

MicroRNA-1179 regulates proliferation and chemosensitivity of human ovarian cancer cells by targeting the PTEN-mediated PI3K/AKT signaling pathway

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Abstract

Introduction: Owing to widespread roles of miRs, the dysregulation of their expression in human tissues has been linked with the development of several diseases such as cancer. The study was designed to investigate the role and therapeutic potential of miR-1179 in ovarian cancer.

Material and methods: Proliferation rate was monitored by MTT assay. Transfections were performed using Lipofectamine 2000 reagent. Cell cycle apoptosis was detected by AO/EB and annexin V/PI staining. Expressions analysis was carried out by qRT-PCR and western blotting. *In vivo* evaluation was carried out in xenografted mouse models.

Results: The results revealed that miR-1179 is considerably upregulated in ovarian cancer cell lines. Inhibition of miR-1179 triggers decrease in the viability via initiation of apoptotic cell death of ovarian PA-1 cancer cells. TargetScan analysis showed PTEN to be the main target of miR-1179 in PA-1 cells. Exploration of PTEN expression in ovarian cancer cell lines revealed up to 9-fold downregulation of PTEN. However, inhibition of miR-1179 in PA-1 cells resulted in upregulation of PTEN expression. In addition, overexpression of PTEN caused a reduction of PA-1 cell viability via induction of apoptotic cell death. However, silencing of miR-1179 could rescue the effects of miR-1179 inhibition on the proliferation of miR-1179. The miR-1179 suppression was accompanied by a significant decline in phosphorylation of PI3K and AKT expression in the PA-1 cells. The *in vivo* study showed that miR-1179 suppression inhibits the xenografted tumor growth.

Conclusions: The results of this study indicate that miR-1179 may prove to be an important therapeutic target for ovarian cancer.

Key words: microRNA, apoptosis, miR-1179, ovarian cancer.

Introduction

Ovarian cancer is one of the prevalent gynecological malignancies in women across the globe. Accounting for about 2.5% of all malignancies in women, it is responsible for 5% of all cancer-related deaths in women [1]. Although the incidence of ovarian cancer has declined over the last few decades the clinical outcome is still far from acceptable [2]. It has

been reported that in the United States alone more than 22 000 new ovarian cancer cases and 14 000 ovarian cancer deaths are recorded annually [3]. The main hurdles in the treatment of ovarian cancer are its late diagnosis and dearth of therapeutic targets. Improvement of prevention through early detection and identification of therapeutic targets may prove beneficial to curb ovarian cancer related mortality [4]. Over the last few decades microRNAs (miRs) have gained importance and are believed to act as therapeutic targets for treating numerous diseases [5]. Consisting of around 20 to 24 nucleotides, miRs act in almost all mammalian biological pathways such as apoptosis, proliferation, regulation of cell cycle and metabolism [6]. The miRs are aberrantly expressed under disease conditions such as cancer [7]. Each miR may modulate the expression of hundreds of mRNAs and as such may affect a wide array of processes, many of which are cancer related [8]. Among miRs, miR-1179 has been shown to be dysregulated in several cancer types and has the potential to act as a therapeutic target for drugs. miR-1179 acts an oncogene in several types of cancers such as esophageal, breast and colorectal cancers to name a few [9–11]. Recently, miR-1179 was shown to regulate the growth and metastasis of pancreatic cancer both *in vitro* and *in vivo* [12]. However, the role and therapeutic potential of miR-1179 is still largely unknown in ovarian cancer. Against this backdrop the present study focused on elucidating the role of miR-1179 in ovarian cancer and exploring its therapeutic potential. It was observed that miR-1179 is aberrantly upregulated in ovarian cancer and suppression of miR-1179 suppresses the proliferation of ovarian cancer cells by targeting the phosphatase and tensin homolog (PTEN) mediated PI3K/AKT pathway. To sum up, the present study shows that miR-1179 may prove an imperative therapeutic target for ovarian cancer treatment.

