MicroRNA-let-7f-1 is induced by lycopene and inhibits cell proliferation and triggers apoptosis in prostate cancer

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Abstract. Previous studies have suggested that lycopene has cytotoxic effects in a variety of types of human cancer. An improved understanding of the mechanisms underlying the anticancer effects of lycopene may provide novel therapeutic targets for cancer treatment. PC3 cells were treated with different concentrations of lycopene for 24 and 48 h, the level of protein kinase B (AKT2) was detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting. Additionally, the expression levels of microRNA (miR)-let-7f-1 were measured using RT-qPCR. miR-let-7f-1 function was analyzed using cell proliferation and apoptosis assays in gain- and loss-of-function experiments. It was observed that lycopene downregulated the expression of AKT2 and upregulated the expression of miR-let-7f-1 in PC3 cells. Re-introduction of miR-let-7f-1 into PC3 cells was able to inhibit cell proliferation and induce apoptosis. Further investigation indicated that miR-let-7f-1 targeted AKT2 in PC3 cells and upregulation of AKT2 could attenuate the effects induced by miR-let-7f-1. The results of the current study indicate that miR-let-7f-1 is involved in the anticancer effects of lycopene and serves an important role in the inhibition of prostate cancer progression through the downregulation of AKT2.

Introduction

Prostate cancer is the most common cancer diagnosed and the second leading cause of cancer-associated mortality in males in the USA, with an estimated 220,800 newly diagnosed cases and 27,540 cases of cancer-associated mortality predicted to occur in 2015 in the USA (1). Lycopene, a carotenoid found in tomatoes, exhibits multiple bioactivities and has been reported to protect against prostate cancer via inhibition of cancer cell proliferation and induction of apoptotic cell death (2-5). A previous study suggested that lycopene regulates the breast cancer cell cycle and apoptosis via the suppression of expression of cell cycle regulatory proteins (6). Additional studies have reported that high intakes of tomato-based products were associated with a 10-20% reduction in the risk of prostate cancer (7,8). However, the molecular mechanisms responsible for the effects of lycopene on prostate cancer remain to be fully elucidated.

MicroRNAs (miRNAs) are a class of endogenous small non-coding RNAs that regulate the expression of target genes at transcriptional and post-transcriptional levels (9,10). Previous studies have reported that miRNAs may function as oncogenes or tumor suppressors in various types of cancer, including lung cancer, breast cancer, hepatocellular carcinoma and gastric cancer (11-14). Additionally, studies have observed aberrant expression of numerous miRNAs in prostate cancer (15-17). In addition, circulating miRNAs in serum or plasma samples have been demonstrated to be associated with patient survival, and may be developed as potential biomarkers for prostate cancer diagnosis and recurrence (18). Let-7f-1 was reported to inhibit proliferation, migration and *in vivo* tumor formation of human glioblastoma cancer cells by downregulating the expression of the oncogenes pan-RAS, N-RAS and K-RAS (19). A previous study demonstrated that miR-let-7f-1 mediated cisplatin resistance via targeting secreted protein, acidic, cysteine-rich (osteonectin) (SPARC), a crucial regulator of multiple cellular signal transduction pathways (20). Let-7f was reported to be upregulated in primary breast cancer using miRNA microarray, however was not validated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis (21). Previous studies have indicated that lycopene inhibited prostate cancer progression though multiple growth factor-mediated signaling pathways (22-25). Therefore, whether specific miRNAs targeting growth factor signalling pathways are involved in the antitumor activity of lycopene remains to be further elucidated.

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Materials and methods

Cell culture and treatment. Human prostate carcinoma cells PC3 (CRL-1435) were purchased from the American Type Culture Collection (Manassas, VA, USA) and were maintained in RPMI 1640 media (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in a 5% CO_2 incubator. Lycopene (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in tetrahydrofuran (Sigma-Aldrich) as 20 mM stock solution and maintained at -20°C. For the experiments, PC3 cells were cultured in serial concentrations of lycopene (10, 20 and 50 μm) and control cultures were treated with water only.

