MicroRNA-519 inhibits hypoxia-induced tumorigenesis of pancreatic cancer by regulating immune checkpoint PD-L1

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Received July 12, 2019; Accepted November 7, 2019

DOI: 10.3892/ol.2019.11234

Abstract. Pancreatic cancer is highly prevalent and exhibits a high incidence and mortality rate. Hypoxia contributes to tumorigenesis and the progression of pancreatic cancer. To the best of our knowledge, the role of microRNA (miR)-519 has not been investigated in hypoxia-induced pancreatic cancer progression. The purpose of the present study was to elucidate the mechanism underlying miR-519-mediated regulation of pancreatic cancer progression. Reverse transcription-quantitative PCR and western blotting were performed to investigate miR-519 and programmed death ligand 1 (PD-L1) mRNA and protein levels, respectively. Additionally, a Transwell assay was performed to examine the invasiveness of PANC-1 and SW1990 cells. Cells were subsequently stained with Annexin V to determine the apoptotic rate of cells. Furthermore, bioinformatics analysis and a dual-luciferase reporter assay were performed to confirm the direct association between miR-519 and PD-L1, and a xenograft experiment was conducted to test the role of miR-519 *in vivo*. The results revealed that the expression levels of miR-519 in pancreatic cancer cells were reduced following hypoxia treatment. Furthermore, transfection with miR-519 mimics inhibited PANC-1 and SW1990 cell invasiveness, and induced apoptosis under hypoxic conditions. PD-L1 was also identified as a downstream target of miR-519, and rescued the miR-519 mimic-attenuated tumorigenesis of pancreatic cancer cells under hypoxic conditions. Additionally, treatment with miR-519 mimics significantly suppressed the tumor growth of PANC-1 cells. The results of the present study indicated a novel mechanism of miR-519-mediated tumorigenesis in pancreatic cancer cells under hypoxic conditions. The conclusions may be crucial for the improvement of future pancreatic cancer treatment.

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Key words: pancreatic cancer, tumorigenesis, hypoxia, microRNA-519, programmed death ligand 1

Introduction

Pancreatic cancer is the fourth most common cause of cancer-associated mortality, and pancreatic ductal adenocarcinoma (PDAC) accounts for 90% of total pancreatic cancer cases (1,2). PDAC exhibits a 5-year survival rate of ~5% and a median survival time of <6 months (3,4). Hypoxia is commonly observed in solid tumors, particularly in pancreatic cancer (5). Previous studies have reported that hypoxia enhances tumor invasion and metastasis, serving as an activator of epithelial-mesenchymal transition (6,7). However, the mechanisms underlying hypoxia-induced tumor progression in pancreatic cancer are yet to be determined.

MicroRNAs (miRNAs or miRs) are implicated in the regulation of a number of biological processes, under normal and pathological conditions (8). They regulate putative downstream targets by binding to their 3'-untranslated region (UTR) sequences (9). It has been demonstrated that the aberrant expression of certain miRNAs influences the genesis and progression of various types of cancer (10). Previous studies have revealed that certain miRNAs, including miR-21, miR-217 and miR-135a, may serve a diagnostic and prognostic role in patients with PDAC (11-13). To the best of our knowledge, no previous study has reported the role of miR-519 in pancreatic cancer.

The immune checkpoint, programmed death ligand 1 (PD-L1; also known as CD274 or B7-H1), has been demonstrated to be activated via binding to its cognate receptor, programmed death 1 (PD-1), in numerous types of cancer, including oral squamous cell carcinoma (14). Upon activation, the PD-L1/PD-1 pathway facilitates escape from T cell-mediated immune function (15). In cancer, high PD-L1 levels are associated with tumor progression and a poor prognosis (16). Recently, PD-L1 has been revealed to influence tumor progression via the inhibition of the T cell-mediated immune response (17,18). Unfortunately, therapies targeting the PD-L1/PD-1 signaling pathway have not yet exhibited a marked effect in pancreatic cancer treatment (19). Therefore, determination of the underlying mechanisms underpinning this phenomenon require further study.

The present study aimed to investigate the molecular mechanisms of hypoxia-induced tumorigenesis in pancreatic cancer. The results revealed that miR-519 suppressed the invasion, and induced the apoptosis, of pancreatic cancer cells via downregulation of PD-L1. The conclusions of the present study may advance the understanding of pancreatic cancer treatment.