Material and methods

Cell lines and culture conditions

The human ovarian cancer cell lines (SK-OV-3, OVACAR-3, PA-1, SW-626, Caov3) and normal ovarian cell line (SV40) were obtained from Sun Yat-sen University Cancer Center (Guangzhou, China). Culturing of the cells was performed in RPMI 1640 medium (Invitrogen). The media was supplemented with 10% FBS and the incubation of the cells was carried out at 37°C in a humidified atmosphere with 5% CO₂.

Expression analysis

The TRIzol reagent (Invitrogen) was used for the extract of RNA from the tissues and cell lines. This was followed by purification of the RNA by

the RNeasy Mini Kit (Qiagen). The complementary DNA was then synthesized using the miScript Reverse Transcription Kit (Qiagen). Afterwards the cDNA was amplified using SYBR Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan). The expression was estimated by the 2^{-ΔΔC_t} method and actin was used as an internal control. The primers for miR-1179 were 5'-GCGGAAGCATTCTTTCATT-3' (sense) and 5'-CAAGGGCTCGACTCCTGTTC-3' (anti-sense) and the primers for PTEN were 5'-ACCAGGACCAGAG-GAAACCT-3' (sense) and 5'-GCTAGCCTCTGGATTGACG-3' (anti-sense).

Cell transfection

The 1179 inhibitor and NC were synthesized by RiboBio (Guangzhou, China). The transfection was then carried out using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. As the PA-1 and SK-OV-3 cells reached 80%, the appropriate concentration of miR-27a inhibitor or miR-NC was transfected into PA-1 and SK-OV-3 cells.

MTT cell viability assay

In brief, the PA-1 and SK-OV-3 cells were subjected to transfection with appropriate miRNA constructs and cultured at 37°C in 96-well plates for 24 h. Subsequently, MTT was added into the cell culture and the formazan crystals formed were dissolved by the addition of DMSO (200 μl). Absorbance was then taken at 570 nm to determine the viability of the PA-1 and SK-OV-3 cells.

Apoptosis assays

In brief, 0.6 × 10⁶ transfected PA-1 and SK-OV-3 ovarian cancer cells were cultured for 24 h at 37°C and then fixed with ethanol (70%) for 20 min. The cells were then subjected to PBS washing and subsequently stained with a solution of AO/EB or DAPI. Finally, the cells were examined under a microscope to detect the induction of apoptosis. For determination of the percentage of apoptosis, the transfected cells were stained with annexin V/PI and subsequently examined by a flow cytometer.

Target identification

The target of miR-1179 was identified with the help of the online software TargetScan version 7.2 (<http://www.targetscan.org>) using default parameters.

Western blotting

The ovarian cancer cells were transfected with appropriate constructs and cultured for 24 h at 37°C. Next the cultures were transferred to centrifuge tubes and centrifuged at 13,000 g for 15 min

at 4°C. The pellets were washed with PBS and then resuspended in lysis buffer. BCA assay was used to determine the concentration of the proteins in each sample. Equal concentrations of the proteins from each sample were loaded and separated on SDS-PAGE (10%) and then transferred to polyvinylidene fluoride membrane. These membranes were then treated with TBS and then exposed to primary antibody (dilution 1 : 1000, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C for 24 h. Afterwards, the cells were treated with HRP-conjugated anti-rabbit secondary antibody and subsequently chemiluminescence reagent was used for visualization of the proteins.

In vivo study

The xenograft male C57 BL/6 mice were maintained in the animal holding capacity of Lianshui County People's Hospital, Huai'an, 223400, China following the National Institutes of Health standards for the care and use of laboratory animals. The animals received free access to the standard diet in well-ventilated rooms with a 12 : 12 dark/light cycle and a temperature of around 27°C. The mice were randomly divided into two groups, and the miR-1179 or NC transfected PA-1 cells were delivered into the left flanks of the mice through subcutaneous injections. The mice were monitored and the growth of the tumors was monitored until the end of the study. The mice were sacrificed at the end of the study (7 weeks) and tumors were harvested and used for further experimentation.

