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MicroRNA-26b-5p enhances T cell responses by targeting PIM-2 in hepatocellular carcinoma

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is a common tumor malignancy threatening a significant number of people worldwide. Although microRNAs (miRNAs) have been shown to play essential role in tumorigenesis, little is known about their role in T cells functions during HCC progression.
Methods: The abundances of miR-26b-5p were detected in HCC tissues or cells, T cells and H22 cells by quantitative real-time polymerase chain reaction (qRT-PCR). Regulation effect of miR-26b-5p on proviral integrations of moloney virus 2 (PIM2) was investigated by qRT-PCR, western blot (WB) and immunohistochemical analysis. The effect of miR-26b-5p and PIM-2 on cytokines secretion in CD4+ and CD8+ cells was evaluated by commercial enzyme linked immunosorbent assays (ELISA) kit. The interaction between miR-26b-5p and PIM-2 was probed by luciferase activity and RNA immunoprecipitation (RIP). H22 subcutaneous model was established to investigate the interaction of miR-26b-5p with HCC and immune competence.
Results: The abundance of miR-26b-5p was decreased in HCC and associated with poor survival. Addition of miR-26b-5p contributed to secretion of tumor necrosis factor α (TNF-α), interferon-γ (IFN-γ), interleukin-6 (IL-6) and IL-2 in CD4+ and CD8+ cells. Interestingly, PIM-2 was negatively regulated by miR-26b-5p and PIM-2 knockdown reversed anti-miR-26b-5p-mediated immunosuppression. Moreover, inhibitory effect of miR-26b-5p on HCC tumorigenesis was dependent on immune competence.
Conclusions: miR-26b-5p enhanced T cells responses by targeting PIM-2 in HCC, uncovering a promising therapeutic opportunity of HCC through reactivating immune system.

1. Introduction

Hepatocellular carcinoma (HCC) is an inflammation-related tumor malignancy with high incidence and approximately 18% 5-year survival rate [1]. Despite many attentions on prevention and diagnosis, the prognosis is still poor because of few effective treatments for HCC at advanced stage [2]. Immunotherapy has been reported to open the possibility for treatment of advanced HCC [3]. CD4+ and CD8+ T cells have been suggested to be critical for anti-tumor responses during HCC progression [4,5]. Immunosuppression was triggered in liver microenvironment against autoimmune liver injury [6]. Immune dysfunction is associated with HCC patients and reactivating the immune system in tumor environment may be one promising therapy measure [2].

MicroRNAs (miRNAs) are a class of short noncoding RNAs and play essential roles in driving tumorigenesis in HCC process [7]. MiRNAs are required for T lymphocyte biology through modulating cell activation, differentiation and development [8]. Moreover, cellular cytokines expression has an impact on the ability of miRNAs regulating immune cells activation [9]. For example, miRNA let7i promotes interleukin-2 (IL-2) secretion in CD4+ T cells, which is the early marker in CD4+ cells activation [10,11]. MiR-568 suppresses T cells activation and function, revealed by IL-2, IL-10 and Transforming growth factor-β (TGF-β) reduction [12]. Recent finding reveals that miR-150 depletion restores immune responses in CD8+ T cells by regulating T cell proliferation as well as differentiation [13]. Absence of miR-155 induces decreased cytokines production and poor survival in CD8+ cells [14]. Addition of miR-15b may damage antitumor responses by inhibiting T cells activation and IL-2 as well as interferon-γ (IFN-γ) secretion [15]. Recently, miR-26b-5p has been regarded as a tumor suppressive in various cancer. For instance, addition of miR-26b-5p inhibits cell proliferation, invasion and metastasis by regulating S100A7 in human intrahepatic cholangiocarcinoma [16]. Moreover, miR-26b-5p suppresses cell migrated and invasive abilities in bladder cancer [17]. Besides, miR-26b-5p has been reported to be associated with cell proliferation,

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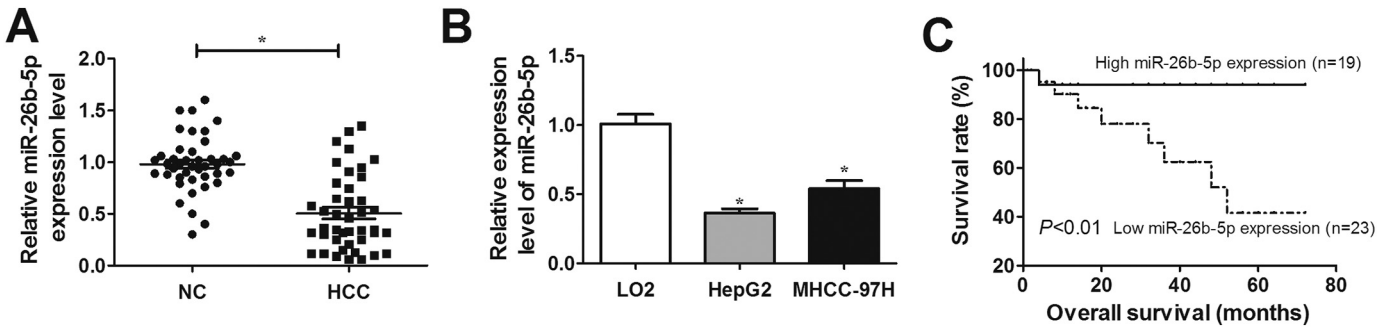

Fig. 1. The abundance of miR-26b-5p was impaired in HCC. (A) The expression of miR-216b-5p was investigated in HCC tissues compared with adjacent normal tissues. $n = 42$. (B) The abundance of miR-26b-5p was detected in HCC cell lines (HepG2 and MHCC-97H) compared with normal hepatic cell line (LO2). (C) The survival rate was detected between low and high miR-26b-5p expression group. The low miR-26b-5p expression group ($n = 23$) was below the mean value of miR-26b-5p expression, and the high miR-26b-5p expression group ($n = 19$) was above the mean value in HCC. Data were the mean ± standard deviation from three independent experiments. $^*p < 0.05$.