RNA extraction and RT-qPCR. Total RNA was isolated from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. For miR-let-7f-1 detection, miRNA was polyadenylated and reverse transcribed into cDNA with the One Step PrimeScript miRNA cDNA Synthesis kit (Takara Biotechnology, Co., Ltd., Dalian, China) in triplicate. For AKT2 detection, cDNA (50 ng) was synthesized using the PrimeScript RT reagent kit (Takara Biotechnology, Co., Ltd.) according to the manufacturer's protocol. RT-qPCR analysis was performed with SYBR Green (Takara Biotechnology, Co., Ltd.) in an ABI 7500 Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The small nuclear RNA U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were used as internal controls for miRNA and mRNA detection, respectively. The forward primers for the miR-let-7f-1 and U6 were synthesized by Biosune Co., Ltd. (Shanghai, China), and the sequences were as follows: miR-let-7f-1, 5' CTATACAATCTATTGCCTTCCC 3' and U6, 5' TGCGGGTGCTCGCTTCGGCAGC 3'. The reverse primers for the miR-let-7f-1 and U6 were universal adaptor primers available in a ready-to-go format in the One Step PrimeScript miRNA cDNA Synthesis kit (D350A; Takara Biotechnology, Co., Ltd.). The primers for the AKT2 and GAPDH genes were obtained from Primerbank (http://pga.mgh.harvard.edu/primerbank/) and the sequences were as follows: AKT2, forward 5'-AGG CACGGGCTAAAGTGAC-3' and reverse 5'-CTGTGTGAG CGACTTCATCCT-3'; and GAPDH, forward 5'-CTGGGCTAC ACTGAGCACC-3' and reverse 5'-AAGTGGTCGTTGAGG GCAATG-3'. The $\Delta\Delta$Cq method (26) was used in the analysis of the PCR data.

Oligonucleotide transfection. miR-let-7f-1 mimics, miR-let-7f-1 inhibitors (anti-miR-let-7f-1) and their corresponding controls, scramble and negative controls (NC), respectively, were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). Cells were transfected with 50 nM oligonucleotides using Superfect™ Transfection Reagent (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. Subsequent to 48 h transfection, cells were harvested and the miR-let-7f-1 expression level was confirmed by RT-qPCR.

Cell proliferation assay and apoptosis assay. Following 24 h cultivation, cells were transfected with 50 μm lycopene. To measure the effect of miR-let-7f-1 mimics or lycopene on cell proliferation, cells ($2x10^3$) were incubated in 96-well plates in 100 μl medium containing 10% FBS. A total of 10 μl WST-1 (Roche Diagnostics GmbH, Mannheim, Germany) was added to each well at the indicated time points and the plate was incubated for another 2 h at 37°C. The absorbance was measured at 450 nm using a microtiter plate reader (Spectra Rainbow; Tecan Group Ltd., Männedorf, Switzerland) according to the manufacturer's instructions. Relative optical density was calculated using a Spectra Rainbow microplate reader (Tecan Group, Ltd., Männedorf, Switzerland) in three independent experiments.

Cell apoptosis assay was determined using the Annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Detection kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. PC3 cells treated as indicated were harvested and resuspended in 100 μl annexin V-FITC labeling solution containing 5 μl annexin V-FITC and 5 μl propidium iodide (BD Pharmingen) and incubated for 30 min at room temperature in dark. Subsequent to incubation, the samples were analyzed by the FC500 Flow Cytometer (Beckman Coulter, Inc., Miami, FL, USA). Each measurement was performed in quadruplicate and each experiment was repeated at least three times.

Western blotting. Whole cell protein lysates were extracted using radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.) containing 200 ml Na_3VO_4, 200 mM NaF, 0.5 M ethylenediaminetetraacetic acid and proteinase inhibitors, for 30 min on ice. The protein concentrations were quantified with Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal amounts of proteins (40 μg) were separated by 10% sodium dodecyl sulfate-polyacrylimide gel electrophoresis (Bio-Rad Laboratories, Inc.) and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA), which were incubated with primary monoclonal antibodies against rabbit AKT2 (1:1,000; 2964), mouse phosphorylated AKT2 (Ser474) (1:1,000; 12694; both Cell Signaling Technology, Inc., Danvers, MA, USA) and rabbit GAPDH (1:1,000; sc-47724; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4°C overnight. The membranes were washed 3 times in Tris-buffered saline with Tween-20 (TBST) and incubated with the corresponding horseradish peroxidase-conjugated secondary bovine anti-mouse (sc-2371)and anti-rabbit (sc-2370) IgG antibodies, (both 1:1,000, Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Subsequent to three washes with TBST, the bound secondary antibody was detected using an enhanced chemiluminescence system (Pierce Biotechnology, Inc., Rockford, IL, USA). The signals were detected using a SuperSignal Protein Detection kit (Pierce Biotechnology, Inc.). The band density of specific proteins was quantified subsequent to normalization with the density of GAPDH using ImageJ (version 1.48; National Institutes of Health, Bethesda, MA, USA.