Statistical analysis

Experiments were done in triplicate and expressed as mean \pm SD. Student's *t*-test using GraphPad prism 7 software was employed for statistical analysis and values of $p < 0.05$ were considered statistically significant.

Results

miR-1179 is upregulated in ovarian cancer cell lines

Gene expression analysis revealed miR-1179 to be significantly upregulated in the ovarian cancer tissues as compared to the adjacent normal tissues. The upregulation of miR-1179 in ovarian cancer tissues was observed to be up to more than 7-fold (Figure 1 A). The expression of miR-1179 was assessed in normal and ovarian cancer cell lines by qRT-PCR (Figure 1 B). The results showed that miR-1179 was significantly enhanced ($p < 0.05$) in all the ovarian cancer cell lines. In addition, miR-1179 was observed to be upregulated in ovarian cancer lines by 3.4-fold in comparison to the normal cell line SV40.

Suppression of miR-1179 inhibits proliferation of ovarian cancer cells by induction of apoptosis

Next, we wanted to understand the role of miR-1179 in ovarian cancer. Therefore, the PA-1 and SK-OV-3 cells were subjected to transfection with either the miR-1179 inhibitor or NC (negative control). The suppression of miR-1179 in PA-1 and SK-OV-3 cells was confirmed by qRT-PCR (Figure 1 C). The viability of the NC and miR-1179 inhibitor transfected PA-1 and SK-OV-3 cells was assessed at various time points by MTT assay. It was found that miR-1179 inhibition resulted in remarkable decline in the viability of PA-1 and SK-OV-3 cells (Figure 1 D). DAPI and AO/EB assay of the NC and miR-1179 inhibitor transfected PA-1 cells was carried out to understand the underlying mechanism and it was revealed that miR-1179 suppression resulted in activation of apoptosis of the PA-1 and SK-OV-3 cells (Figure 1 E). Furthermore, annexin V/PI staining showed that the apoptotic cell percentage increased from 3.7 and 4% in NC to about 37.1 and 21.2% in the miR-1179 inhibitor transfected PA-1 and SK-OV-3 cells (Figure 1 E). These results unequivocally suggest that miR-1179 suppression inhibits PA-1 cell proliferation by initiation of apoptotic cell death.

miR-1179 suppression enhances chemosensitivity of PA-1 cells to 5-fluorouracil

Next we investigated the effects of miR-1179 inhibition on the chemosensitivity of the PA-1 ovarian cancer cells. Hence, miR-1179 inhibitor, 5-FU treated (2.5 μ M), or miR-1179 transfected plus 5-fluorouracil treated cells (2.5 μ M) were subjected to MTT assay and the cell viability was monitored at different time points. It was found that viability of the miR-1179 inhibitor transfected plus 5-FU treated PA-1 cells decreased significantly ($p < 0.05$) as compared to the miR-inhibitor transfected or 5-FU treated cells (Figure 2). Taken together, these results indicate miR-1179 inhibition increases the sensitivity of the PA-1 cells to the anticancer drug 5-FU.

miR-1179 targets PTEN in ovarian cancer cells

The target of miR-1179 in ovarian cancer cells was identified by online TargetScan analysis. The results indicated PTEN as the main target of miR-1179 in PA-1 ovarian cancer cells (Figure 3 A). The PTEN expression was also checked in all the ovarian cancer tissues and cell lines. It was observed that relative to the expression of PTEN was significantly downregulated in both the ovarian cancer tissues and cell lines (8.5-fold) in the ovarian