Materials and methods

Cell culture and hypoxic treatment. The human pancreatic cancer PANC-1 and SW1990 cell lines were purchased from the American Type Culture Collection and cultured at 37˚C in RPMI1640 medium (HyClone; GE Healthcare Life Sciences), supplemented with 1% penicillin/streptomycin and 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). PANC-1 and SW1990 cells were then plated separately either into cell culture dishes or plates, and cultured under normoxic (94% N_2, 5% CO_2 and 1% O_2) or hypoxic conditions (95% N_2 and 5% CO_2 with 100 μM $CoCl_2$).

Cell transfection. PD-L1 cDNA was subcloned and ligated into pCMV vectors. Lipofectamine 2000® (Thermo Fisher Scientific, Inc.) was subsequently used to incorporate 2 μg pCMV-PD-L1 into 5x10^5 PANC-1 and SW1990 cells. Next, ~48 h after transfection, the cells were harvested to perform subsequent experiments. Negative control (NC) mimic, NC inhibitor, miR-519 mimics or miR-519 inhibitors (Shanghai GenePharma Co., Ltd.) were transfected into PANC-1 or SW1990 cells using Lipofectamine® RNAiMAX reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The sequences were as follows: NC mimic, 5'-GGUUCGUAC GUACACUGUUCA-3'; miR-519 mimic, 5'-CUCUAGAGG GAAGCGCUUUCUG-3'; NC inhibitor, 5'-CCAUCAGUCCCA AAUCCA-3'; miR-519 inhibitor, 5'-CCAGAGGGAAGCGCC G-3'. NC mimic and NC inhibitor represented non-targeting sequences. At 36 h after transfection, the cells were subject to subsequent experiments.

Immunofluorescence. PANC-1 and SW1990 cells were fixed using 4% paraformaldehyde, and 0.5% Triton X-100 was used to permeabilize cells. Cells were then incubated with Annexin V-fluorescein isothiocyanate antibodies (cat. no. 556547; 1:20; BD Pharmingen; BD Biosciences) for 1 h at room temperature. Apoptosis rates were subsequently determined by calculating the ratio of Annexin V-positive cells to the total cell number under a fluorescent microscope (Olympus Corporation) with a magnification of x400.

Bioinformatics analysis. The downstream target of miR-519 was predicted using TargetScan online program version 7.2 (http://www.targetscan.org/).

Dual-luciferase reporter assay. The wild-type and mutant 3'-UTR sequences of PD-L1 were subcloned and ligated into pGL3 vectors. The 3'-UTR sequence containing the pGL3 vector (Promega Corporation) was co-transfected with Lipofectamine 2000® (Thermo Fisher Scientific, Inc.) into PANC-1 cells with miRNA (NCs, miR-519 mimics or miR-519 inhibitor). Luciferase activities were measured using a Dual-Luciferase Reporter system (Promega Corporation) at 48 h after transfection. The Firefly luciferase activities were normalized to *Renilla*.

Transwell invasion assay. A total of ~1x10^5 cells were resuspended in 200 μl DMEM (HyClone; GE Healthcare Life Sciences). Subsequently, the medium was transferred to the top chamber with 8.0-μm pore membranes (EMD Millipore) pre-coated with Matrigel for 30 min at 37˚C. DMEM (~350 μl) supplemented with 20% FBS (Gibco; Thermo Fisher Scientific, Inc.) was plated in the lower chamber. Invasive cells were stained with crystal violet at room temperature for 2 h following incubation for 48 h. Cell images were obtained using an inverted light microscope with magnification of x400. Statistical analysis was subsequently performed using GraphPad prism software 6.0 (GraphPad Software, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted as previously described. Takara-PrimeScriptTM RT reagent Kit (Takara Bio, Inc.) was used to reverse transcribe RNA into cDNA according to the manufacturer's protocol. The reverse transcription thermocycling program was 37˚C for 15 min followed by 85˚C for 5 sec. SYBR®-Green dye (Roche Diagnostics) was used to perform qPCR according to the manufacturer's protocol. The thermocycling program was: Step 1, 95˚C for 30 sec; step 2, 95˚C for 3 sec; step 3, 60˚C for 30 sec (step 2-3, 40 cycles); and step 4, holding at 10˚C. GAPDH and U6 were utilized as housekeeping genes for the detection of PD-L1 and miR-519, respectively. Relative expression levels of genes were calculated using the $2^{-\Delta\Delta Ct}$ method (20). The primer sequences were as follows: miR-519 forward, 5'-CATGCTGTGACCCTCCAAAG-3' and reverse, 5'-GAGAAAACAAACAGAAAGCGCT-3'; PD-L1 forward, 5'-CTGAACGCCCCATACAACAA-3' and reverse, 5'-CTTGGAATTGGTGGTGGTGG-3'; GAPDH forward, 5'-GAGAAGTATGACAACAGCCTC-3' and reverse, 5'-ATG GACTGTGGTCATGAGTC-3'; and U6 forward, 5'-CTCGCT TCGGCAGCACATATACTA-3' and reverse, 5'-ACGAAT TTGCGTGTCATCCTTGCG-3'.