Bioinformatics and luciferase assay. In silico analyses were performed to determine the putative miRNAs able to target AKT2. TargetScan 6.2 software (http://www.targetscan.org) was used and the 3'untranslated region (UTR) target regions
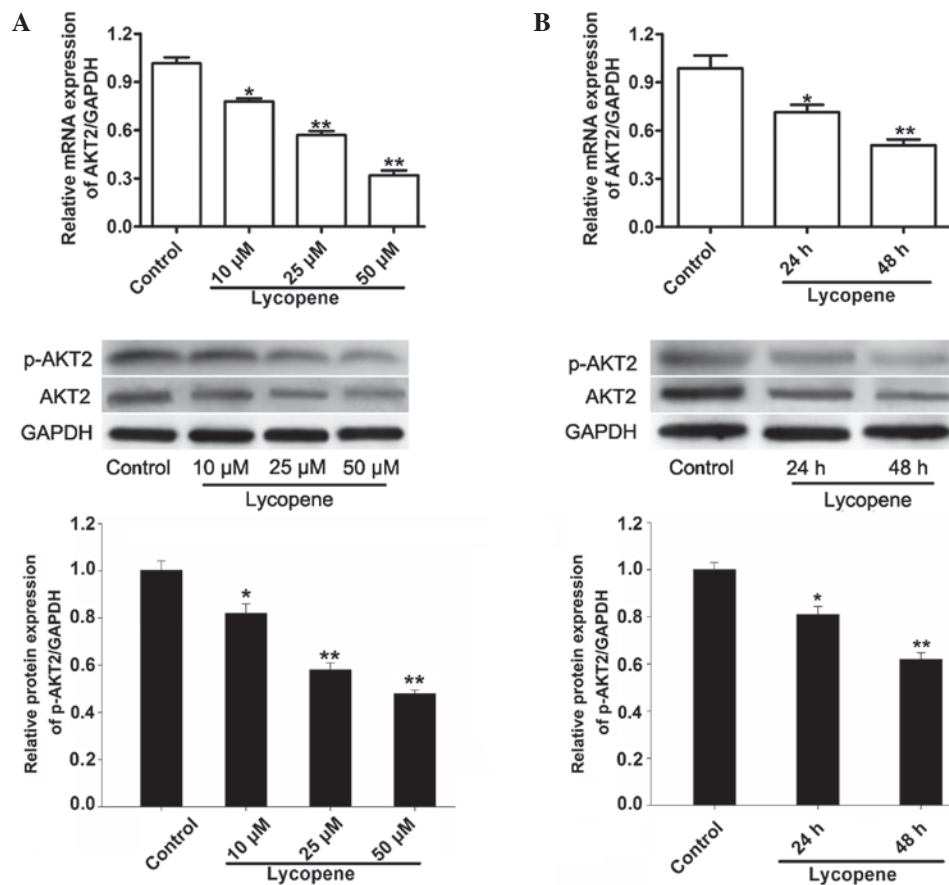
Figure 1. Lycopene reduced the expression of AKT2 in PC3 cells. (A) PC3 cells were treated with different doses of lycopene as indicated for 48 h, then the mRNA expression levels of AKT2 were evaluated by RT-qPCR (upper panel) and the protein levels of AKT2 were evaluated by western blotting (lower panel). (B) PC3 cells were treated with 25 μM lycopene for 24 and 48 h, then the mRNA expression levels of AKT2 were evaluated by RT-qPCR and the protein levels of AKT2 were evaluated by western blotting. GAPDH was used as the loading control. *P<0.05, **P<0.01. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; p-AKT2, phosphorylated-AKT2.

were selected in order to determine miRNA recognition elements which were involved in cell proliferation.

The full length 3'UTR of the AKT2 gene was PCR-amplified from genomic DNA and inserted into the XhoI and NotI sites of the psi-CHECK2 vector (Promega Corporation, Madison, WI, USA), downstream of the luciferase gene, to generate the plasmids AKT2-UTR-WT. The sequences of primers used were: Forward 5'-AAACTCGAGGCAGTCTGCCCACGC AGA-3' and reverse 5'-AAAGCGGCCGCCAGGACTGCT GGTAGCACCA-3'. AKT2-UTR-MUT plasmids were generated from AKT2-UTR-WT by mutating the miRNA binding site using a Quick Change Site-Directed Mutagenesis kit (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA) with the following primers: 5'-TGGGCACAGGCC TGGCGGGGGTCATCTTTTTAGTGCCTCTC-3' (forward) and 5'-GAGAGGCACTAAAAAGATGACCCCGCCAGG CCTGTGCCCA-3' (reverse). All constructs were verified by sequencing.