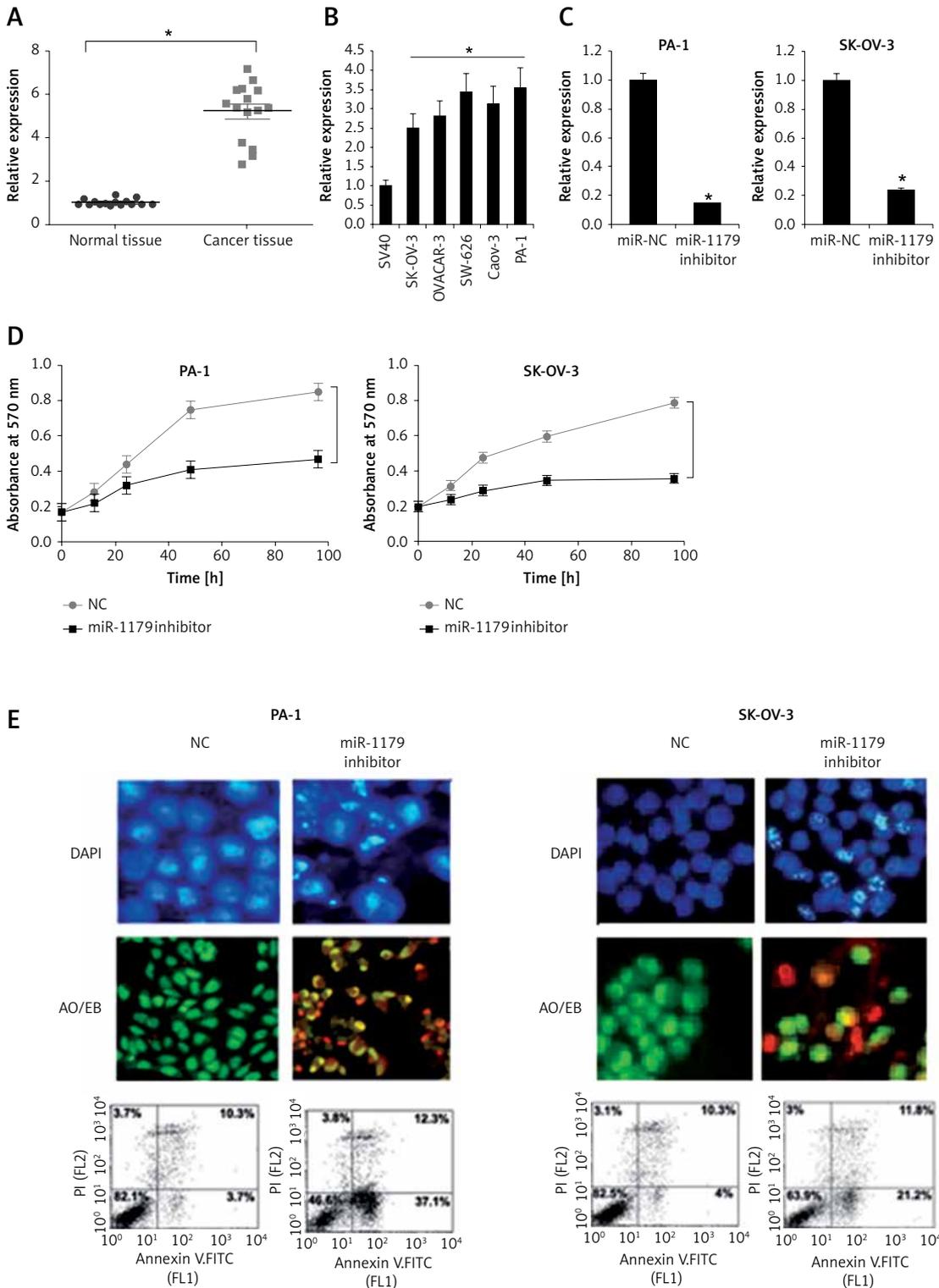


Figure 1. Suppression of miR-1179 inhibits proliferation of ovarian cancer cell lines: **A** – qRT-PCR analysis showing the expression of miR-1179 in ovarian cancer tissues and adjacent normal tissues; **B** – qRT-PCR analysis showing the expression of miR-1179 in ovarian cancer cell lines; **C** – qRT-PCR analysis showing the expression of miR-1179 in NC or miR-1179 inhibitor transfected PA-1 and SK-OV-3 ovarian cancer cells; **D** – Cell viability of the NC or miR-1179 inhibitor transfected PA-1 and SK-OV-3 ovarian cancer cells; **E** – DAPI, AO/EB or annexin V/PI staining of NC or miR-1179 inhibitor transfected PA-1 and SK-OV-3. The experiments were carried out in three biological replicates and expressed as mean ± SD (**p* < 0.05)

