MicroRNA-425 inhibits proliferation of chronic lymphocytic leukaemia cells through regulation of the Bruton's tyrosine kinase/phospholipase Cγ2 signalling pathway

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Abstract. The present study aimed to investigate the effects of microRNA (miR)-425 on the proliferation of chronic lymphocytic leukaemia (CLL) cells and the possible underlying mechanisms. The expression of miR-425 was determined in the B lymphocytes of CLL patients and in normal B lymphocytes by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In addition, MEC-1 cells transfected with miR-425 negative control (NC) or miR-425 mimic were examined. The cell proliferation of different groups was evaluated using an MTT assay, and cell cycle distribution was evaluated using flow cytometry analysis. A dual-luciferase reporter assay was used to verify whether Bruton's tyrosine kinase (BTK) was a target of miR-425. Furthermore, the expression levels of BTK, phospholipase Cγ2 (PLCγ2), Ki-67 and proliferating cell nuclear antigen (PCNA) were determined by RT-qPCR and western blotting. The results revealed that the expression of miR-425 was significantly downregulated in B lymphocytes obtained from CLL patients as compared with that in normal B lymphocytes. When cells were transfected with miR-425 mimic, the proliferation of MEC-1 cells was significantly inhibited at 24, 48 and 72 h compared with the proliferation of control cells. Additionally, the ratio of G0/G1 cells was significantly increased and the ratio of G2/M cells was significantly decreased in miR-425-overexpressing cells compared with that in control cells. The luciferase reporter assay revealed that miR-425 binds to the 3'-untranslated region of BTK mRNA. Finally, BTK, PLCγ2, Ki-67 and PCNA expression was significantly inhibited at the mRNA and protein level in cells where miR-425 was upregulated. In conclusion, miR-425 inhibits the proliferation of MEC-1 cells, potentially by inhibiting BTK/PLCγ2 signalling, and Ki-67 and PCNA expression levels. These results provide a deeper insight for understanding the development of CLL and suggest a potential novel target for the treatment of CLL patients.

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Introduction

Chronic lymphocytic leukaemia (CLL), the most common leukaemia in adults (1), is a malignancy characterised by the accumulation of mature monoclonal B cells in the peripheral blood, the lymphoid organs and the bone marrow of patients with a circulatory $CD5^+CD19^+CD20^{dim}Ig^{dim}CD23^+CD43^+CD27^+$ surface phenotype (2,3). Despite advances in CLL therapy over the years, the survival rate of CLL patients remains low (4).

Although CLL pathophysiology is complicated, B-cell receptor signalling is considered a driving factor of CLL tumour cell survival (5). Bruton's tyrosine kinase (BTK), which is downstream of B-cell receptors, serves an important role in the activation of pathways associated with CLL cell survival (6), including protein kinase B (3), extracellular signal-regulated kinase (7) and nuclear factor-kB pathways (8). It has previously been reported that inhibition of BTK may be used as a treatment strategy in untreated and relapsed CLL patients (9).

MicroRNAs (miRNAs) are small endogenous RNAs involved in various biological processes and diseases, including tumourigenesis, cell proliferation, apoptosis and autophagy (10,11). It has been demonstrated that miRNAs are differentially expressed between normal and tumour cells, including in CLL (12). miRNAs are involved in the proliferation and apoptosis of CLL cells (13); however, a deeper insight into the function of miRNAs in CLL is lacking. miR-425 is an miRNA associated with numerous diseases and bioprocesses, including anti-angiogenesis (14), tumourigenesis (15) and inflammatory cytokine production (16). HE *et al* (17) reported that miR-425 contributes to tumour development by inhibiting the tumour suppressor catenin α-3 in hepatocellular carcinoma. miR-425 is also reportedly upregulated in gastric cancer (18). However, to the best of our knowledge, no previous study has focused on the role of miR-425 in CLL.

In the present study, the effect of miR-425 on the proliferation of CLL cells and the possible mechanism whereby this regulation occurs were assessed, identifying the BTK/phospholipase Cγ2 (PLCγ2) signalling pathway. The current study provides a deeper insight for understanding the development of CLL and identifies a novel potential therapeutic target for CLL patients.