Xenograft tumor experiment. Immunodeficient mice (n=8; 4 males and 4 females; NOD-SCID; age, ~6 weeks; weight, 20-22 g) were utilized, and purchased from Shanghai SLAC Laboratory Animal Co., Ltd. PANC-1 cells (1x10^6) suspended in RPMI1640 medium were injected subcutaneously into right armpit of each mouse following anesthetic treatment with 2% isoflurane (Baxter). The mice were housed in a specific-pathogen-free room with enough distilled water and food, under controlled conditions (25˚C; 40-60% humidity; 10 h light/14 h dark). Animal health and behavior were monitored daily. Body temperature and weight, behavioral changes, pathological changes (such as autonomous tumors, observed using micro-Computer Tomography imaging technology) and blood oxygen saturation were the criteria used to determine whether animals should be euthanized. Mice were sacrificed by decapitation 4 weeks after injection. Animal death was verified by cardiac and respiratory arrest, muscle relaxation and lack of reflection. Murine tumors were subsequently removed and weighed. No premature mortalities or significant decreases in body weight were observed during the experiment. The maximum percentage weight of the tumor compared with total body weight was 2%. The protocol of the present study was approved by the Animal Welfare Committee of The Third Affiliated Hospital of Soochow University (Changzhou, China).

Statistical analysis. All experiments were performed in triplicate and data are presented as the mean ± standard deviation. Statistical analysis was performed using GraphPad prism software 6.0 (GraphPad Software, Inc.). Comparisons between two groups were performed using a two-tailed Student's t-test and comparisons among multiple groups were performed

