti-miR-25 did not induce apoptosis of HepG2-CSCs directly, it significantly enhanced the effect of TRAIL on inducing the cell death through the apoptosis pathway. In addition, the transfection of PTEN siRNA rescued the HepG2-CSCs from apoptosis induced by the combination with TRAIL and anti-miR-25 (Fig. 6A). Furthermore, we found knockdown of miR-25 promoted the activation of caspase-9 and caspase-3 induced by the TRAIL treatment in the HepG2-CSCs. Moreover, the transfection of PTEN siRNA inhibited the cleavage of these caspases induced by the combination with TRAIL and anti-miR-25 (Fig. 6B). These results indicate that knockdown of miR-25 in HepG2-CSCs enhanced the TRAIL-induced apoptosis through the PTEN pathway.
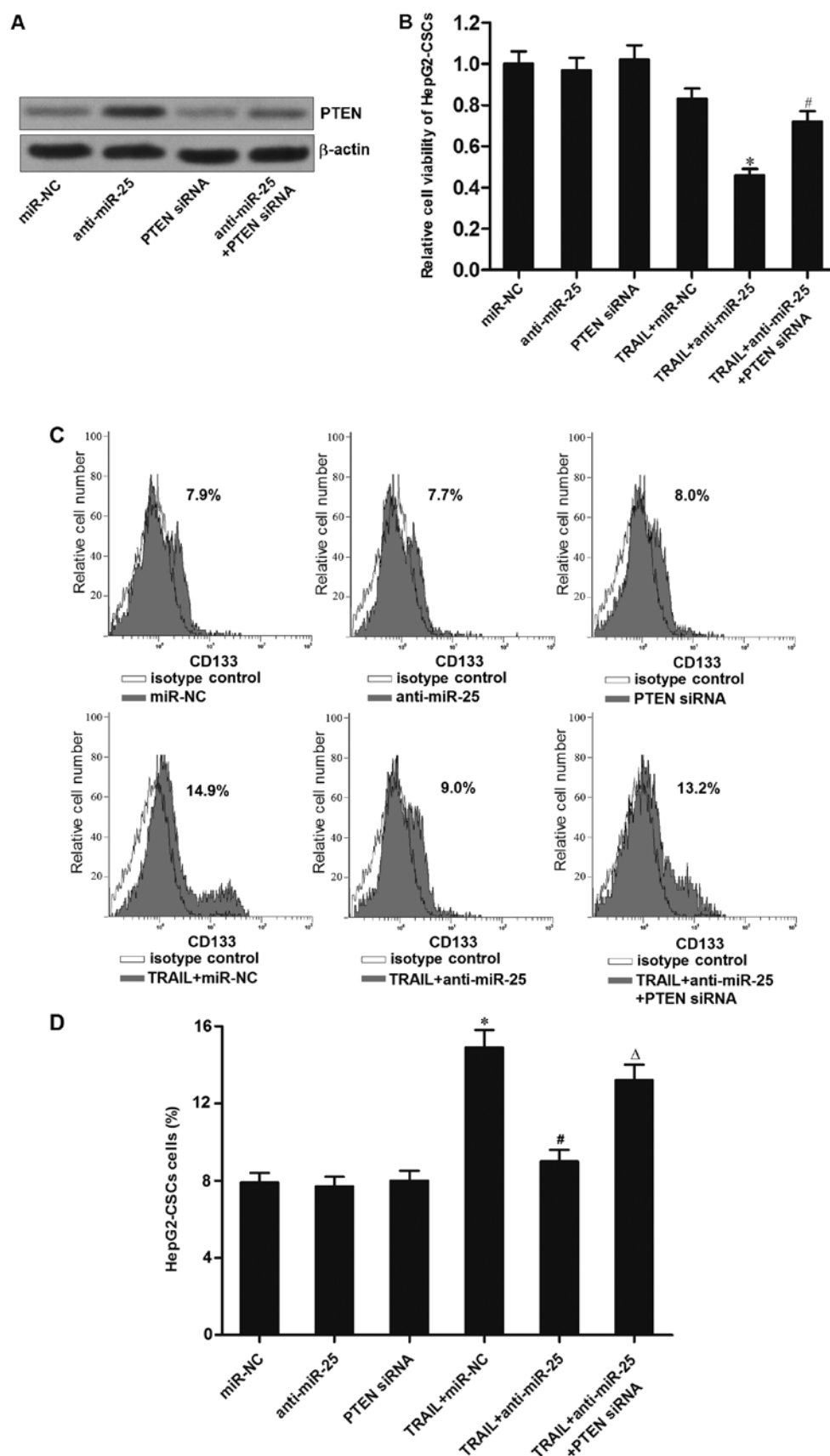
Figure 5. Phosphatase and tensin homologue (PTEN) pathway is essential to the promotion of anti-miR-25 on the tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL)-induced cell death in HepG2-CSCs. (A) The upregulation of PTEN induced by anti-miR-25 was inhibited by PTEN siRNA in HepG2-CSCs. (B) The promotion of anti-miR-25 on the TRAIL (10 ng/ml)-induced cell death