Materials and methods

Clinical samples and cell culture. Normal and CLL B lymphocytes were isolated from peripheral blood collected from 15 healthy individuals and 15 CLL patients, respectively, using the MagCellect Human B Cell Isolation kit (cat. no. MAGH103; R&D Systems) as described previously (19). In addition, the CLL cell line MEC-1 was purchased from ATCC (Manassas, VA, USA), and cells were maintained in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% foetal bovine serum (FBS) under 37˚C and 5% CO_2. The present study was approved by the Ethics Committee of Hunan Provincial People's Hospital (Changsha, China). Informed consent was obtained from participants.

Cell transfection. The miR-425 negative control and miR-425 mimic were purchased from RiboBio Co., Ltd. (Guangzhou, China). In addition, a recombinant plasmid was used to overexpress BTK in MEC-1 cells. Briefly, the BTK promoter was cloned and inserted into the pcDNA3.1 vector (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) to form pcDNA3.1-BTK. MEC-1 cells were cultured to 30-50% confluence in 96-well plates and transfected with miR-425 mimic, pcDNA3.1-BTK or pcDNA3.1-negative control (NC; 50 nmol/l). Samples were also co-transfected with miR-425 mimic and pcDNA3.1-BTK (50 nmol/l for each) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in serum-free Opti-Minimum Essential Medium (Gibco; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

MTT assay. Cells were inoculated into 96-well plates at a density of $1x10^3$ cells/well and incubated for 24, 48 or 72 h. An MTT assay was then performed to evaluate cell viability at 24, 48 and 72 h after transfection, as described previously (20). Briefly, 25 ml MTT solution (5 mg/ml) was added to each well and incubated at 37˚C for 4 h. Following centrifugation at a speed of 1,200 x g for 5 min at room temperature, the supernatant was then replaced with 180 ml dimethyl sulfoxide, and absorbance at 490 nm was evaluated using a Synergy-HT Multi-Detection microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Cell cycle measurements. Cell cycle distribution was evaluated using flow cytometry analysis, as described previously (21). Briefly, cells in different groups were harvested by trypsinization, washed twice with ice cold PBS and then fixed with 70% ethanol overnight at 4˚C. Next, cells were resuspended in 100 μl PBS containing a final concentration of 50 μg/ml RNase A for 30 min at room temperature. Propidium iodide from the FITC Annexin V kit (BD Biosciences, San Jose, CA, USA) was used to stain the cells at a concentration of 20 μg/ml for 20 min. A flow cytometer (BD-LSR; BD Biosciences) was used to detect the DNA content, while the cell cycle distribution was analysed using the Cell Quest software, version 5.1 (BD Biosciences).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the B lymphocytes or MEC-1 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). A High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was then used to convert RNA into cDNA. qPCR reactions were performed using SYBR Green Master Mix (Solarbio Science & Technology Co., Ltd., Beijing, China) in an Exicycler™ 96 (Bioneer Corporation, Daejeon, Korea). The thermocycling conditions were as follows: Initial activation at 95˚C for 15 min, followed by 40 cycles of denaturation at 94˚C for 15 sec, annealing at 55˚C for 30 sec and extension at 72˚C for 30 sec. The Primers utilized for the reverse transcription of mature miR-425-5p and U6 were as follows: hsa-miR-425-5p stem-loop RT primer, 5'-CTCAACTGGTGTCGTGGAGTC GGCAATTCAGTTGAGTCAACGGG-3'; U6-RT, 5'-CAA CTGGTGTCGTGGAGTCGGC-3'. The primers utilized in PCR were as follows: miR-425-5p forward, 5'-ACACTCCAG CTGGGAATGACACGATCACTCC-3' and reverse, 5'-TGG TGTCGTGGAGTCG-3'; BTK forward, 5'-GAGAAGCTG GTGCAGTTGTAT-3', and reverse, 5'-GGCCGAAATCAG ATACTTTAAC-3'; PLCγ2 forward, 5'-GCTGCTTCACCA TCCTATATG-3', and reverse 5'-GGAACTTGGCACTGC TCACT-3'; Ki-67 forward, 5'-GAGAATCTGTGAATCTGG GTAA-3', and reverse, 5'-CAGGCTTGCTGAGGGAAT-3'; proliferating cell nuclear antigen (PCNA) forward, 5'-ACT CGTCTCATGTCTCCTTG-3', and reverse, 5'-TCATTCATC TCTATGGCAACAG-3'; GAPDH forward, 5'-CACCCACTC CTCCACCTTTG-3', and reverse, 5'-CCACCACCCTGT TGCTGTAG-3'. The relative RNA levels were calculated by the $2^{-\Delta\Delta Cq}$ method (22). U6 and GAPDH were utilized as an internal control.