For the luciferase reporter assay, PC3 cells were cultured in 96-well plates and incubated at 37˚C for 24 h prior to transfection. Luciferase reporter constructs and miR-let-7f-1 mimics or miR-let-7f-1 inhibitors were transfected using Superfect™ transfection reagent (Qiagen, Inc.). Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega Corporation) 48 h post-transfection.

Renilla luciferase activity was normalized to firefly luciferase activity. All transfection experiments were conducted in triplicate and repeated 3 times independently.

Statistical analysis. Data were expressed as the mean ± standard deviation of at least three independent experiments. Statistical analyses were analyzed using Student's two-tailed t-test. All analyses were performed with SPSS software, version 15.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Lycopene downregulated the expression of AKT2. Previous reports have demonstrated that lycopene was able to inhibit the growth of human colon cancer cells and breast cancer cells by altering PI3K/Akt signaling pathways (27-29), which served a central role in the promotion of cell proliferation and the inhibition of cell death (30,31). We investigated whether the expression of AKT2 was reduced by lycopene. As indicated in Fig. 1, lycopene reduced the expression of AKT2 in a time- and dose-dependent manner at mRNA and protein levels. The mRNA levels of AKT2 in the PC3 cells were reduced by 19, 42 and 67% in response to treatment with 10, 25 and 50 μM lycopene at 48 h compared with the
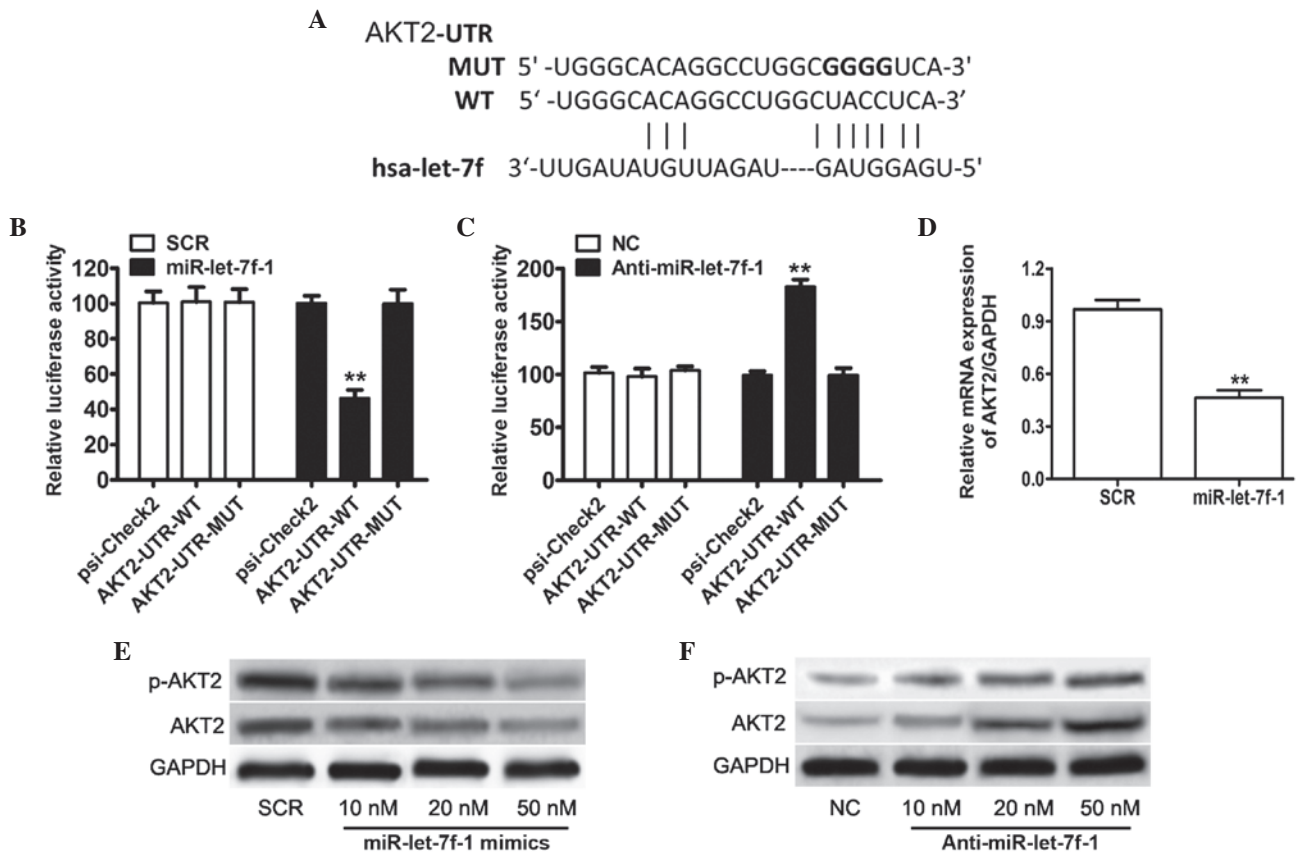
Figure 2. AKT2 was identified as target gene of miR-let-7f-1. (A) A schematic representation indicating the putative target site for miR-let-7f-1 in the AKT2 mRNA 3'UTR. (B) Luciferase assays on PC3 cells transfected with the psi-CHECK2 control, AKT2-UTR-WT or AKT2-UTR-MUT reporters and miR-let-7f-1 mimic oligonucleotides or SCR. (C) Luciferase assays on PC3 cells transfected with the psi-CHECK2 control, AKT2-UTR-WT or AKT2-UTR-MUT reporters and miR-let-7f-1 inhibitor oligonucleotides or NC. (D) The relative mRNA expression levels of AKT2 were determined by reverse transcription-quantitative polymerase chain reaction. (E) Endogenous protein levels of AKT2 and phosphorylated AKT2 in PC3 cells subsequent to transfection with miR-let-7f-1 and scramble control oligonucleotides were detected by western blotting. (F) Western blot assay indicating the expression levels of AKT2 and p-AKT2 in PC3 cells following transfection with the miR-let-7f-1 inhibitor and NC. **P<0.01. miR, microRNA; UTR, untranslated region; WT, wild-type; MUT, mutant; NC, negative control; p-AKT2, phosphorylated AKT2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SCR, scramble.