was inhibited by the PTEN siRNA in HepG2-CSCs. *P<0.05 vs. TRAIL+miR-NC group; #P<0.05 vs. TRAIL + anti-miR-25 group. (C) The effect of anti-miR-25, TRAIL and PTEN siRNA on changing the percentage of the HepG2-CSCs population was evaluated by flow cytometry using CD133 antibody. (D) The inhibition of anti-miR-25 on the TRAIL (10 ng/ml)-induced enrichment of HepG2-CSCs population was abolished by the PTEN siRNA in HepG2 cells. *P<0.05 vs. TRAIL + miR-NC group; #P<0.05 vs. TRAIL + anti-miR-25 group.
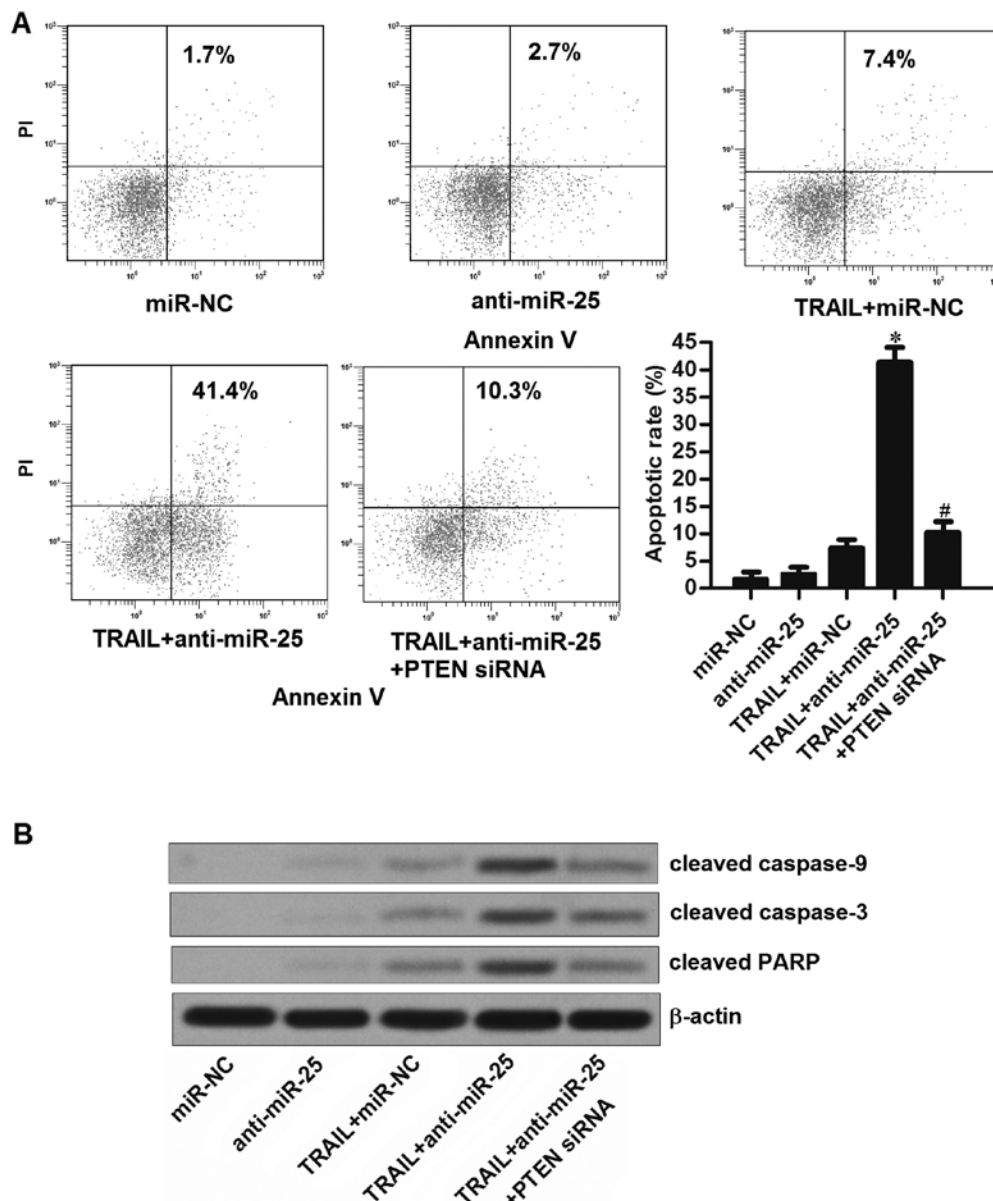
Figure 6. Knockdown of miR-25 in HepG2-CSCs enhanced the tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL)-induced apoptosis through the phosphatase and tensin homologue (PTEN) pathway. (A) The anti-miR-25 enhanced the TRAIL (10 ng/ml)-induced apoptosis in HepG2-CSCs, which could be inhibited by the transfection of PTEN siRNA. *P<0.05 vs. TRAIL + miR-NC group; #P<0.05 vs. TRAIL + anti-miR-25 group. (B) The cleavage of caspase-9, caspase-3 and its substrate of PARP was evaluated by western blot analysis after the HepG2-CSCs were treated with anti-miR-25, TRAIL and PTEN siRNA.