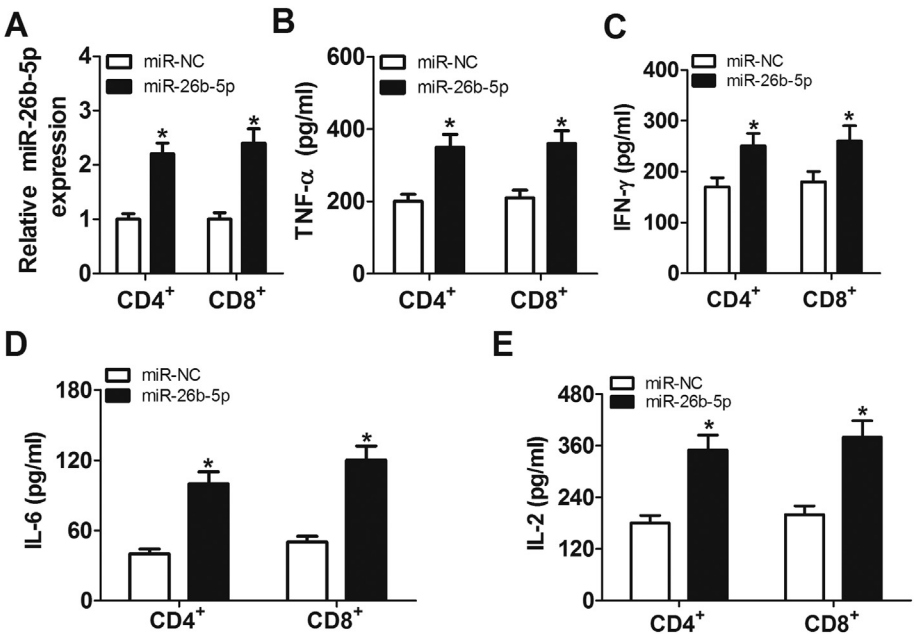

Fig. 2. Addition of miR-26b-5p contributed to cytokines secretion in CD4+ and CD8+ T lymphocytes. (A) Altered expression of miR-26b-5p was detected by qRT-PCR in CD4+ and CD8+ cells with transfection of miR-26b-5p mimics. (B, C, D, and E) The effect of miR-26b-5p addition on TNF-α, IFN-γ, IL-6 or IL-2 secretion was investigated in CD4+ and CD8+ cells, respectively. Data were the mean ± standard deviation from three independent experiments. $^*p < 0.05$.

angiogenesis as well apoptosis in HCC [18]. However, the connection between tumor immunosuppression and miR-26b-5p remains elusive.

The proviral integrations of moloney virus 2 (PIM2), a member of PIM kinase family, inhibits cell viability and accelerates apoptosis by miR-135-5p regulating in fibroblast cells [19]. Additionally, PIM-2 inhibition contributes to skin allograft survival through regulating T cells function and apoptosis pathway [20]. Moreover, PIM-2 knockdown modulates T cells responses and tumor immunity in graft versus host disease [21]. In HCC, PIM-2 has been reported to inhibit HCC cells apoptosis through the nuclear factor kB (NF-kB) pathway [22]. However, there is no direct evidence in support of the interaction between PIM-2 and T cells responses in HCC. In the current study, we investigated the effect of miR-26b-5p and PIM-2 on cytokines secretion in CD4+ and CD8+ cells. Moreover, we developed H22 subcutaneous model of normal or nude mice to uncover the importance of immune competence.

2. Materials and methods

2.1. Specimens

The study was accepted by the Institutional Research Ethics Committee of the First People's Hospital of Shangqiu and written informed consent was obtained from all patients. In this study, a total of 42 HCC tissues and adjacent normal tissues were collected from patients without history of radio and chemotherapy before operation and kept in liquid nitrogen until required. Patients were classified into high and low miR-26b-5p expression groups according to the statistical analysis of miR-26b-5p abundance for survival rate assay in cancer tissues. So, the low miR-26b-5p expression group ($n = 23$) was defined as those below the mean value of miR-26b-5p expression, whereas the high miR-26b-5p expression group ($n = 19$) consisted of those above the mean value in HCC tissues.

2.2. Cell culture and transfection

The normal hepatic cell line (LO2) and HCC cell lines (HepG2, MHCC97-H and H22) were obtained from American Tissue Culture Collection (ATCC, Manassas, VA, USA). All cells were growth in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Gibco), 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C in 5% CO_2 during the study.

CD4+ and CD8+ T lymphocytes were purified from human or mouse using commercial T cell isolation kit (Thermo Fisher, Wilmington, DE, USA) as previously reported [23]. The purity of CD4+