control (Fig. 1A, upper panel). Meanwhile the protein levels of AKT2 and phosphorylated AKT2 in the PC3 cells were significantly reduced by 18, 42 and 52% following treatment with 10, 25, and 50 μM lycopene at 48 h (Fig. 1A, lower panel). Treatment of PC3 cells with 25 μM lycopene for 24 h or 48 h also significantly reduced AKT2 mRNA expression levels by 29 and 49% (Fig. 1B, upper panel) and protein levels by 19 and 38% (Fig. 1B, lower panel).

miR-let-7f-1 regulates AKT2 expression through direct binding to its 3'UTR. It is known that that miRNAs exert their biological functions by binding to the 3'-UTR and inhibiting expression of their target genes (9). For this reason, bioinformatics were used to predict the potential miRNAs targeting AKT2. By searching TargetScan, AKT2 was identified as the target of miR-let-7f-1 with the highest possibility.

To confirm the negative regulation of miR-let-7f-1 on AKT2, the 3'-UTR of AKT2 was cloned into a luciferase reporter construct (Fig. 2A). The reporter assay indicated that overexpression of miR-let-7f-1 triggered a marked reduction of luciferase activity of the AKT2-UTR-WT plasmid by 54% in PC3 cells compared with the scramble control, without alteration in luciferase activity of AKT2-UTR-MUT (Fig. 2B). By contrast, inhibition of miR-let-7f-1 dramatically led to a

marked increase of luciferase activity of AKT2-UTR-WT by 91%, without alterations in luciferase activity of AKT2-UTR-MUT (Fig. 2C). Consistent with the reporter assay, a significant reduction of AKT2 mRNA by 50% was observed in miR-let-7f-1-overexpressed cells compared with the scramble control in PC3 cells (Fig. 2D). Furthermore, western blot analysis demonstrated that overexpression of miR-let-7f-1 following administration of 10, 20 and 50 nM miR-let-7f-1 mimics was able to downregulate the expression of AKT2 and phosphorylated AKT2 by 35, 45 and 66%, respectively (Fig. 2E). miR-let-7f-1 knockdown at 10, 25 and 50 nm anti-miR-let-7f-1 treatment resulted in a marked increase in AKT2 and phosphorylated AKT2 expression by 42, 69 and 88%, respectively, as compared with the NC group (Fig. 2F). These data indicate that AKT2 is likely to be regulated by miR-let-7f-1 in prostate cancer at transcriptional and post-transcriptional levels.