Plasmid construction and dual-luciferase reporter assay. The predicted binding of BTK and miR-425 was obtained via bioinformatic prediction using targetscan 5.1 software (http://www.targetscan.org; Whitehead Institute for Biomedical Research, Cambridge, MA, USA). Wild-type (WT) constructs of the BTK 3'-untranslated region (3'-UTR) contained the predicted miR-425 binding region. Mutant (MUT) constructs of BTK 3'-UTR lacked the predicted miR-425 responsive element. Each were inserted downstream of the firefly luciferase gene in the pmiR-REPORT™ plasmid (Guangzhou RiboBio Co., Ltd., Guangzhou, China). MEC-1 cells were co-transfected with the luciferase reporter plasmids and miR-425 mimics/miR-425-NC. Following transfection for 48 h, luciferase activity was measured by a Dual-Luciferase Reporter System (Promega Corporation, Madison, WI, USA) using an Centro LB 960 microplate luminometer (Berthold Technologies, Bad Wildbad, Germany). All reactions were repeated in triplicate for at least three independent experiments.

Western blotting. Western blotting was used to test the protein expression levels of BTK, PLCγ2, Ki-67 and PCNA,

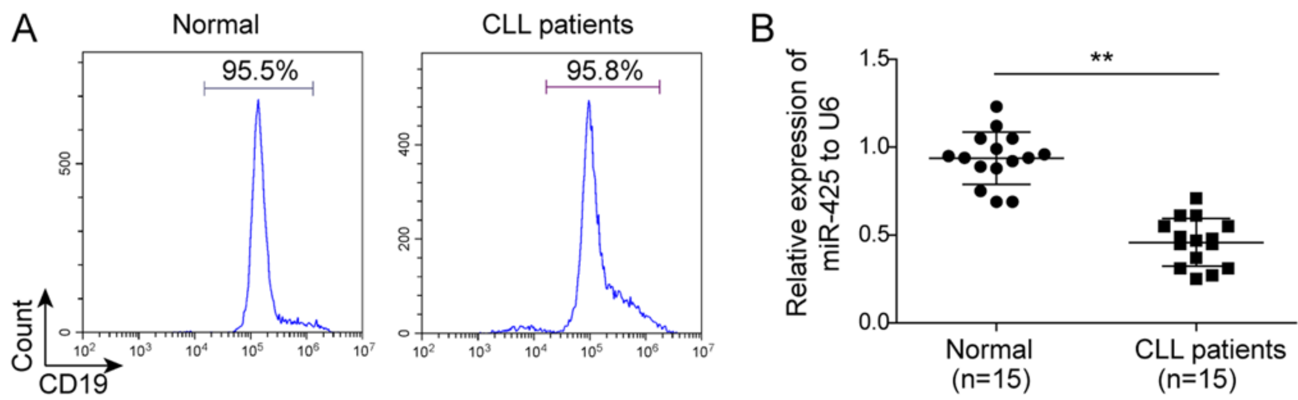

Figure 1. miR-425 is significantly downregulated in B lymphocytes of CLL patients compared with its expression in normal B lymphocytes. (A) Purity of B lymphocyte cultures was assessed by flow cytometry. (B) Expression of miR-425 in the B lymphocytes of CLL patients and normal B lymphocytes, determined by reverse transcription-quantitative polymerase chain reaction. All experiments were conducted in triplicate. **P<0.01 vs. normal group. miR, microRNA; CLL, Chronic lymphocytic leukaemia.