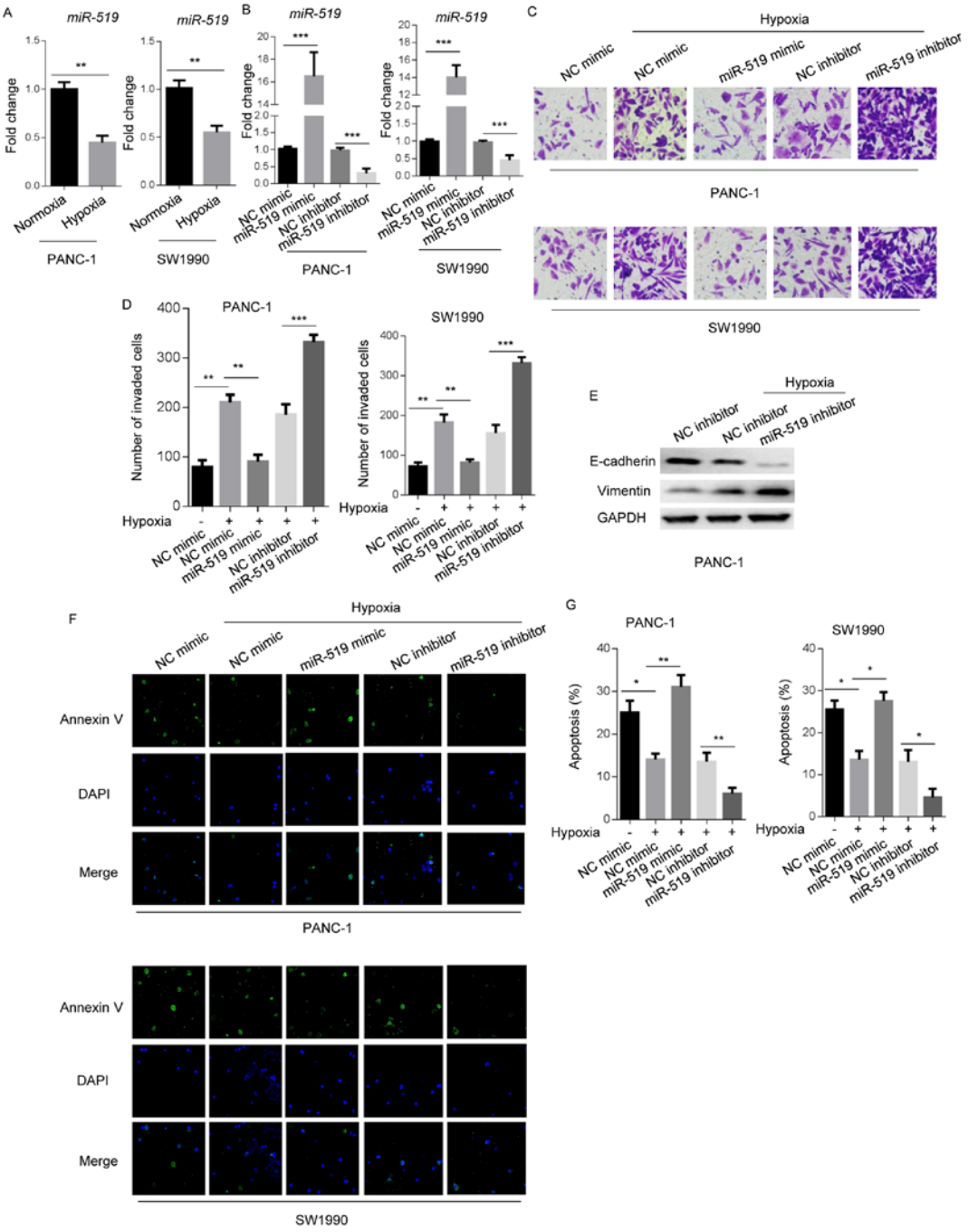

Figure 1. miR-519 attenuates the hypoxia-induced tumorigenesis of pancreatic cancer cells. miR-519 levels in PANC-1 or SW1990 cells were determined via reverse transcription-quantitative PCR under (A) normoxic or hypoxic conditions; or (B) following transfection with NCs, miR-519 mimics or miR-519 inhibitors. (C) Invasive abilities of PANC-1 or SW1990 cells transfected with NCs, miR-519 mimics or miR-519 inhibitors were examined by performing a Transwell assay under normoxic or hypoxic conditions. Magnification, x200. (D) Statistical analysis of invasive cell numbers. (E) E-cadherin and vimentin expression were examined using western blotting in PANC-1 cells transfected with either the NC inhibitor or miR-519 inhibitor under normoxic or hypoxic conditions. GAPDH was used as the loading control. (F) Apoptosis of NC-, miR-519 mimic- or miR-519 inhibitor-transfected PANC-1 and SW1990 cells was determined using an immunofluorescence assay following staining with Annexin V under normoxic or hypoxic conditions, merge denotes that Annexin V was merged with DAPI pictures. Magnification, x200. (G) Statistical analysis of apoptosis. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001, as indicated. miR, microRNA; NC, negative control.

using ANOVA (Tukey's post-hoc test). *P<0.05 was considered to indicate a statistically significant difference.

Results

miR-519 attenuates the hypoxia-induced tumorigenesis of pancreatic cancer cells. To investigate the influence of miR-519 on the tumorigenesis and progression of pancreatic cancer cells, miR-519 expression in PANC-1 and SW1990 cells was determined under normoxic and hypoxic conditions. The results of RT-qPCR revealed that miR-519 expression was significantly reduced in both cell lines when they were cultured in a hypoxic environment (Fig. 1A), indicating that miR-519 may serve a suppressive role in hypoxia-induced phenotypes of pancreatic cancer.