cancer cell lines (Figures 3 B, C). However, as the PA-1 cells were transfected with the miR-1179 inhibitor, the expression of PTEN was remarkably enhanced as depicted by the western blot analysis (Figure 3 D). The impact of the PTEN overexpression on the viability of the ovarian cancer PA-1 cells was also investigated. It was found that the overexpression of PTEN (Figure 3 E) caused a significant ($p < 0.05$) decline in the viability of the PA-1 ovarian cancer cells (Figure 3 F). The decline in viability was again found to be due to the induction of apoptosis as indicated by DAPI, AO/EB and annexin V/PI staining (Figures 3 G, H).

PTEN rescues growth inhibitory effects of miR-1179 on PA-1 cells

Since miR-1179 suppression and PTEN overexpression showed similar effects on the viability of the PA-1 cells, we wanted to determine whether PTEN silencing could rescue the growth inhibitory effects of miR-1179 suppression in PA-1 cells. Intriguingly, it was observed that PTEN silencing in the miR-1179 inhibitor transfected PA-1 cells increased the viability of the PA-1 cells, suggesting that the inhibitory effects of the miR-1179 silencing are directly due to PTEN overexpression (Figure 4 A).

miR-1179 modulates PI3K/AKT pathway in PA-1 cells

As miR-1179 was found to target PTEN and PTEN is a strong regulator of the PI3K/AKT signaling pathway, we determined the expression of AKT, p-AKT, PI3K and p-PI3K in NC, miR-1179 inhibitor and miR-1179 inhibitor mimics plus si-PTEN transfected PA-1 cells. It was found that miR-1179 inhibition caused a significant decline in the phosphorylation of AKT and PI3K. However, PTEN silencing could at least partially rescue the effects of miR-1179 inhibition on the phosphorylation (Figure 4 B).

miR-1179 inhibition suppresses the xenografted tumor growth *in vivo*

The effects of miR-1179 inhibition on the growth of xenografted tumors in mice were also examined (Figure 4 C). It was found that the miR-1179 inhibition in the xenografted tumors reduced the tumor weight as well as the volume time dependently (Figures 4 D, E).

Discussion

Ovarian cancer accounts for 0.152 million deaths annually and around 0.3 million new ovarian cases are diagnosed annually worldwide [13–15]. The clinical outcome is unsatisfactory due its diagnosis

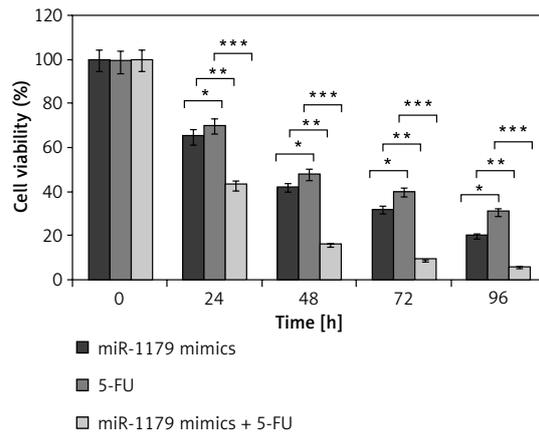


Figure 2. Effect of miR-1179 suppression on the chemosensitivity of PA-1 ovarian cancer cells to 1.5 μ M concentration of 5-fluorouracil as determined by MTT assay. The experiments were carried out in three biological replicates and expressed as mean \pm SD (* $p < 0.05$)