miR-let-7f-1 inhibited the proliferative abilities and induced apoptosis in vitro. Previous studies have demonstrated that miR-let-7f-1 was involved in the suppression of cell proliferation (19,20), thus it is hypothesized that the expression of miR-let-7f-1 is augmented by lycopene in PC3 cells. In order to test whether lycopene induced the upregulation of miR-let-7f-1,
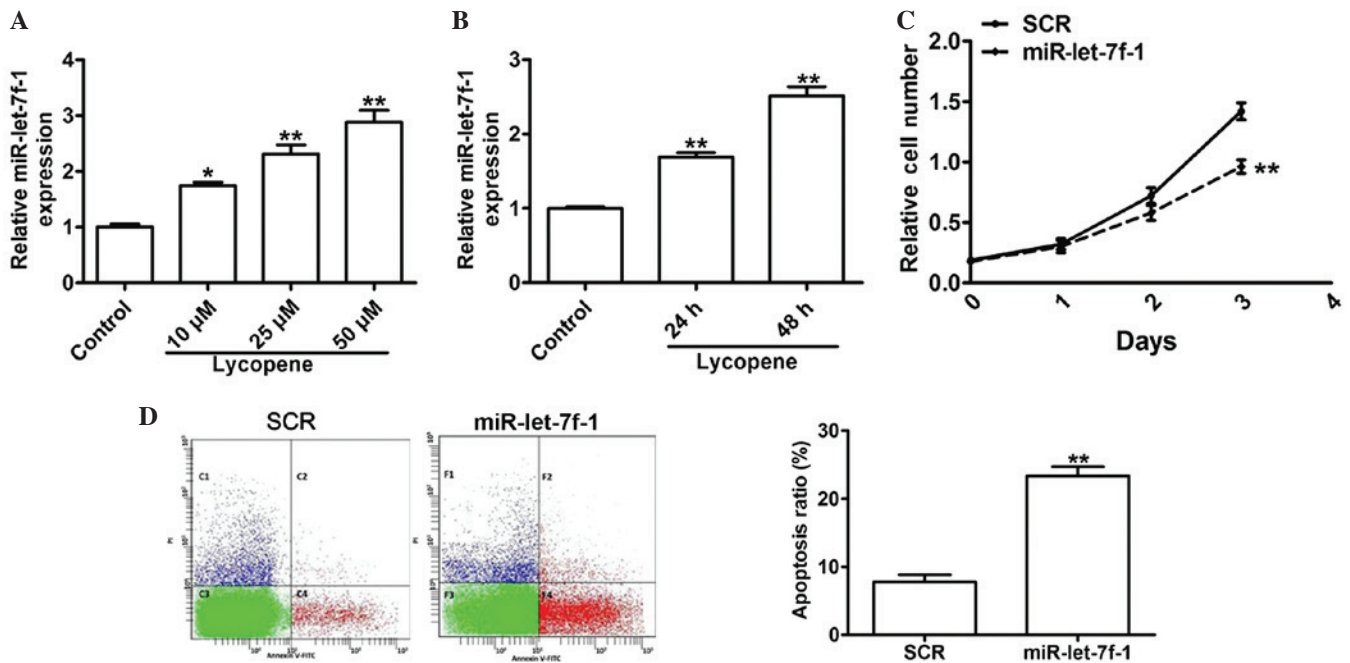
Figure 3. Effect of miR-let-7f-1 on growth and apoptosis of PC3 cells. (A) The relative expression of miR-let-7f-1 was determined by RT-qPCR in PC3 cells pretreated with lycopene (10, 25 and 50 μM) for 48 h. (B) RT-qPCR was used to determine the expression of miR-let-7f-1 in PC3 cells pretreated with 25 μM lycopene for 24 or 48 h. (C) The growth rate of the cells was detected using the WST-1 assay. (D) Flow cytometry analysis of apoptosis in PC3 cells transfected with SCR or miR-let-7f-1. *P<0.05; **P<0.01. miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SCR, scramble.
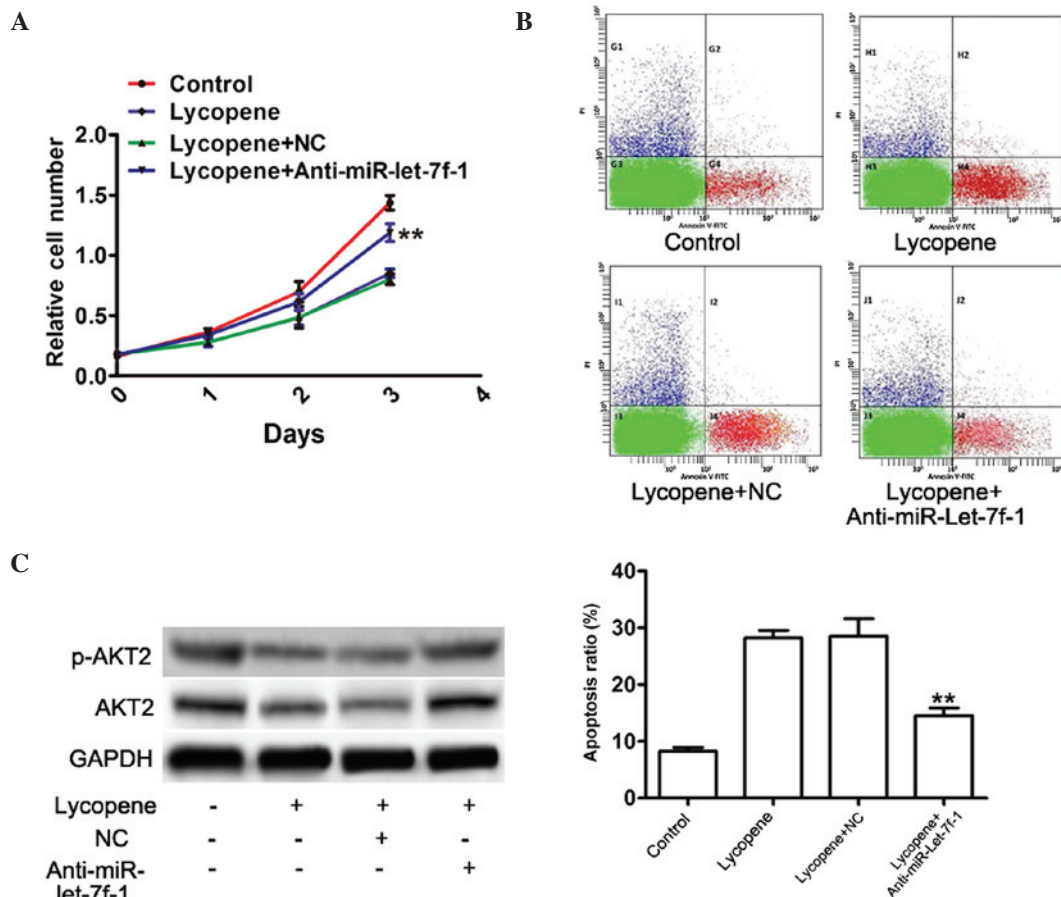