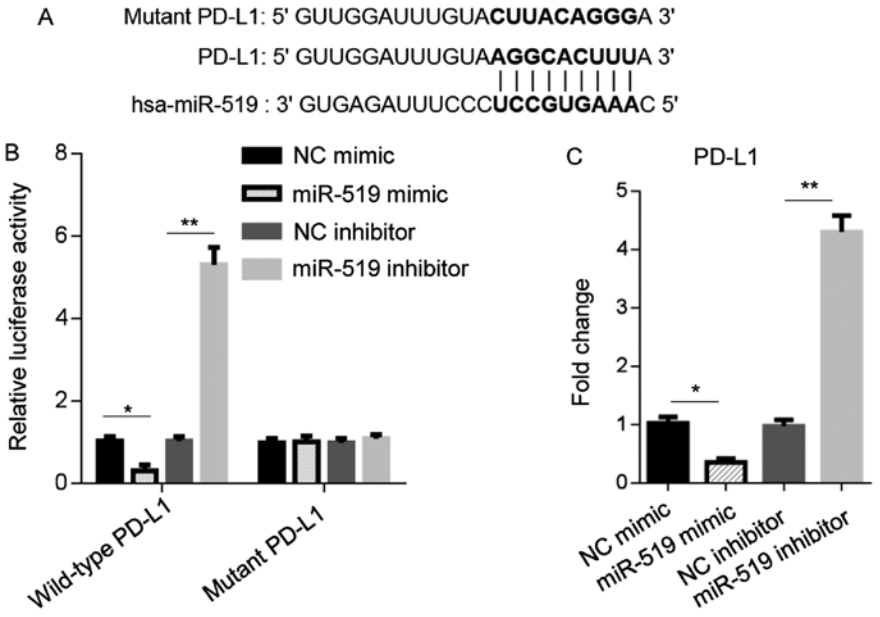

Figure 2. miR-519 binds to and regulates PD-L1. (A) Binding sites at the 3'-UTR region of PD-L1 mRNA and miR-519 are indicated. (B) Luciferase activity was measured in PANC-1 cells co-transfected with 3'-UTR-fused pGL3 vectors and miRs (NC, miR-519 mimic or miR-519 inhibitor). (C) PD-L1 levels were determined via reverse transcription-quantitative PCR in PANC-1 cells transfected with NCs, miR-519 mimics or miR-519 inhibitors. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01, as indicated. miR, microRNA; PD-L1, programmed death ligand 1; UTR, untranslated region; NC, negative control.

Invasion was subsequently assessed using a Transwell assay in cells with indicated oligonucleotides. The results revealed that hypoxia significantly increased the invasiveness of both cell lines transfected with NC mimic (Fig. 1C and D). Furthermore, treatment with miR-519 mimics reduced the number of invasive cells when compared with the NC group, under hypoxic conditions. Furthermore, treatment with miR-519 inhibitors promoted cell invasion (Fig. 1B-D). Additionally, it was revealed that hypoxia and miR-519 inhibitor reduced E-cadherin expression, whereas vimentin expression was elevated by hypoxia treatment and miR-519 inhibitor (Fig. 1E). The apoptosis of PANC-1 and SW1990 cells was examined via Annexin V staining. As hypothesized, hypoxic treatment decreased Annexin V signals produced in pancreatic cancer cells. In addition, miR-519 mimics elevated Annexin V positive cell numbers when compared with NC treated cells under hypoxic conditions. The miR-519 inhibitor reduced the apoptosis of pancreatic cancer cells (Fig. 1F and G). The results revealed that miR-519 suppressed the hypoxia-induced tumorigenesis of pancreatic cancer cells.

miR-519 binds to PD-L1 and regulates its expression. To investigate the downstream targets of miR-519 in pancreatic cancer, bioinformatics analysis was performed. The results revealed that miR-519 was associated with PD-L1, as direct binding to its 3'-UTR regions was predicted using the TargetScan online program (Fig. 2A). To assess whether miR-519 was directly associated with PD-L1 and regulated its expression, a dual-luciferase reporter assay was performed. The results demonstrated that the miR-519 mimic significantly reduced luciferase activity in PANC-1 cells transfected with wild-type 3'-UTR-pGL3 vectors. Additionally, the miR-519 inhibitor promoted luciferase activity (Fig. 2B). To validate these results, RT-qPCR was performed and revealed that miR-519 mimics decreased PD-L1 expression by 60-70%, whereas transfection with the miR-519 inhibitor upregulated PD-L1 by 4.4-fold in PANC-1 cells (Fig. 2C). Overall, the results indicated that miR-519 directly regulated PD-L1 expression and activity via binding to its 3'-UTR region.

PD-L1 mediates the miR-519-attenuated tumorigenesis of pancreatic cancer cells under hypoxic conditions. After it was revealed that PD-L1 represents a molecular downstream target of miR-519 in PANC-1 and SW1990 cells, the present study aimed to confirm whether PD-L1 is responsible for miR-519-associated tumorigenesis in pancreatic cancer cells. The mRNA levels of PD-L1 in PANC-1 and SW1990 cells were detected under normoxic and hypoxic conditions. RT-qPCR analysis revealed that hypoxia significantly increased PD-L1 expression (2-3-fold; Fig. 3A). These data indicated that PD-L1 may promote hypoxia-induced phenotypes of pancreatic cancer.