Knockdown of miR-25 enhances the TRAIL-induced apoptosis via PTEN/PI3K/Akt/Bad signaling pathway. Since the PTEN is a natural inhibitor of PI3K and negatively regulates the Akt (16), we next investigated the relationship between the anti-miR-25 and the PTEN/PI3K/Akt signaling. Transfection of anti-miR-25 inhibited the phosphorylation of PI3K and Akt (Fig. 7A). In contrast, the single TRAIL treatment did not alter the activity of PI3K or Akt. Previous studies have demonstrated that Bad is one of the substrates for Akt (17). As we observed, the anti-miR-25 induced significant inhibition of Bad phosphorylation, which may be the molecular mechanism by which the anti-miR-25 promotes the apoptosis in HepG2-CSCs (18). The dephosphorylation of Bad induced by the anti-miR-25, knockdown of miR-25 significantly increased the Bad-Bcl-2 heterodimer and the Bad-Bcl-xL heterodimer in HepG2-CSCs (Fig. 7B), subsequently inactivating the anti-apoptotic proteins of Bcl-2 and Bcl-xL. Studies have demonstrated that the inactivation of Bcl-2 and Bcl-xL promotes the release of mitochondria-derived apoptogenic compounds (19). We therefore detected the cytochrome *c*, Smac/DIABLO, and AIF in the cytoplasm of HepG2-CSCs. The levels of all of these apoptogenic compounds were