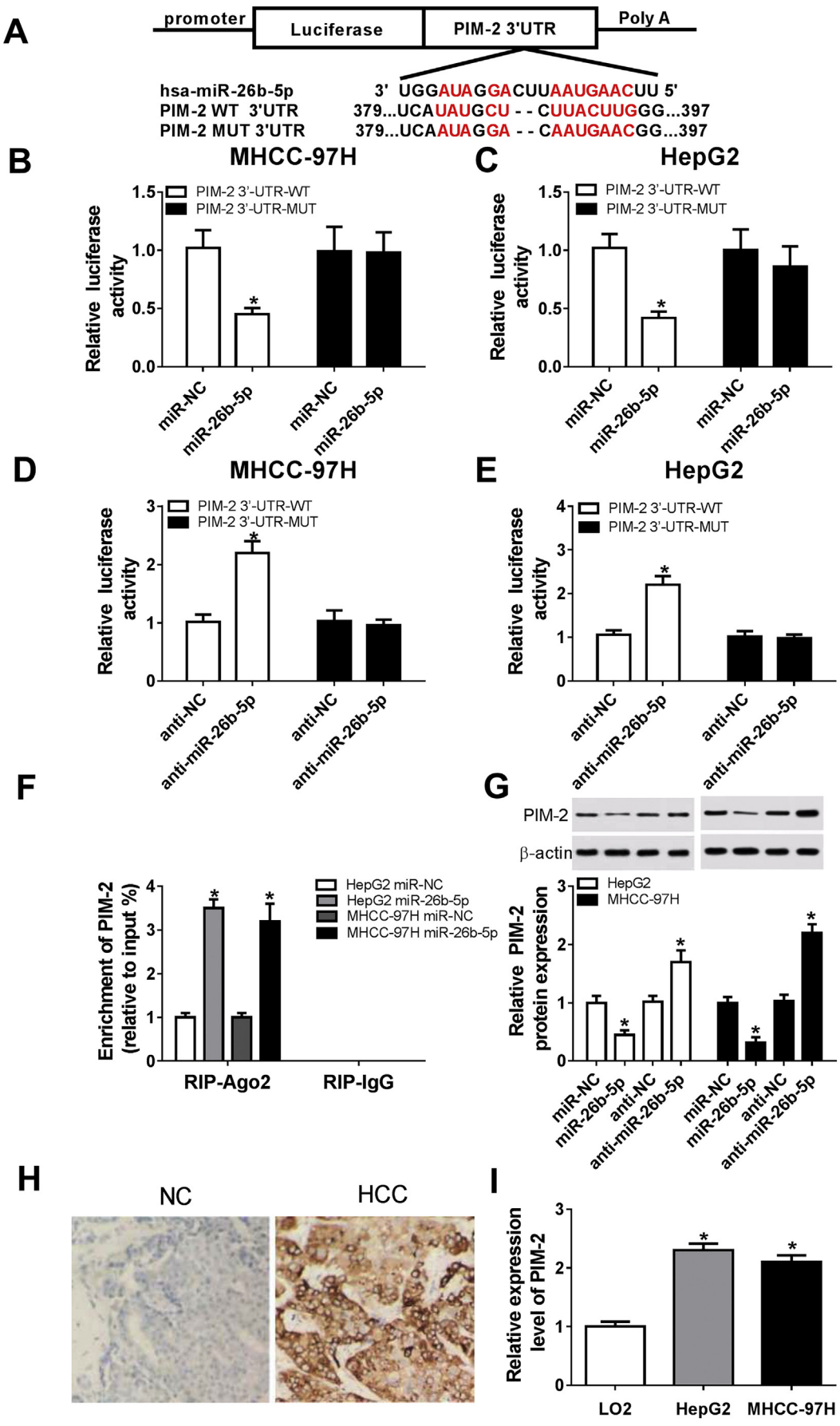

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Fig. 3. PIM-2 was a target gene of miR-26b-5p. (A) The potential binding sites of miR-25b-5p and PIM-2 was predicted by TargetScan. (B and C) Luciferase activity was investigated in MHCC-97H and HepG2 cells with miR-26b-5p transfection compared with miR-NC. (D and E) Luciferase activity was detected in HCC cells with anti-miR-26b-5p treatment. (F) Ago2 RIP assay was conducted in HCC cells. (G) The effect of miR-26b-5p on PIM-2 expression was investigated in HepG2 and MHCC-97H cells by WB. (H and I) The expression of PIM-2 was measured in HCC tissues and cells by immunohistochemical analysis or qRT-PCR. Data were the mean ± standard deviation from three independent experiments. *$p < 0.05$.

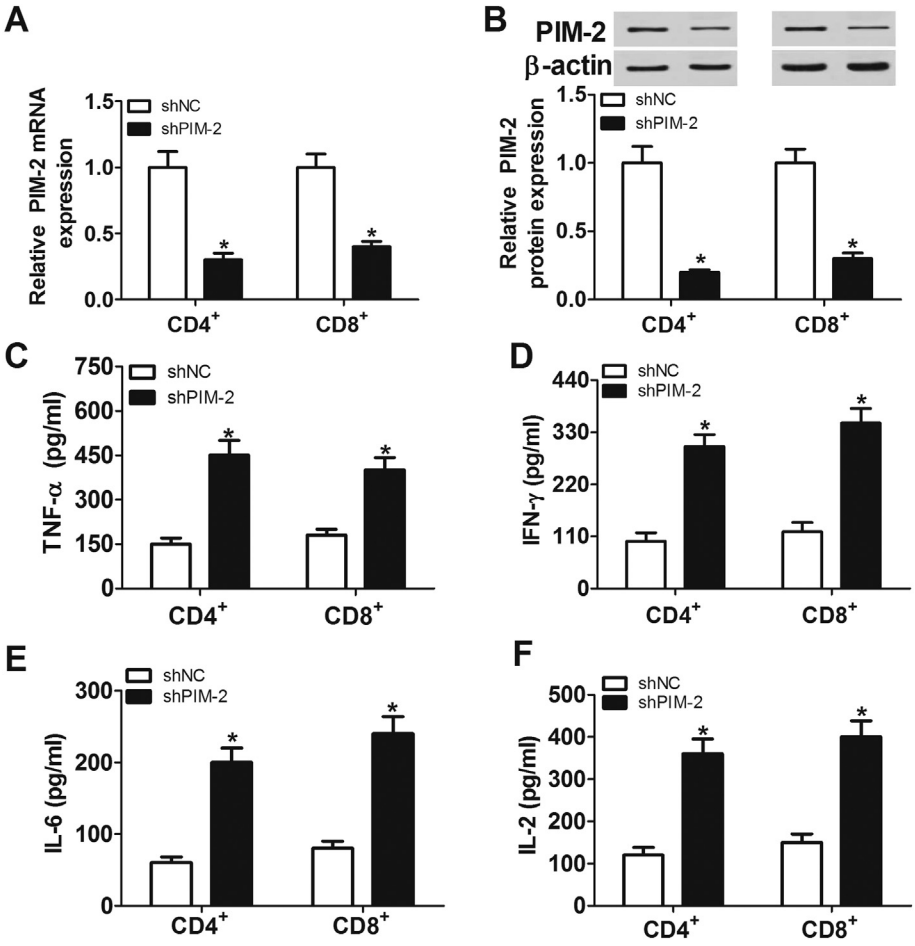

Fig. 4. PIM-2 knockdown increased cytokines secretion in T cells. (A) Altered expression of PIM-2 mRNA was detected in T cells with shPIM-2 transfection. (B) Inhibitory effect of shPIM-2 on PIM-2 protein was detected in CD4+ and CD8+ cells. (C, D, E, and F) The effect of shPIm-2 on secretion levels of TNF-α, IFN-γ, IL-6, and IL-2 were examined in CD4+ and CD8+ cells, respectively. Data were the mean ± standard deviation from three independent experiments. *$p < 0.05$.

and CD8+ T lymphocytes was over 96%. Human or murine miR-26b-5p mimics, miR-26b-5p inhibitor (anti-miR-26b-5p), negative control (miR-NC or anti-miR-NC), short hairpin RNA against PIM-2 (shPIM-2), and shNC were obtained from GenePharma (Shanghai, China). To investigate the effect of miR-26b-5p and PIM-2 on T lymphocytes, transfection was performed in purified CD4+ and CD8+ cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