The miR-519 mimic decreased PD-L1 expression compared with the NC mimic (Fig. 3B). In addition, PD-L1 level was higher when PD-L1 was overexpressed in miR-519 mimic PANC-1 and SW1990 cells (Fig. 3B). Furthermore, the Transwell assay demonstrated that PD-L1 significantly increased the number of invasive miR-519 mimic cells under hypoxic conditions (Fig. 3C and D). miR-519 mimic increased the E-cadherin level and decreased the vimentin level, which was partially restored by PD-L1 overexpression. However, PD-L1 attenuated the number of pancreatic cancer cells that stained positive for Annexin V, indicating that PD-L1 functioned as an effector of miR-519 and served an apoptosis-inhibiting role in pancreatic cancer cells, under hypoxic conditions (Fig. 3F and G). The present results indicated that, when exposed to hypoxic conditions, PD-L1

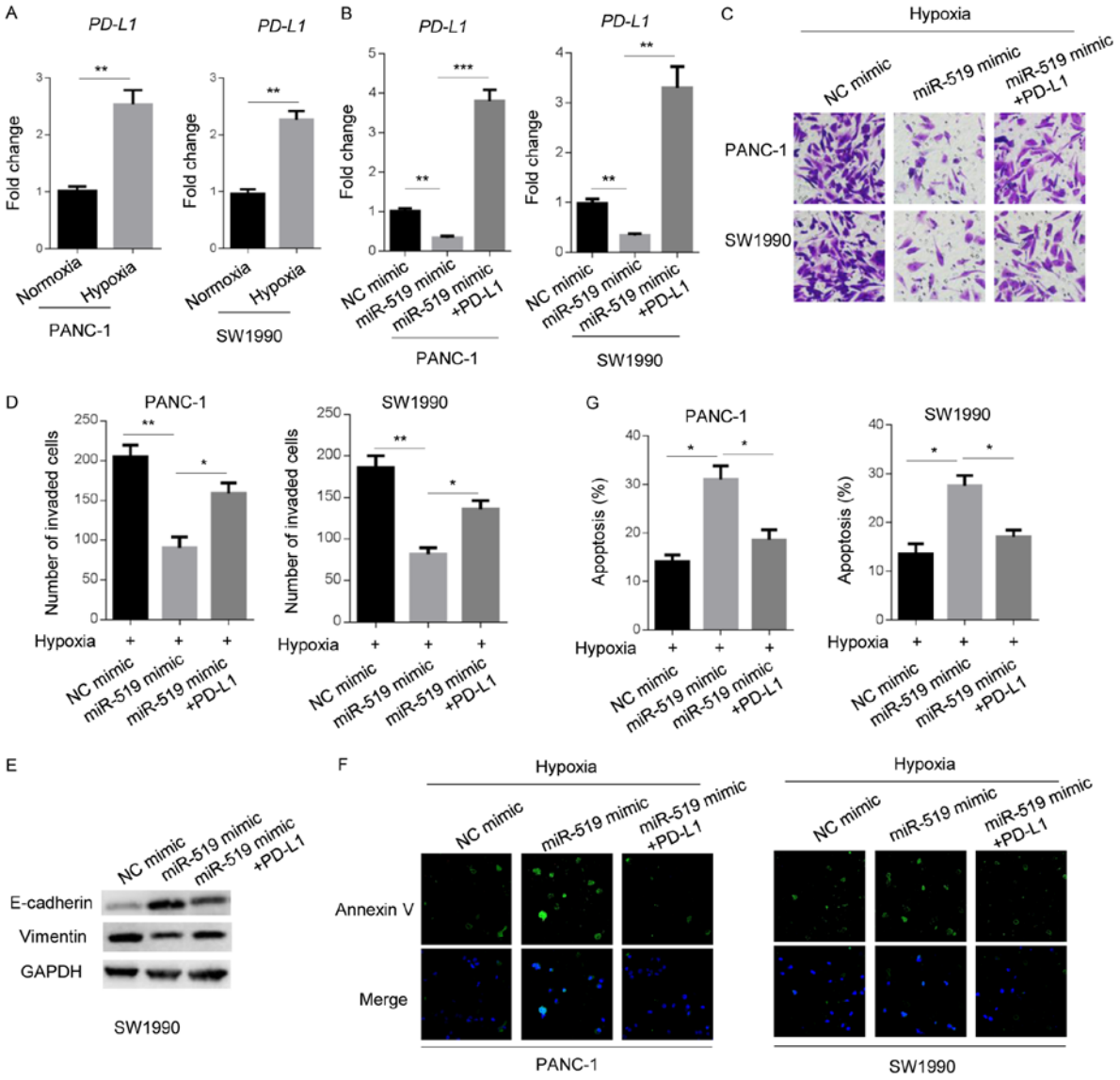

Figure 3. PD-L1 mediates the miR-519-attenuated tumorigenesis of pancreatic cancer cells under hypoxia. PD-L1 mRNA levels were determined in PANC-1 or SW1990 cells via reverse transcription-quantitative PCR (A) under normoxic or hypoxic conditions, or (B) when transfected with NCs, miR-519 mimics, or miR-519 mimics and PD-L1. (C) Invasive abilities of NC, miR-519 mimic, or miR-519 mimic and PD-L1-transfected PANC-1 or SW1990 cells were examined by performing a Transwell assay under hypoxic conditions. Magnification, x200. (D) Statistical analysis of invasion assay. (E) E-cadherin and vimentin expression were examined in SW1990 cells transfected with NC mimic, miR-519 mimic, miR-519 mimic and PD-L1 by western blotting under hypoxic conditions. GAPDH was used as the loading control. (F) Apoptosis of PANC-1 or SW1990 cells transfected with NCs, miR-519 mimics, or miR-519 mimics and PD-L1 were examined using an immunofluorescence assay with Annexin V staining under hypoxic conditions. Magnification, x200. (G) Statistical analysis of apoptosis assay. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001, as indicated. PD-L1, programmed death ligand 1; miR, microRNA; NC, negative control.

served as an effector of the miR-519-attenuated tumorigenesis of pancreatic cancer.

miR-519 and PD-L1 levels are dysregulated in vivo. To determine whether miR-519 and PD-L1 were dysregulated in an animal model, a xenograft tumor experiment was performed to assess the role of miR-519 in the tumorigenesis of PANC-1 cells. Cells treated with miR-519 mimics formed smaller tumors compared with NC-treated cells (Fig. 4A-C). In addition, the results demonstrated that miR-519 and PD-L1 levels were up- and downregulated, respectively, in mouse tumors with overexpressed miR-519 mimic (Fig. 4D and E). The present results indicated that miR-519 and PD-L1 are aberrantly expressed in mouse models, *in vivo*.

Discussion

The present study characterized the critical roles of miR-519 and PD-L1 in hypoxia-induced pancreatic cancer cell tumorigenesis (Fig. 4F). The results also revealed that miR-519 interacted with PD-L1 and regulated its expression. Additionally, miR-519 treatment inhibited invasiveness and tumor growth in a mouse model, and induced pancreatic cancer cell apoptosis by negatively regulating PD-L1. Clinically, it was determined that miR-519 and PD-L1 were aberrantly expressed in human pancreatic tumors compared with adjacent paracancerous tissues.

Pancreatic cancer is a highly malignant form of cancer, which represents the fourth most common cause of cancer-associated