with β-actin used as a loading control. Briefly, protein was extracted from MEC-1 cells using radioimmunoprecipitation assay buffer (Vazyme, Piscataway, NJ, USA). Protein was quantitated using a protein assay reagent Colorimetric assay kit (cat. no. 5000002) from Bio-Rad (Hercules, CA, USA). Samples were then subjected to 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes. Following blocking with 5% non-fat milk at room temperature for 1 h, membranes were incubated with the following primary antibodies (all purchased from Abcam, Cambridge, UK) at 4˚C overnight: Anti-BTK (cat. no. ab137503; 1:1,000), anti-PLCγ2 (cat. no. ab227129; 1:500), anti-Ki67 (cat. no. ab16667; 1:500), anti-PCNA antibodies (cat. no. ab29; 1:1,000) and β-actin (cat. no. ab8226; 1:1,000). Next, membranes were incubated with a horseradish peroxidase-conjugated anti-rabbit (ab6721) or anti-mouse (ab6785) immunoglobulin G secondary antibody (Abcam) at 37˚C for 45 min. Films were scanned using Super Signal West Pico Chemiluminescent Substrate kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Relative protein expression was quantified using Image-Pro Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. Data are expressed as the mean ± standard deviation. Comparisons between two groups were performed using Student's t-test. Comparisons among three or more groups were conducted using one-way analysis of variance with Tukey's post-hoc test. The results were considered to be statistically significant when the P-value was <0.05. All calculations were performed using SPSS software (version 18.0; SPSS, Inc., Chicago, IL, USA).

Results

Downregulation of miR-425 in B lymphocytes of CLL patients. Initially, the expression of miR-425 was compared between the B lymphocytes of CLL patients and normal B lymphocytes using RT-qPCR. The B lymphocytes were isolated from peripheral blood collected from healthy individuals and CLL patients, and the purity was >95%, as indicated in Fig. 1A. As shown in Fig. 1B, the expression of miR-425 was significantly downregulated in the B lymphocytes of CLL patients compared with that in normal B lymphocytes (P<0.05).

Upregulation of miR-425 inhibits the proliferation of MEC-1 cells. To further investigate the effects of miR-425 on CLL cells, an miR-425 mimic was used to transfect the CLL cell line MEC-1, and its effect on cell proliferation was subsequently investigated. As shown in Fig. 2A, transfection with the miR-425 mimic significantly enhanced the expression of miR-425, as expected, compared with that in the control groups (P<0.05). Furthermore, when miR-425 was upregulated, the proliferation of MEC-1 cells was significantly inhibited at 24, 48 and 72 h compared with the proliferation of control cells (P<0.05; Fig. 2B). Next, the effect of miR-425 on cell cycle distribution in MEC-1 cells was evaluated by flow cytometry analysis. The results demonstrated that the ratio of G0/G1 cells was significantly increased in miR-425-overexpressing cells, while the ratio of G2/M cells was significantly decreased, compared with that in the controls (P<0.05; Fig. 2C). These results indicate that overexpression of miR-425 significantly inhibits the proliferation of MEC-1 cells.

BTK expression is regulated by miR-425. As reported in a previous study (23), miR-425 may target the BTK gene. In the present study, a dual-luciferase reporter assay was used to confirm whether BTK was a target of miR-425. As presented in Fig. 3A and B, luciferase activity significantly decreased when cells were transfected with miR-425 mimics of WT BTK 3'-UTR vectors, while no significant difference was identified in MUT BTK 3'-UTR. Next, the expression of BTK in cells transfected with miR-425 mimic and in control cells was measured. The results revealed that the expression of BTK was significantly inhibited at the mRNA and protein levels when miR-425 was upregulated by mimic transfection (Fig. 3C and D), indicating that the expression of BTK is negatively regulated by miR-425.

miR-425 inhibits the proliferation of MEC-1 cells by regulating the BTK/PLCγ2 signalling pathway. To investigate