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

Total RNA from tissues or cells was isolated using Trizol reagent (Invitrogen) following the manufacturer's instructions. Then 1 μL of sample was introduced into a NanoDrop Spectrophotometer (NanoDrop, Wilmington, DE, USA) for quality evaluation of RNA. Subsequently, total RNA was used for cDNA synthesis by commercial Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Synthesized cDNA was diluted and then used for qRT-PCR using SYBR Green Real-time PCR Master Mix (Toyobo, Tokyo, Japan) following the amplification instructions. All primers were listed as follows: human: PIM-2 (Forward, 5'-CGTAG CTAGTCGTCATACGTAGC-3′; Reverse, 5'-CGTAGTACGTCGTATGTCGTAGT C-3′), β-actin (Forward, 5'-AGAGCT ACGAGCTGCCTGAC-3′; Reverse, 5'-AGC ACTGTGTTGGCGTACAG-3′), murine: PIM-2 (Forward, 5'-ATGTTGACCAAGC CTCTGCAGG-3′; Reverse, 5'-GCTCAAGGACCAGCATGAAGC-3′), tumor necrosis factor α (TNF-α) (Forward, 5'-GCCCAAGGCGCCACATCTCC-3′; Reverse, 5'-TTGGGGACCGATCACCCCGA-3′), IFN-γ (Forward, 5'-CCCACAGCTCC AGC GCCAAG-3′; Reverse, 5'-TCAGCAGCGACTCCTTTTCCGC-3′), IL-6 (Forward, 5'-TCACAGAAGGAGTGGCTAAGGACC-3′; Reverse, 5'-ACGC ACTAGGTTTG CCGAGTAGAT-3′), IL-2 (Forward, 5'-GCACCCACTTCA AGCTCCA-3′; Reverse, 5'-AAATTTGAAGGTGAGCATCCTG-3′), β-actin (Forward, 5'-CAACCGTGAAA AGATGACCC-3′; Reverse, 5'-GTCTCCGG AGTCCATCACAA-3′). For miRNA expression assay, cDNA was synthesized by All-in-one miRNA qRT-PCR Reagent Kits (GeneCopoeia, Rockville, MD, USA). Primers of human or murine miR-26b-5p and U6 were ordered from GeneCopoeia. Results were analyzed by $2^{-\Delta\Delta Ct}$ method and β-actin or U6 small RNA was used as housekeeping gene.

2.4. Enzyme linked immunosorbent assays (ELISA)

The cell culture medium was collected and the level of TNF-α, IFN-γ, IL-6 or IL-2 was detected on a microplate reader using special

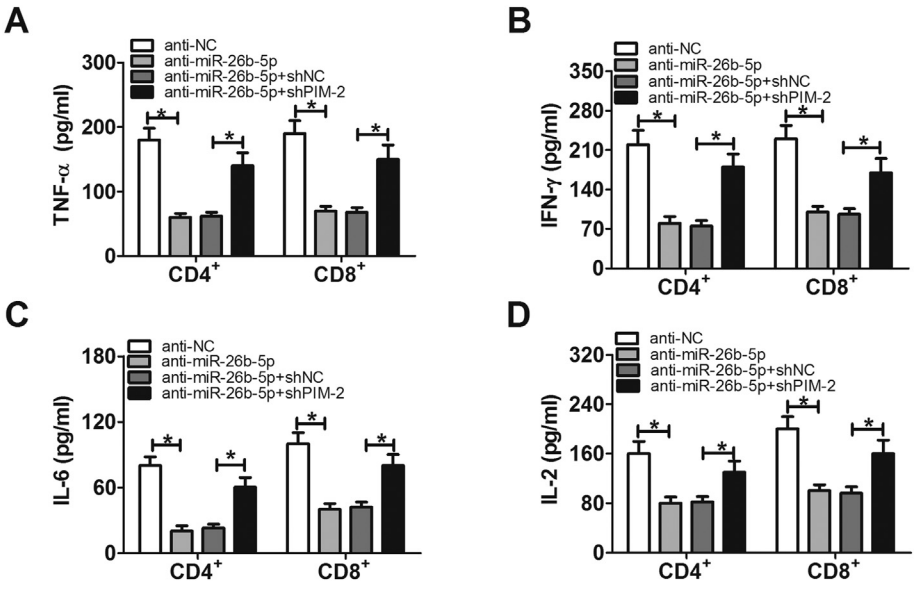

Fig. 5. PIM-2 depletion reversed anti-miR-26b-5p-mediated inhibitory effect on cytokines secretion in T cells. The effect of anti-miR-26b-5p and shPIM-2 on TNF-α (A), IFN-γ (B), IL-6 (C), and IL-2 (D) were detected in CD4+ and CD8+ cells. Data were the mean ± standard deviation from three independent experiments. *$p < 0.05$.

commercial ELISA Kit (Invitrogen) following the manufacturer's instructions. In brief, medium supernatant were added on the microwell plate and incubated in biotin and streptavidin-HRP buffer, followed by amplification reaction for 1 h. After the substrate reaction, color response was stopped by stop buffer and intensity of color was assayed at 450 nm with reference wave length at 620 nm with a microplate reader (Bio-Rad, Hercules, CA, USA).

2.5. Luciferase assays

TargetScan analysis demonstrated putative binding sites of miR-26b-5p and 3′ untranslated regions (3'-UTR) sequences of PIM-2. The wild or mutant sequence of 3'-UTR of PIM-2 were amplified and cloned into pGL3 luciferase reporter vector or control vector (Promega, Madison, WI, USA) to generate the wild-type plasmid (PIM-2-WT) or mutant-type plasmid (PIM-2-MT), respectively. WT or MT luciferase reporter plasmids, Renilla luciferase plasmid as well as miR-26b-5p mimics or anti-miR-26b-5p were transfected in HepG2 and MHCC-97H cells using Lipofectamine 2000 according to the manufacturer's protocols. Then cells were lysed and used for the luciferase activities analysis using Luciferase Assay Kit (GeneCopoeia, Rockville, MD, USA) after 48 h. Firefly luciferase activity was normalized by Renilla luciferase activity.