at advanced stages and unavailability of therapeutic targets. In addition, the development of drug resistance in cancer cells forms another major hurdle in the treatment of ovarian cancer [3]. It has been reported that miRNAs modulate the expression of the majority of the human genes and regulate a diversity of cellular processes [16]. Because of the importance of the miRNAs in cellular and physiological processes, several studies have revealed the potential of miRNAs as therapeutic targets [17]. Herein, the role and therapeutic implications of miR-1179 were investigated in ovarian cancer. The results revealed that miR-1179 was aberrantly upregulated in the ovarian cancer cells. Previous studies have indicated that dysregulated expression of miR-1179 may be associated with the development of gastric cancer [18]. Moreover, miR-1179 has been shown to be dysregulated in glioblastoma and also regulates its proliferation and metastasis [19]. Inhibition of miR-1179 in PA-1 ovarian cancer cells leads to a remarkable decrease in the proliferation rate of PA-1 ovarian cancer cells via induction of apoptotic cell death. Studies carried out previously have shown that miR-1179 suppresses induction of apoptosis of pancreatic cancer cells by targeting E2F5 [12]. In the current study it was also found that miR-1179 increases the chemosensitivity of PA-1 ovarian cancer cells to 5-FU, which is also supported by previous studies wherein several miRNAs have been found to control the chemosensitivity of the cancer cells to anticancer drugs [20]. *In silico* analysis indicated PTEN to be the main target of miR-1179. Herein, we observed that PTEN is highly downregulated in ovarian cancer and miR-1179 inhibition could upregulate the expression of PTEN. Additionally, PTEN silencing could also inhibit the