significantly increased in the cytoplasm after the HepG2-CSCs were co-treated with TRAIL and anti-miR-25 (Fig. 7C). In addition, ROS which is an important apoptosis inducer (20) was also generated after the combination treatment with TRAIL and anti-miR-25 (Fig. 7D). Taken together, our data strongly suggests that the anti-miR-25 promotes the TRAIL-induced apoptosis which is regulated by the PTEN/PI3K/Akt/Bad signaling pathway.
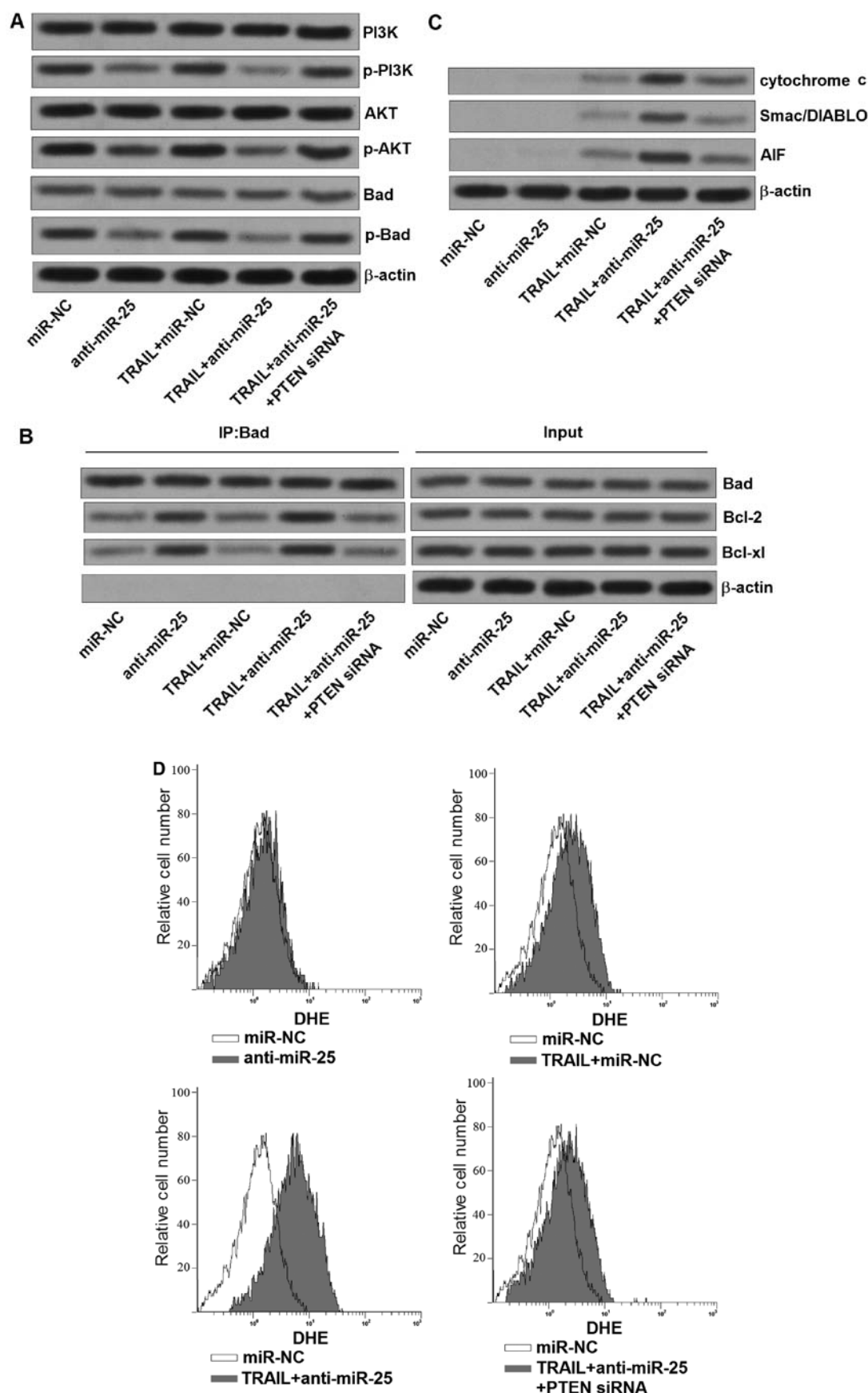
Figure 7. Knockdown of miR-25 enhances the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-induced apoptosis via phosphatase and tensin homologue (PTEN)/PI3K/Akt/Bad signaling pathway. (A) Western blot analysis was performed to evaluate the phosphorylation of PI3K, Akt and Bad in HepG2-CSCs treated with anti-miR-25, TRAIL (10 ng/ml), and PTEN siRNA. (B) Co-immunoprecipitation was performed to detect the Bad-Bcl-2 heterodimer and the Bad-Bcl-xL heterodimer in HepG2-CSCs treated with anti-miR-25, TRAIL and PTEN siRNA. (C) After the mitochondria were removed, the proteins of cytochrome c, Smac/DIABLO and AIF in the cytoplasm of HepG2-CSCs were detected by western blot analysis. (D) The generation of reactive oxygen species (ROS) in HepG2-CSCs was measured by dihydroethidium (DHE) staining.