2.6. RNA immunoprecipitation (RIP)

Argonaute 2 (Ago2) immunoprecipitation was conducted in HepG2 and MHCC-97H cells with transfection of miR-26b-5p mimics. Subsequently, transfected cells were lysed in cell lysis buffer and added to magnetic beads (Thermo Fisher) bound with anti Ago2 or IgG antibody for 2 h at 4 °C. After washed with PBS, the RNA-protein-beads complexes were isolated by Trizol reagent. The abundance of PIM-2 in extracted products was investigated by qRT-PCR.

2.7. Western blots (WB)

Total proteins were prepared in cell lysis buffer containing 1% protease inhibitor (Thermo Fisher). Then proteins were quantified by BCA assay kit (Sigma, St. Louis, MO, USA) according to the instructions and then denatured at 98 °C for 5 min. Subsequently, equal amounts of proteins were loaded onto SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with blocking reagent (Thermo Fisher) for 1 h at room temperature and then incubated overnight at 4 °C with primary antibodies against PIM-2 or β-actin (Cell Signaling Technology, Danvers, MA, USA). After washed with Tris-buffer saline containing 0.1% Tween 20 (TBST), membranes were hatched with horseradish peroxidase (HRP) conjugated secondary antibodies (CST) for 2 h at room temperature. Densitometry analysis of protein bands was realized by enhanced chemiluminescence (ECL) chromogenic substrate (GE Healthcare, Amersham, UK) using Image Lab software (Bio-Rad, Hercules, CA, USA).

2.8. Cell proliferation assay

Cell proliferation was assessed using cell counting kit-8 (CCK-8) assay. H22 cells with miR-26b-5p transfection were seeded into 96-well plates with 1×10^4 cells per well overnight. Then cell medium was removed and CCK-8 assay (Sigma) was conducted for 2 h at 37 °C. The absorbance was measured at 450 nm with a microplate reader.

2.9. Animals and H22 subcutaneous model

Every effort was performed in accordance with the guiding principles approved by the Animal Research committee of the First People's Hospital of Shangqiu. SPF C57BL/6 and nude mice were obtained from Vital River Laboratory Animal Technology (Beijing, China) and hosed in specific pathogen-free microisolator cages with a 12 h light/dark cycle for one week. All animals were free access to water and food during the experiments.

To induce subcutaneous model of HCC, H22 cells were collected for tumor engraftment by injection subcutaneously after acclimatization. Tumors were examined every other day and tumor volume was calculated with slide calipers by (length × width × height)/2. Once the size reached palpable size, miR-26b-5p or miR-NC was delivered systemically by intravenously or intratumorally injection every other day. Survival duration was evaluated in different groups during the study. Reaching end points, CD4+ and CD8+ T lymphocytes were purified from mouse using commercial T cell isolation kit. Then tumor specimens were collected for molecular analyses.

2.10. Immunohistochemical analysis

Tumor sections were prepared from each model. After blocking endogenous peroxidase using 3% H_2O_2, sections were incubated with primary antibodies against PIM-2 for 2 h. Subsequently, cells were