Figure 4. Knockdown of miR-let-7f-1 partly rescued lycopene-dependent suppression of cell functions and AKT2 expression. (A) PC3 cells were treated as indicated and were measured by the WST-1 assay at different time periods. (B) Apoptotic cells were determined by fluorescence-activated cell sorting. (C) Western blot analysis of AKT2 and p-AKT2 protein expression in PC3 cells treated as indicated. GAPDH was regarded as the endogenous control. **P<0.01 vs. NC. miR, microRNA; NC, negative control; p-AKT2, phosphorylated AKT2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

miR-let-7f-1 expression was detected by RT-qPCR in PC3 cells treated with lycopene. The RT-qPCR assay indicated that the expression of miR-let-7f-1 was enhanced by 74, 131 and 188% in response to lycopene treatment at 10, 25 and 50 μM, in a dose-dependent manner (Fig. 3A). A significant increase of 69 and 151% in miR-let-7f-1 expression was also observed in response to 25 μm lycopene treatment at 24 and 48 h, in a time-dependent manner (Fig. 3B). In order to investigate the role of miR-let-7f-1 in prostate cancer cells, miR-let-7f-1 mimics were transfected into PC3 cells. A proliferation assay was performed to evaluate cell growth and the data demonstrated that the increased expression of miR-let-7f-1 induced significant inhibition on cell proliferation by 7, 20 and 32% at days 1, 2 and 3, respectively (Fig. 3C). Apoptosis assays demonstrated that PC3 cells that were transfected with miR-let-7f-1 mimics exhibited an increase in the apoptotic rate by 200%, as compared with those of the scramble controls (Fig. 3D). These data indicated that miR-let-7f-1 induced by lycopene may effectively inhibit the growth and enhance apoptotic levels of PC3 cells *in vitro*.