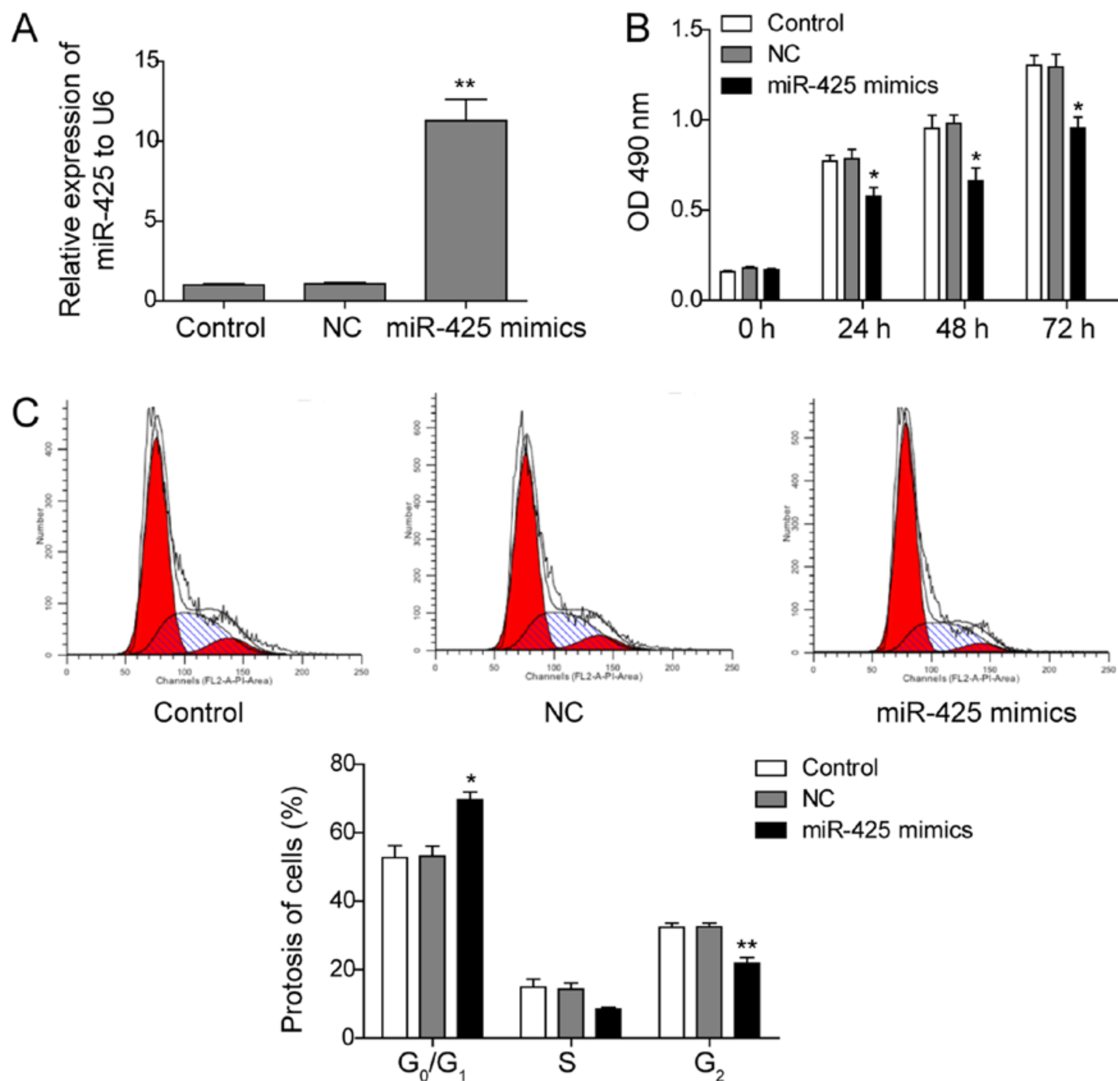

Figure 2. Upregulation of miR-425 significantly inhibits the proliferation of MEC-1 cells. (A) miR-425 expression in cells transfected with miR-425 mimic or NC, and untreated cells, examined by reverse transcription-quantitative polymerase chain reaction at 48 h after transfection. (B) Proliferation of cells in different experimental groups examined by MTT assay at 24, 48 and 72 h after transfection. (C) Cell cycle distribution in different experimental groups, assessed by flow cytometry analysis. All experiments were conducted in triplicate. Statistical analysis was conducted using Student t-test for comparisons between two groups and analysis of variance for comparisons among three groups. *P<0.05 and **P<0.01 vs. control and NC groups. miR, microRNA; NC, negative control.