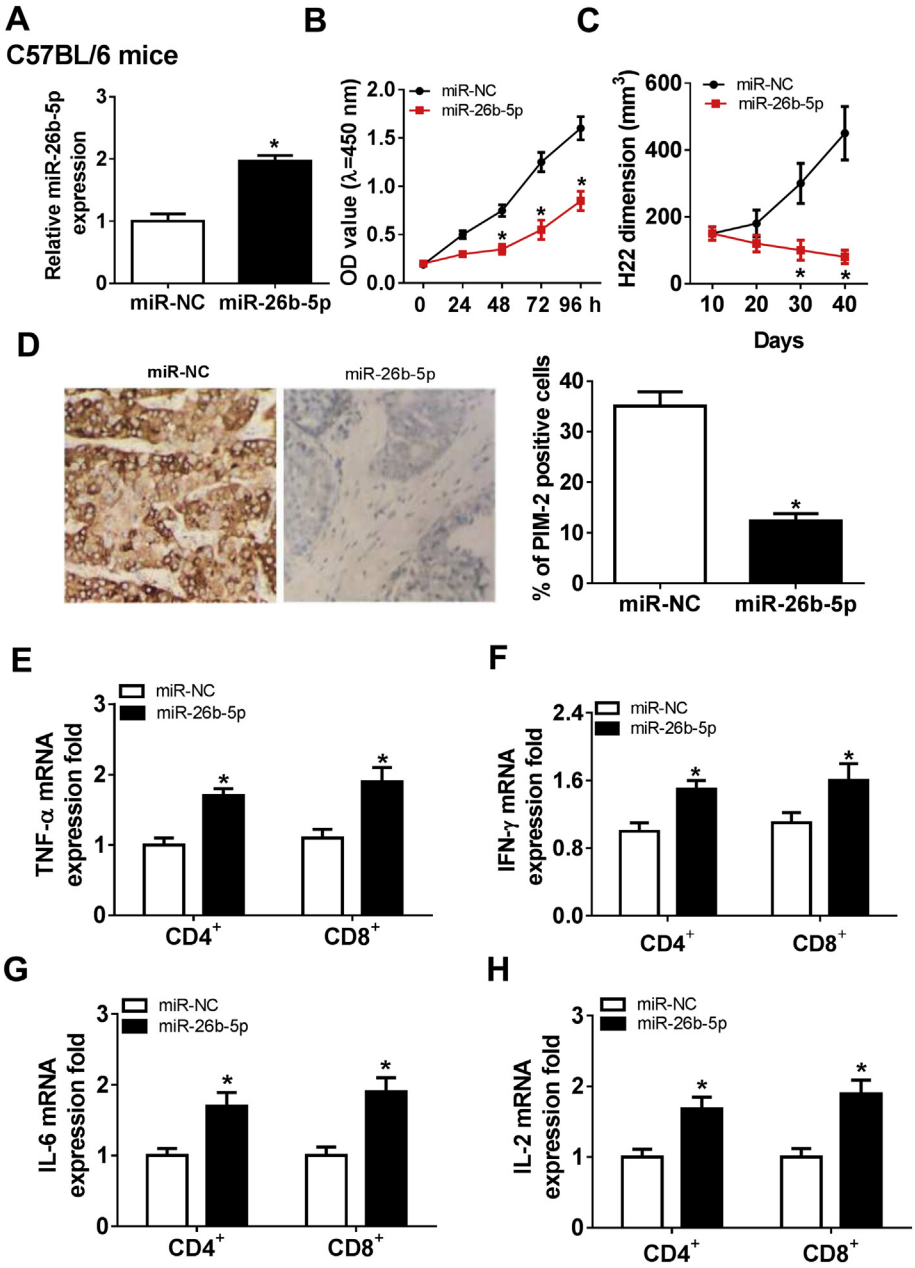

Fig. 6. Addition of miR-26b-5p inhibited subcutaneous H22 tumors in C57BL/6 mice. (A) Mimics induced miR-26b-5p expression in H22 cells. (B) Cell proliferation was analyzed in H22 cells with miR-26b-5p or miR-NC transfection by CCK-8. (C) The effect of miR-26b-5p on H22 tumors growth was investigated in C57BL/6 mice. (D) PIM-2 positive cells were examined in tumor tissues after miR-26b-5p treatment. (E-H) TNF-α, IFN-γ, IL-6 and IL-2 expression levels were detected in CD4+ and CD8+ cells from C57BL/6 mice with miR-26b-5p treatment. Data were the mean ± standard deviation from three independent experiments. * $p < 0.05$.

incubated with horseradish peroxidase (HRP) conjugated IgG secondary antibody for 30 min after rinsed with PBS, followed by incubated with DAB staining and hematoxylin (Sigma). The PIM-2 positive cells were examined under a light microscopy (Olympus) and analyzed using Image Lab software.

2.11. Statistical analysis

Data were presented as the mean ± standard deviation from more than three independent experiments. Student's *t*-test or one-way analysis of variance (ANOVA) was used to evaluate the differences using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). Statistically significant was considered as p values < 0.05.

3. Results

3.1. Low miR-26b-5p expression is associated with HCC

The major roles of miR-26b-5p vary in different conditions and the expression of miR-26b-5p was investigated in HCC tissues and cells. As a result, a great loss of miR-26b-5p expression was observed in HCC tissues compared with adjacent normal samples (Fig. 1A). Moreover, miR-26b-5p level was detected in HCC cell lines and normal hepatic cells. Result showed lower expression of miR-26b-5p in HepG2 and MHCC-97H cells than that in LO2 cell (Fig. 1B). Furthermore, the patients with HCC were classified into high and low miR-26b-5p expression groups according to the mean value. Based on the clinical data of 42 HCC samples, low miR-26b-5p expression was correlated with poor survival rate of patients with HCC (Fig. 1C).

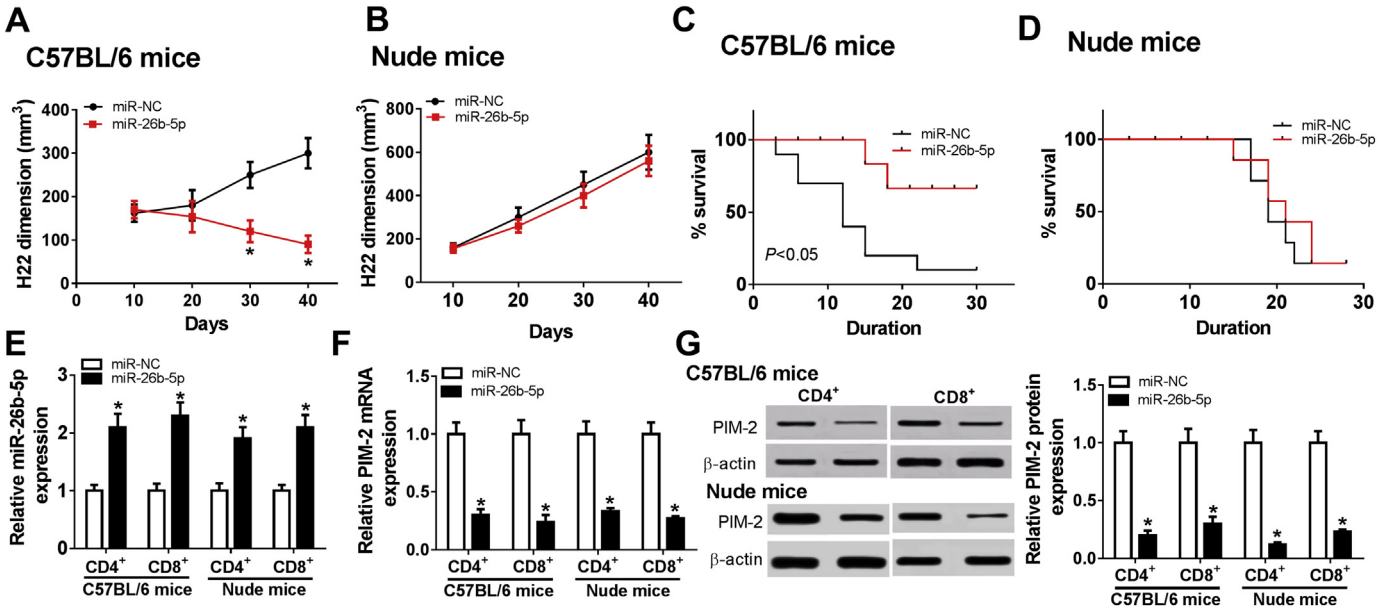

Fig. 7. The therapeutic effect of miR-26b-5p was dependent on immune competence. (A and B) The effect of miR-26b-5p on H22 tumors growth was evaluated in C57BL/6 and nude mice, respectively. (C and D) MiR-26b-5p treatment affected the survival duration in C57BL/6 and nude mice. (E) The expression of miR-26b-5p was measured in CD4+ and CD8+ cells from C57BL/6 and nude mice after treatment of miR-26b-5p or miR-NC. (F and G) The effect of miR-26b-5p on PIM-2 expression was detected CD4+ and CD8+ cells from C57BL/6 and nude mice after treatment of miR-26b-5p or miR-NC at transcriptional and protein levels. Data were the mean ± standard deviation from three independent experiments. *$p < 0.05$.