Anti-miR-let-7f-1 oligonucleotides partly reversed the downregulation of AKT2 induced by lycopene in prostate cancer PC3 cells. To further confirm that lycopene downregulated the expression of AKT2 by upregulation of miR-let-7f-1, PC3 cells were treated 50 μM lycopene, followed by transfection with 50 nM anti-miR-let-7f-1 oligonucleotides for 48 h. Using the WST-1 assay, it was demonstrated that anti-miR-let-7f-1 attenuated the inhibition of cell proliferation caused by lycopene in PC3 cells by 18, 21 and 28% by days 1, 2 and 3, respectively (Fig. 4A). In addition, knockdown of miR-let-7f-1 in PC3 cells incubated with lycopene resulted in significantly reduced cell apoptosis (47%; Fig. 4B), indicating reversal of the increased effects of lycopene on PC3 cell apoptosis. In addition, anti-miR-let-7f-1 oligonucleotides partly abolished the inhibitory effect of lycopene on AKT2 and phosphorylated AKT2 protein expression (96%; Fig. 4C). Thus, it was confirmed that miR-let-7f-1 was a key mediator of growth inhibition and apoptotic enhancement of lycopene in prostate cancer.

Discussion

Lycopene, which is naturally present in tomato carotenoids, has been suggested to exhibit potential anticancer activity in several types of human cancer, including prostate, colon and breast cancer (4,5,27). In addition, lycopene has been reported to inhibit the development of certain cases of chemically induced carcinogenesis (32,33). Epidemiological and clinical studies have suggested that increased consumption of tomato products and greater blood concentrations of lycopene are associated with a reduced risk of prostate cancer (34,35). However, the inhibitory effect and possible molecular mechanisms of lycopene, including cell proliferation arrest and/or apoptosis induction, remain poorly understood.

In the current study, a novel molecular mechanism of lycopene in cancer control was proposed. For the first time, to the best of our knowledge, it was demonstrated that lycopene may inhibit cellular proliferation progression and induce apoptosis in PC3 cells, via upregulating miR-let-7f-1 expression and inhibiting the expression of AKT2. It has been previously

reported that AKT2, an important member of the PI3K signaling pathway, is activated in prostate cancer (30,36). Subsequently, the expression of AKT2 was investigated. Upon treatment with lycopene, a time- and dose-dependent reduction in AKT2 expression was observed, with the greatest inhibition observed when PC3 cells were treated with 50 μm for 48 h, indicating that the growth-inhibitory activity of lycopene likely reduced the mRNA and protein levels of AKT2. However, the mechanism by which lycopene downregulates AKT2 expression remains unclear. Previous studies have indicated that miRNAs act as fine-tuning regulators of protein expression (9,10). With an aim to identify miRNAs regulating AKT2 expression in prostate cancer, it was identified that miR-let-7f-1 could significantly downregulate AKT2 expression, which was supported by the luciferase reporter assay.

Therefore, it is notable to examine whether miR-let-7f-1 had an effect on cell proliferation and cell apoptosis. It was identified that the upregulation of miR-let-7f-1 was able to significantly inhibit the growth of PC3 cells. Upregulation of miR-let-7f-1 could also enhance apoptosis of PC3 cells. To the best of our knowledge, the current study was the first to investigate the roles and possible mechanisms of miR-let-7f-1 upregulation in PC3 cells induced by lycopene. In addition, it remains unclear whether miR-let-7f-1 is required to reduce the level of phosphorylated Akt in PC3 cells pretreated with lycopene. The data of the current study indicated that knockdown of miR-let-7f-1 could partly reverse the inhibitory effects of lycopene on cell proliferation and AKT2 expression in PC3 cells. In addition, lycopene-induced apoptotic induction was abrogated by knockdown of miR-let-7f-1 in PC3 cells. Taken together, it is suggested that lycopene-mediated growth inhibition and apoptosis is mediated through downregulating AKT2 and at both the mRNA and protein levels.

In conclusion, the data of the current study indicate that lycopene downregulates AKT2 expression via an miRNA pathway. These observations suggest that lycopene may be a potential anticancer compound with therapeutic applications.

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