the mechanisms underlying the inhibitory effect of miR-425 on the proliferation of MEC-1 cells, the expression levels of proteins associated with the BTK/PLCγ2 signalling pathway were measured by western blotting in different experimental groups. The results indicated that upregulation of miR-425 significantly inhibited the expression levels of BTK and its downstream protein PLCγ2, as compared with the control treatment (P<0.05; Fig. 4A and B). The levels of the proliferation-associated proteins Ki-67 and PCNA were also downregulated in miR-425-overexpressing cells. Subsequently, BTK overexpression was conducted by plasmid transfection, and western blotting indicated that BTK protein level was successfully increased (Fig. 4C). An MTT assay was then used to assess whether BTK overexpression reduces the inhibition of cell proliferation in response to miR-425. As indicated in Fig. 4D, following 24 h culture, the optical density value was significantly higher in the miR-425 mimic + BTK-treated group at all time points (24, 48 and 72 h), as compared with that in the group treated with miR-425 mimic alone, indicating that BTK overexpression partially rescued the inhibitory effect of miR-425 on cell proliferation. These results suggest that miR-425 regulates the BTK/PLCγ2 signalling pathway to further inhibit expression of Ki-67 and PCNA, inhibiting the proliferation of MEC-1 cells.

Discussion

As the most common leukaemia in adults, CLL remains a worldwide health problem and accounts for ~30% of all leukaemia cases in Western countries (24). Recently, Bottoni *et al* (23) demonstrated that BTK protein expression was targeted and reduced most strongly by miR-210 and miR-425 in CLL, and that miR-425 was expressed at lower levels in primary CLL samples in comparison with that in normal B cells. However, whether miR-425 affects CLL

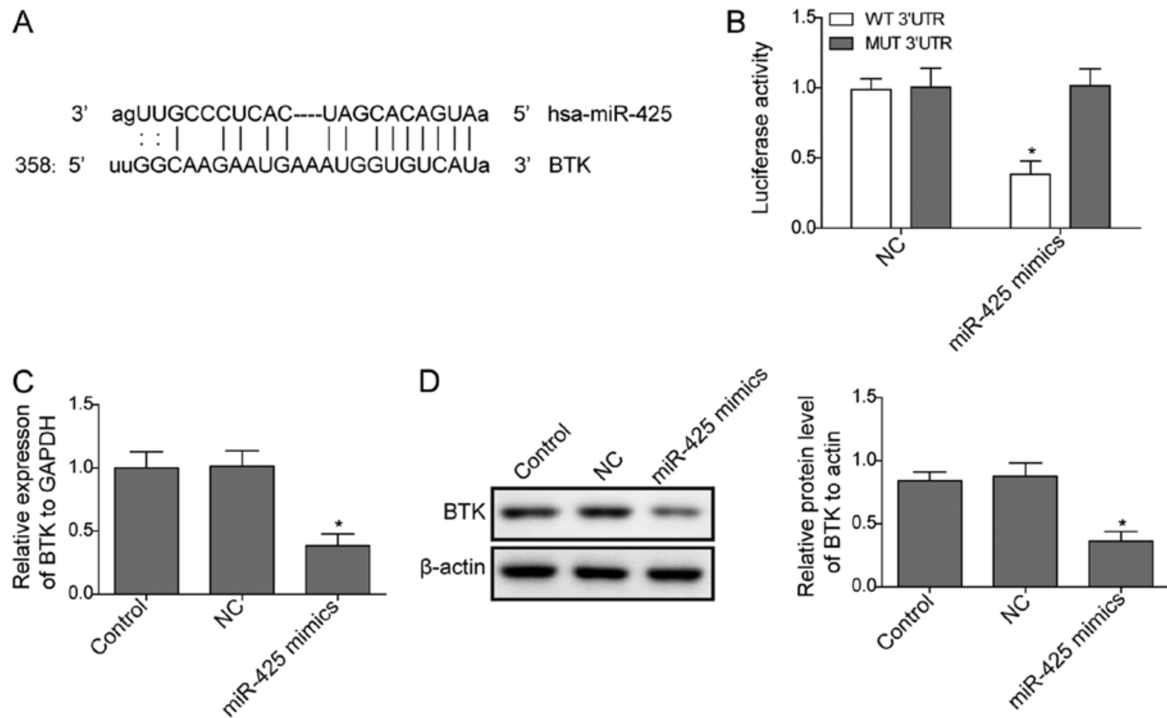

Figure 3. Expression of BTK is regulated by miR-425. (A) Binding mode for miR-425 and BTK. (B) Luciferase activity was measured by a dual-luciferase reporter assay. (C) Expression of BTK mRNA in cells transfected with miR-425 or NC, as determined by reverse transcription-quantitative polymerase chain reaction. (D) Expression of BTK protein in cells transfected with miR-425 or NC, measured by western blotting. All experiments were conducted in triplicate. Statistical analysis was conducted using Student t-test. *P<0.05 vs. control and NC groups. miR, microRNA; BTK, Bruton's tyrosine kinase; WT, wild-type; MUT, mutated; 3'-UTR, 3'-untranslated region; NC, negative control.