3.2. Addition of miR-26b-5p promotes cytokines secretion in CD4+ and CD8+ cells

Seeing that altered miR-26b-5p was required for HCC, we further investigated whether miR-26b-5p might affect function of T lymphocytes. To validate the effect of miR-26b-5p on cytokines secretion, the mimics or miR-NC were introduced into CD4+ and CD8+ cells. As a result, altered miR-26b-5p expression was realized in CD4+ and CD8+ cells in response to mimics, revealed by enhanced miR-26b-5p abundance (Fig. 2A). The effector response of T lymphocytes was associated with cytokines secretion, including TNF-α, IFN-γ, IL-6 or IL-2 production. The level of TNF-α was increased in miR-26b-5p transfected CD4+ and CD8+ cells (Fig. 2B). Similarly, addition of miR-26b-5p induced more secretion of IFN-γ, IL-6 as well as IL-2 in CD4+ and CD8+ cells than miR-NC treatment (Fig. 2C-E).

3.3. PIM-2 is a target of miR-26b-5p

Functional miRNA was realized by regulating target gene in many cancers. Hence, we expected to probe a gene targeted by miR-26b-5p. Intriguingly, bioinformatics analysis described potential binding sites of miR-26b-5p and 3'-UTR of PIM-2, indicating that PIM-2 might be regulated by miR-26b-5p (Fig. 3A). Therefore, luciferase activity and RIP analyses were conducted to prove the interaction. Accumulation of miR-26b-5p reduced the luciferase activity of MHCC-97H and HepG2 cells with PIM-2-WT transfection, respectively, whereas the activity showed little change in PIM-2-MUT transfected cells (Fig. 3B and C). However, introduction of anti-miR-26b-5p induced elevated luciferase activity of MHCC-97H and HepG2 cells with PIM-2-WT transfection rather than PIM-2-MUT treatment (Fig. 3D and E). Moreover, the abundance of enriched PIM-2 by Ago2 was significantly increased in HepG2 and MHCC-97H cells with miR-26b-5p transfection compared with miR-NC group, whereas IgG showed little enrichment (Fig. 3F). In addition, miR-26b-5p overexpression resulted in decreased while its knockdown induced elevated expression of PIM-2 protein in HepG2 and MHCC-97H cells (Fig. 3G). Besides, the expression of PIM-2 in HCC tissues and cells were detected by immunohistochemical analysis or qRT-PCR. Results displayed PIM-2 was highly expressed in HCC tissues and cells compared with that in their corresponding control (Fig. 3H and I).

3.4. PIM-2 depletion reverses anti-miR-26b-5p-mediated inhibitory on cytokines secretion in CD4+ and CD8+ cells

In view of above efforts, we further investigated the effect of PIM-2 on T lymphocytes. To probe the interaction of PIM-2 with cytokines secretion, shPIM-2 was introduced into CD4+ and CD8+ cells. Unsurprisingly, the mRNA level of PIM-2 was dramatically impaired in CD4+ and CD8+ cells suffering from PIM-2 knockdown (Fig. 4A). Likewise, shPIM-2 treatment induced a great loss of PIM-2 abundance at protein level compared with shNC group (Fig. 4B). Functional assay demonstrated that PIM-2 depletion contributed to the secretion of TNF-α, IFN-γ, IL-6 and IL-2 in CD4+ and CD8+ cells, respectively (Fig. 4C-F). In addition, the levels of TNF-α, IFN-γ, IL-6 and IL-2 were inhibited in anti-miR-26b-5p treated T cells compared with anti-NC did (Fig. 5). Moreover, abrogation of PIM-2 ablated the effect of miR-26b-5p absence on these cytokines in CD4+ and CD8+ cells (Fig. 5).

3.5. MiR-26b-5p inhibits subcutaneous HCC growth in mice

Given that miR-26b-5p was involved in immune responses, we next probed whether miR-26b-5p might afford anti-tumor efficacy. Firstly, we detected the effect of miR-26b-5p on H22 murine HCC cells proliferation in vitro. The abundance of miR-26b-5p was indeed increased in H22 cells with mimic's transfection (Fig. 6A). Moreover, abundant presence of miR-26b-5p limited cell proliferation in H22 cells compared with miR-NC treatment (Fig. 6B). Accordingly, H22 cells were implanted into normal C57BL/6 mice and then treated them by tail vein with miR-26b-5p and miR-NC. The subcutaneous HCC tumor was progressive growth in miR-NC treated C57BL/6 mice, whereas the tumor size was effectively suppressed in miR-26b-5p treated group (Fig. 6C). Furthermore, immunohistochemical assay displayed that PIM-2 expression was obviously repressed in miR-26b-5p treated group (Fig. 6D). To evaluate whether miR-26b-5p addressed immune

activation of effector T cells in tumor microenvironment, we detected cytokines production in T cells. Strong increase of TNF-α, IFN-γ, IL-6 and IL-2 mRNA expression was showed in CD4+ and CD8+ cells in miR-26b-5p treated group compared with control (Fig. 6E-H).

3.6. The inhibitory effect of miR-26b-5p requires immune competence

To uncover whether the therapeutics was mediated by immune system, H22 cells were introduced into normal C57BL/6 or nude mice by injection subcutaneously and then treated then with miR-26b-5p or miR-NC by intratumorally injection. Consistent with intravenously treatment, intratumorally administration of miR-26b-5p also limited tumor growth in C57BL/6 mice (Fig. 7A). However, miR-26b-5p failed to show a plausible therapeutic role, revealed by similar growth curves, indicating that anti-tumor effect of miR-26b-5p on HCC was involved in immune system (Fig. 7B). Moreover, the effect of miR-26b-5p on established tumors was investigated by survival duration. In C57BL/6 mice, miR-26b-5p treatment obviously enhanced survival duration and protected survival at same duration compared with miR-NC insult (Fig. 7C). Nevertheless, the efficacy was again lost in immune incompetent system (Fig. 7D). In addition, the expressions of miR-26b-5p and PIM-2 were measured in CD4+ and CD8+ cells from C57BL/6 and nude mice after treatment of miR-26b-5p or miR-NC. Results showed high expression of miR-26b-5p in CD4+ and CD8+ cells in miR-26b-5p group compared with that in miR-NC group (Fig. 7E). Besides, addition of miR-26b-5p evidently inhibited PIM-2 expression in CD4+ and CD8+ cells from C57BL/6 or nude mice at mRNA and protein levels (Fig. 7F and G).