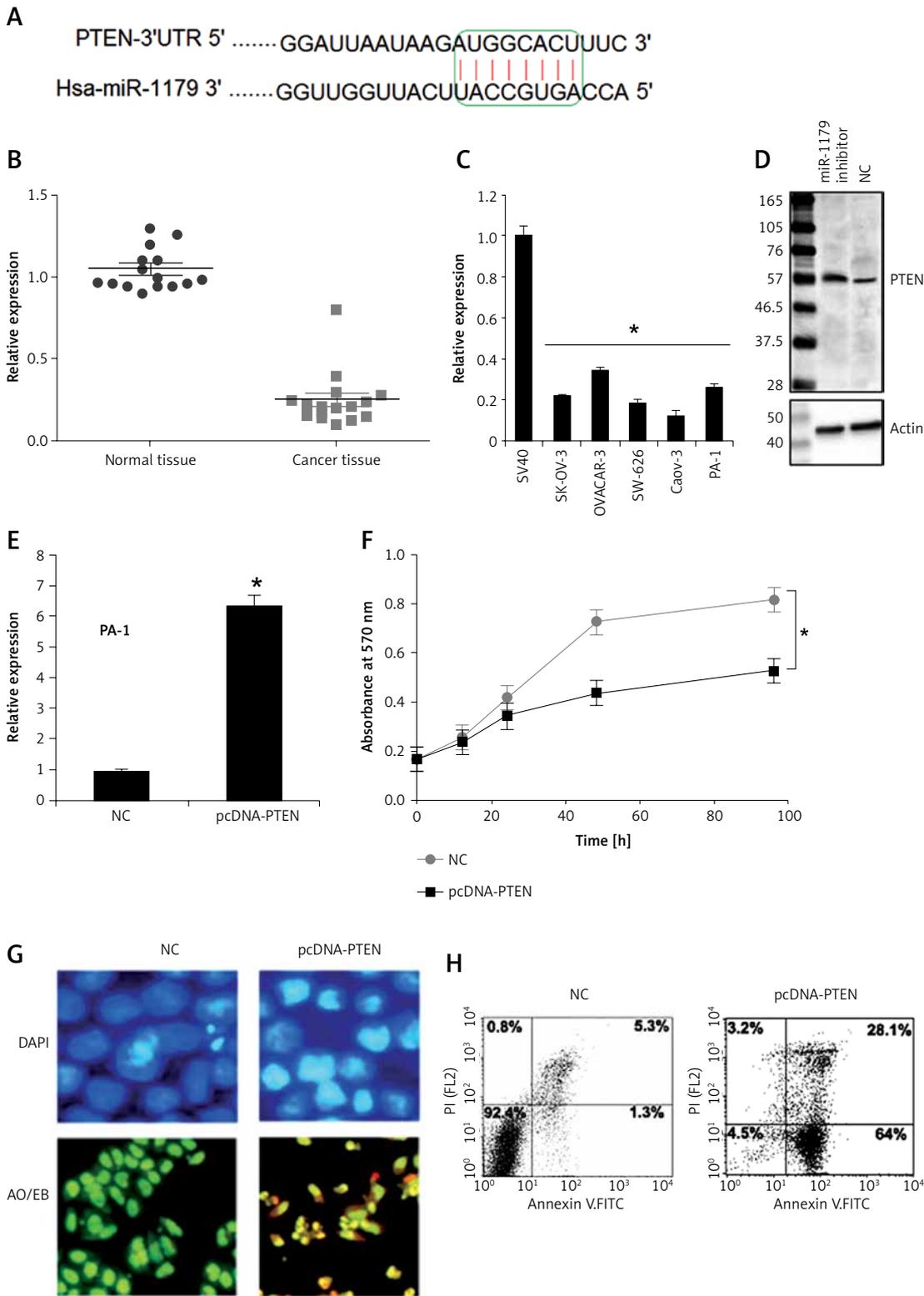


Figure 3. miR-1179 exerts its effects by targeting PTEN: **A** – TargetScan analysis showing PTEN as the target of miR-1179; **B** – qRT-PCR analysis showing the expression of PTEN in ovarian cancer tissues and normal adjacent tissues; **C** – qRT-PCR analysis showing the expression of PTEN in ovarian cancer cell lines; **D** – western blot analysis showing the expression of PTEN in NC or miR-1179 inhibitor transfected PA-1 cells; **E** – qRT-PCR analysis showing the expression of PTEN in NC or pcDNA-PTEN transfected PA-1 ovarian cancer cells; **F** – cell viability of the NC or miR-pcDNA-PTEN transfected PA-1 ovarian cancer cells; **G** – DAPI and AO/EB staining of NC or pcDNA-PTEN transfected PA-1 ovarian cancer cells; **H** – Annexin V/PI staining of NC or pcDNA-PTEN transfected PA-1 ovarian cancer cells. The experiments were carried out in three biological replicates and expressed as mean \pm SD (* $p < 0.05$)