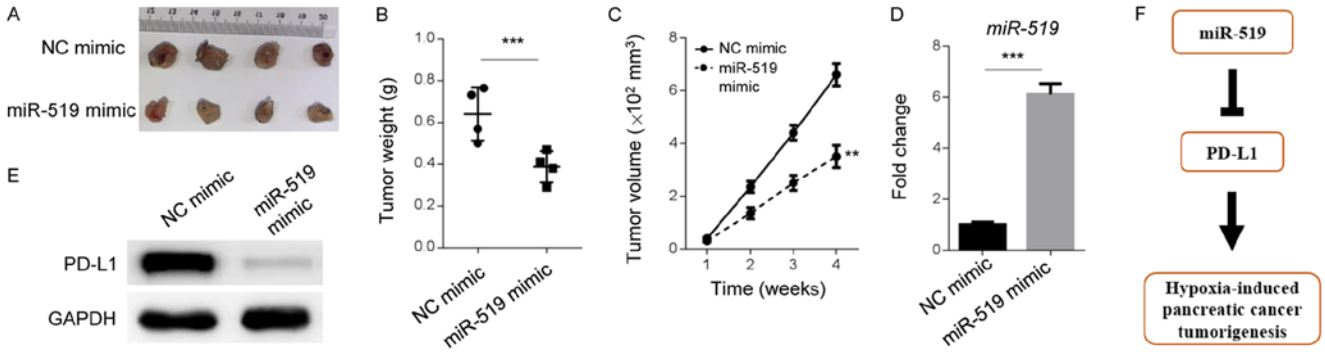

Figure 4. miR-519 and PD-L1 expression are dysregulated *in vivo*. (A) Xenograft assays revealed the *in vivo* tumor growth of PANC-1 cells transfected with NC mimics or miR-519 mimics (n=4). Statistical analysis of tumor (B) weight and (C) volume. (D) miR-519 levels were determined by reverse transcription-quantitative PCR. (E) Western blot analysis showed PD-L1 protein levels in cells with NC mimics or miR-519 mimics. GAPDH was used as the loading control. (F) Working model describing the role of miR-519/PD-L1 in pancreatic cancer. Data are presented as the mean ± standard deviation of three independent experiments. **P<0.01, ***P<0.001 vs. negative control. miR, microRNA; PD-L1, programmed death ligand 1; NC, negative control.

mortality worldwide (21). As early diagnosis is challenging and the prognosis is poor, surgery and chemotherapy remain the most effective and common therapeutic strategies for pancreatic cancer treatment (22,23). Recently, researchers and clinicians have demonstrated that immune checkpoint inhibitors have an efficacy of 50% in phase I clinical trials of patients with pancreatic cancer (24). However, objective responses were not observed. The present study indicated that miR-519 inhibited the tumorigenesis of pancreatic cancer via the PD-L1 signaling pathway. The results may catalyze the development of novel approaches by targeting miR-519 and PD-L1.

MicroRNAs have been demonstrated to be aberrantly expressed and implicated in hypoxia-induced tumor phenotypes (25). Hypoxic conditions are also associated with the upregulation of miR-21 expression in pancreatic cancer cells (26). Similarly, the present study revealed that miR-519 was modulated by hypoxia. Certain research groups have demonstrated that various miRNAs, including miR-212 and miR-224, promote pancreatic cancer progression via hypoxia-inducible factor 1α (27,28). By contrast, the present study revealed that miR-519 inhibited the tumorigenesis of pancreatic cancer cells, in accordance with prior conclusions (29,30). Additionally, the present study investigated and confirmed the inhibitory role of miR-519 under hypoxic conditions.

It has been demonstrated that immune checkpoint inhibitors, such as the cytotoxic T-lymphocyte-associated protein 4 receptor antibody Ipilimumab, increase the overall survival rate of patients with pancreatic cancer, when used in combination with GVAX (31,32). Furthermore, anti-PD-L1 drugs alone have exhibited less efficacy for the treatment of pancreatic cancer (33). Therefore, the synergistic therapeutic mechanisms of immune checkpoint inhibitors and chemotherapy or radiotherapy may represent a promising area for the development of therapeutics (34). The present study determined that PD-L1 served as a mediator of miR-519 in pancreatic cancer cells, indicating that miR-519 and PD-L1 targeting may facilitate the development of improved pancreatic cancer treatments.

In conclusion, the present study demonstrated a novel interaction between miR-519 and PD-L1, which influenced genesis and growth of pancreatic tumors. The *in vitro* and *in vivo* experiments performed represent solid foundations for explaining miR-519-mediated tumorigenesis. The current results may aid the development of an effective therapeutic method for patients with pancreatic cancer.

Acknowledgements

The authors would like to thank Dr Guo Jing (Tongji University School of Medicine) for the critical reading of this manuscript and for providing helpful suggestions.

Funding

No funding was received.

Availability of data and materials

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

Authors' contributions

KN, DZ and HC designed the study. KN, DZ, CC, YuY and HC collected and analyzed the data. KN and HC drafted and wrote the manuscript. YoY and SL were involved in the interpretation of data and critically revised the manuscript. All authors had intellectual input into the study and approved the final version of the manuscript.

Ethics approval and consent to participate

All animal protocols were approved by the Animal Care and Use Committee at the The Third Affiliated Hospital of Soochow University (Jiangsu, China). All procedures in the animal studies were performed in accordance with the ethical standards of the institution.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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