4. Discussion

HCC is the dominant type of liver cancer with poor prognosis and treatment. Since immunotherapy is becoming a popular topic in HCC, there is still a need to explore another driver of HCC process for the desired anticancer response through unleashing immunosuppression and restoring antitumor immune responses. In this study, we first provided the view on that addition of miR-26b-5p enhanced T cell responses by targeting PIM-2 in HCC. Here we found miR-26b-5p expression was impaired in HCC tissues and cells. Moreover, low miR-26b-5p expression was associated with poor survival. This is also in agreement with former work that suggested low expression of miR-26b-5p was correlated with poor outcome of HCC, revealed by which miR-26b-5p inhibited cell growth and promoted apoptosis [18]. Similarly, in another study, investigators have reported that miR-26b-5p was downregulated and addition of miR-26b-5p blocked epithelial mesenchymal transition, migration as well as invasion via regulating SMAD1 in HCC [24]. Further efforts are needed to demonstrate whether miR-26b-5p addressed the biological roles in T cells of tumor microenvironment.

T lymphocytes are the key drivers in cell immunity and can be classified into CD4+ and CD8+ cells which modulate immune responses through varying pathway. CD4+ T cells apoptosis has been suggested to be associated with HCC development [4]. IFN-γ and IL-2 production were inhibited in tumor-infiltrating CD8+ T cells from HCC patients, compromising anti-tumor immunity [5]. Immunosuppression resulted in HCC development and clinical trials of HCC immunotherapy have been performed involving in cytokines introduction of TNF-α, IFN-γ, IL-4 and IL-2 [25]. Moreover, T cells and varying target genes have a vital role in HCC immunotherapy [25]. MiRNAs have been regarded as potential modulators of T cell biology through mediating function, proliferation and differentiation in varying cancers [8]. For example, miR-155, miR-146a and miR-181a have been shown to control T cell biology in different aspects [8]. Besides, miR-146a exacerbated immune suppression and drug resistance in colorectal cancer, revealed by increased TNF-β and IL-10 expressions as well as regulatory T cells population [26]. Let 7a compromised T cell proliferation and blocked IFN-γ production via negatively regulating signal transducer and activator of transcription 3 (STAT3) expression in psoriasis patients [27]. MiR-31 led to T cells dysfunction in chronic viral infection, whereas miR-31 exhaustion promoted IFN-γ secretion [28]. Depletion of miR-15a/16 contributed to cytokines secretion by modulating mTOR level, resulting in strong anti-tumor immune responses of glioma infiltrating CD8+ T cells [29]. In miR-126 knockdown mice, activation and proliferation were triggered in CD4+ T cells. In addition, miR-126 deficiency induced IFN-γ expression by targeting insulin receptor substrate 1 (IRS-1) [30]. Contrary to above miRNAs, enrichment of miR-26b-5p enhanced TNF-α, IFN-γ, IL-6 and IL-2 secretions in CD4+ and CD8+ cells, uncovering miR-26b-5p unleashed immunosuppression and facilitated T cell activation in our study. This is also consistent with the functions of miR-155 and miR-4443, respectively. Anti-tumor immunity was defective by miR-155 deletion in T cells, uncovered by reduced intra-tumor IFN-γ expression and enhanced tumor growth [31]. Overexpression of miR-4443 in CD4+ cells increased T cell proliferation and IL-1β, IL-6 as well as IL-17 expression by targeting tumor necrosis factor receptor associated factor 4 (TRAF4), indicating that miR-4443 promoted T cell function in grave disease [32].

Usually, functional miRNAs were afforded by targeting mRNA, resulting in reduced abundances of related protein [33]. MiR-26b-5p has been shown to target S100A7 in human intrahepatic cholangiocarcinoma, therefore regulating cell proliferation, invasion and metastasis [16]. Besides, former work demonstrated the trichorhinophalangeal syndrome 1 (TRPS1) was targeted by miR-26b-5p and involved in development of breast cancer with radiation treatment [34]. PIM-2 was a member of PIM kinase family which played key role in regulating cell viability and apoptosis in fibroblast cells [19]. Addition of PIM-2 protected HepG2 survival ability and prevented apoptosis, disclosing PIM-2 induced liver tumorigenesis [35]. Moreover, PIM-2 played essential role in HCC progression by regulating cell apoptosis through NF-kB pathway [22]. However, little is known about interaction between miR-26b-5p and PIM-2 in HCC. Bioinformatics analysis provided potential binding sites of miR-26b-5p and PIM-2, indicating that PIM-2 might be a target of miR-26b-5p. Hence, we validated the prediction by luciferase activity and RIP in HCC cells, revealed by miR-26b-5p negatively regulating PIM-2 just like miR-135-5p did [19]. Furthermore, we found that PIM-2 depletion could enhance and reverse miR-26b-5p deficiency-mediated T cells responses, such as TNF-α, IFN-γ, IL-6 and IL-2 production in CD4+ and CD8+ cells. This is also in agreement with the effect of PIM-2 on CD4+ and CD8+ T cells in graft versus host disease, which showed that PIM-2 knockdown might be responsible for T cells responses as well as tumor immunity, uncovered by that PIM-2 inhibited T cell viability, whereas PIM-2 deficiency triggered anti-tumor responses [21]. Likewise, PIM-2 exhaustion in skin allograft, prolonged cell survival by regulating T cell apoptosis [20].

MiR-26b-5p and PIM-2 were associated with HCC development through regulating T cells responses. This is also suggesting the importance of T cell. We further hypothesized that immune competence might be required for miR-26b-5p-mediated potential strategy to augment antitumor activity. To test this hypothesis, we injected H22 cells into normal C57BL/6 or nude mice, respectively, and found that the therapeutic effect of miR-26b-5p required immune competence. Consistent with our effort, presence of abundant miR-124 enhanced T cell function in glioma and this effect would be lost on immune incompetent [23].

5. Conclusions

In conclusion, this study provided insight into the relevance between miR-26b-5p and HCC progression. Here we found miR-26b-5p triggered T cell responses by targeting PIM-2 in HCC. Moreover, immune competence was required for miR-26b-5p-mediated effect of HCC treatment. These data uncover that miR-26b-5p might present a potential therapeutic targeting opportunity of HCC immunotherapy.

Conflicts of interest

The authors have no conflict of interest to declare.

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