Discussion

Previous studies have demonstrated that miRNAs are associated with the sensitivity of cancer cells to the antitumor treatment. For instance, overexpression of miR-122 in HCC could reverse the resistance to doxorubicin by inhibiting the glucose metabolism of cancer cells (21). Knockdown of miR-221 could enhance the TRAIL-induced apoptosis by upregulating the expression of BIM gene in breast cancer (22). The tumor suppressor of miR-101 could sensitize the HCC cells to the induction of apoptosis via targeting the gene of Mcl-1 (23). It is clear that the dysregulation of miRNAs should be considered responsible for the low-sensitivity of cancer cells to the chemotherapy.

miR-25 has been reported to act as an onco-miRNA in various cancers, such as ovarian cancer, breast cancer and gastric cancer. High levels of miR-25 in plasma predicted poor prognosis in patients with gastric cancer (24-26). However, the role of miR-25 in the cancer stem cells of HCC remains unclear. In the present study, we demonstrate that the miR-25 is dysregulated in the LCSCs. Moreover, we proved that the knockdown of miR-25 could significantly increase the sensitivity of HepG2-CSCs to the treatment of TRAIL *in vitro* and *in vivo*. Interestingly, knockdown of miR-25 could inhibit the effect of TRAIL on enriching the HepG2-CSCs population, because the absence of miR-25 promoted the killing of HepG2-CSCs induced by TRAIL. These findings may provide a potential use of miR-25 antagonist as a therapeutic strategy for reducing the drug-resistance of LCSCs.

PTEN is a well-known tumor suppressor in multiple cancers, which inhibits the tumorigenicity by negatively regulating the highly oncogenic PI3K/Akt-signaling pathway (27). Previous studies have demonstrated that the gene of PTEN is dysregulated in various cancers, and the change of PTEN is one of the critical factors for tumorigenesis (28,29). Recently, it was reported that PTEN is regulated by miRNAs in cancers, and the miRNAs/PTEN axis has been confirmed to be associated with the tumor growth, migration, invasion and chemosensitivity (30-32). In this study we also indicated that the gene of PTEN is downregulated in the CSCs of HepG2. Moreover, the miR-25/PTEN axis determined the sensitivity of HepG2-CSCs to TRAIL.

BCL2 associated agonist of cell death (Bad) is a pro-apoptosis protein belonging to the Bcl-2 family, which is also a substrate of Akt. The non-phosphorylated Bad promotes apoptosis by conjugating to the anti-apoptotic proteins of Bcl-2 and Bcl-xL, subsequently inactivating them. However, when Bad is phosphorylated by Akt, it will separate from the heterodimer of Bcl-2/Bcl-xL, and bind to 14-3-3 scaffold proteins in the cytoplasm with an inactive form. Thus, the free Bcl-2 and Bcl-xL could inhibit the apoptosis pathway (18,33). Our present study demonstrates that the knockdown of miR-25 increases the expression of PTEN, thus inhibiting the PI3K/Akt signaling pathway followed by keeping the Bad in a non-phosphorylated form. Benefiting from this, TRAIL is able to induce the mitochondrial apoptosis (34) in HepG2-CSCs, leading to the release of mitochondria-derived apoptogenic compounds such as cytochrome *c*, Smac/DIABLO, AIF and the ROS. Finally, the combination with anti-miR-25 and TRAIL induces the cleavage of caspases and the occurrence of apoptosis.

In conclusion, we have provided several pieces of evidence to prove that the miR-25 is associated with the sensitivity of liver cancer stem cells to TRAIL-induced apoptosis. Mechanistically, we demonstrate that the knockdown of miR-25 promotes TRAIL-induced apoptosis by inhibiting the PI3K/Akt/Bad signaling pathway through the miR-25/PTEN axis. The combination with anti-miR-25 and TRAIL may represent a novel strategy for the treatment of LCSCs.

Acknowledgements

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