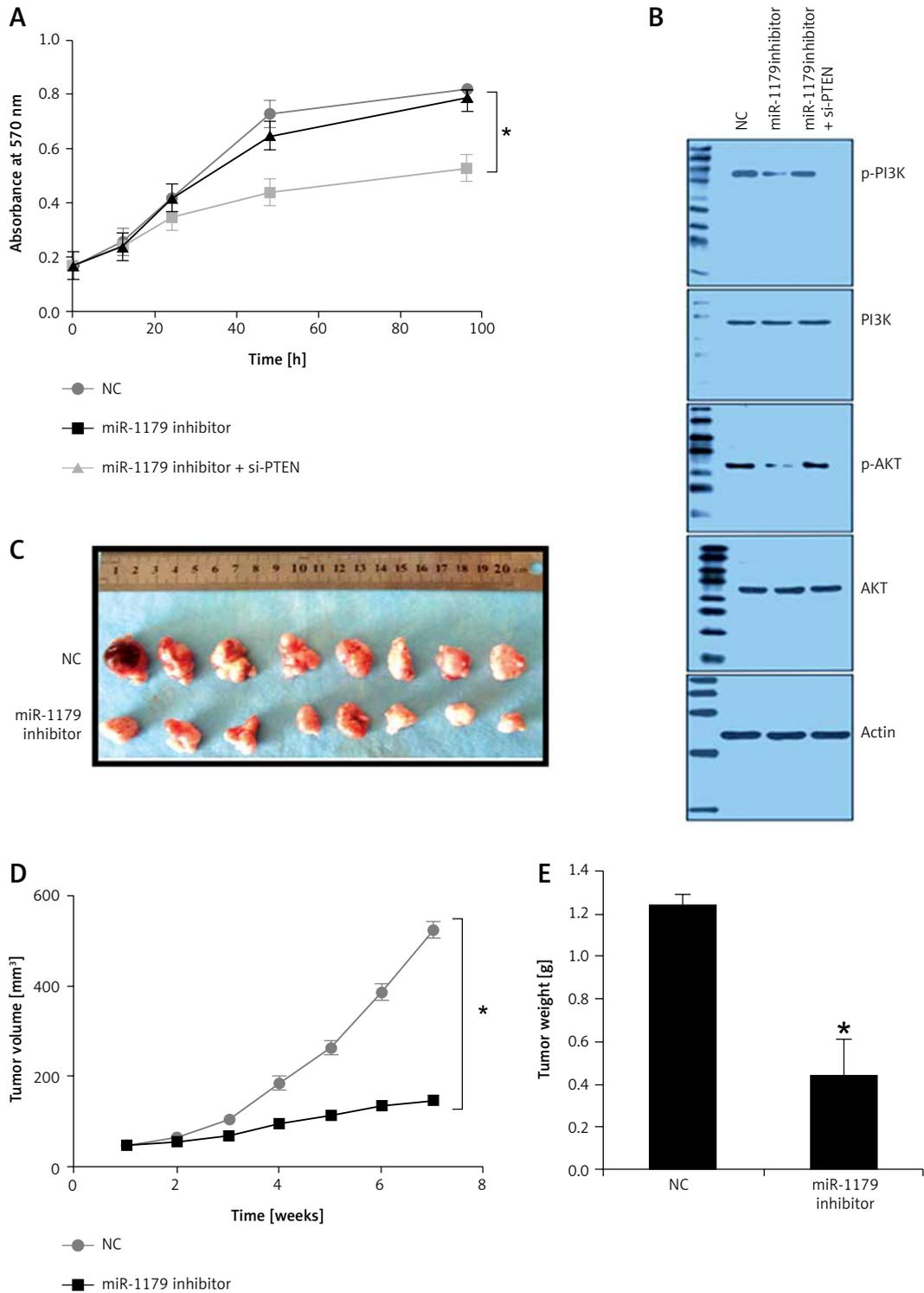


Figure 4. A – Effect of PTEN silencing on the viability of the miR-1179 transfected cells, B – expression of p-PI3K, PI3K, p-AKT and AKT in NC, miR-1179 mimics and miR-1179 mimics + Si-PTEN transfected cells, C – images of the NC and miR-1179 inhibitor xenografted tumors, D – effect of miR-1179 suppression on the tumor volume, E – effect of miR-1179 suppression on the tumor weight. The experiments were carried out in three biological replicates and expressed as mean \pm SD ($*p < 0.05$)

growth of PA-1 ovarian cancer cells via induction of apoptosis similar to that of miR-1179 overexpression. Further, PTEN silencing was found to at least partially rescue the effects of miR-1179 inhibition on the proliferation of the PA-1 cells. Studies have shown PTEN is a negative regulator of PI3K/AKT [21] and therefore we examined the effects of NC, miR-1179 inhibitor and miR-1179 inhibitor plus si-PTEN on the phosphorylation of PI3K and AKT, and it was found that miR-1179 inhibition could block the phosphorylation of PI3K and AKT. However, PTEN silencing could prevent these effects of miR-1179 inhibition. These observations are well supported by a previous investigation wherein miR-1179 was shown to regulate the proliferation of non-small lung cancer cells via the AKT signaling pathway [22]. Previous studies also have shown that miR-1179 regulates the growth of pancreatic xenografted tumors *in vivo* [12]. Therefore we investigated the effects of miR-119 in xenografted tumors *in vivo*, and the results showed that miR-1179 inhibition could suppress the growth of the xenografted tumors, indicative of the therapeutic potential of miR-1179 in the management of ovarian cancer.

In conclusion, miR-1179 is upregulated in human ovarian cancer cells. Suppression of miR-1179 inhibits the proliferation of ovarian cancer cells by inducing apoptosis via targeting of the PTEN-mediated PI3K/AKT pathway. Henceforth, miR-1179 may prove to be an essential therapeutic target for the treatment of ovarian cancer.

Conflict of interest

The authors declare no conflict of interest.

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