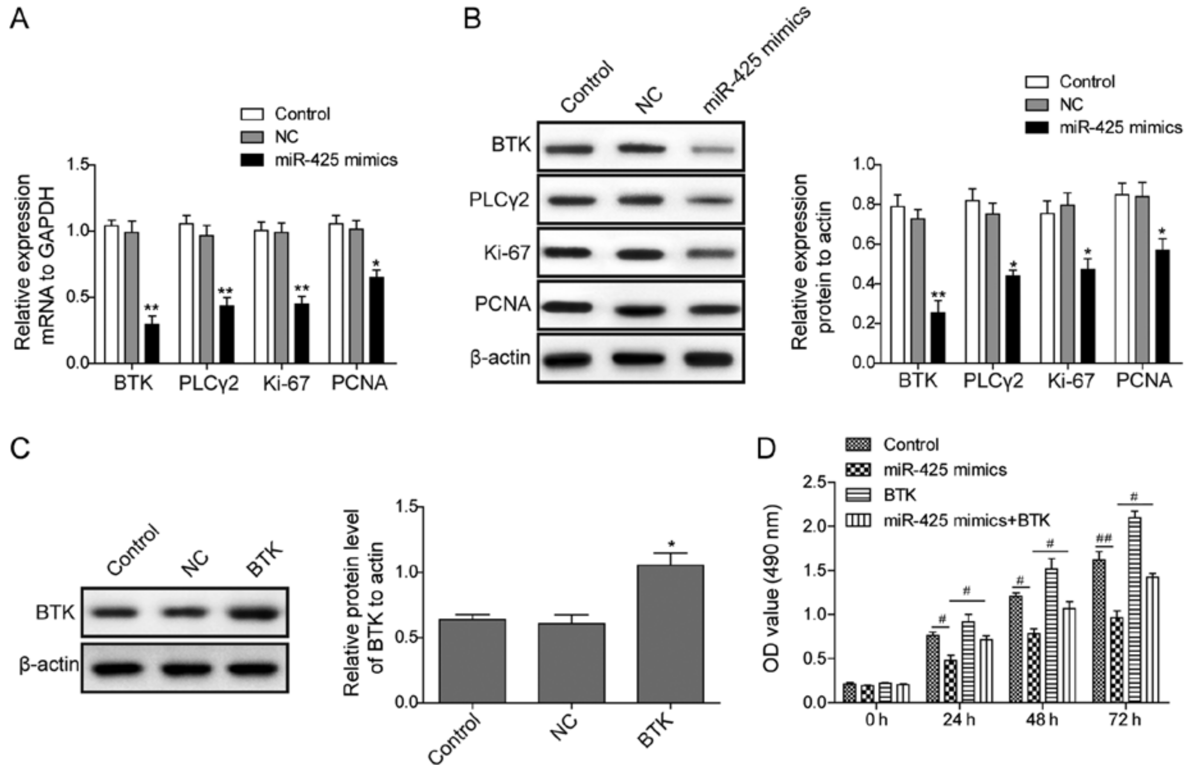

Figure 4. miR-425 inhibits the proliferation of MEC-1 cells through regulation of the BTK/PLCγ2 signalling pathway. (A) mRNA and (B) protein expression levels of BTK, PLCγ2, Ki-67 and PCNA in cells transfected with miR-425 or NC at 48 h after transfection, as detected by reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. (C) BTK protein expression levels in cells transfected with pcDNA3.1-BTK or pcDNA3.1-NC, and control cells, detected by western blotting at 48 h after transfection. (D) Proliferation of cells in different experimental groups, determined by MTT assay at 24, 48 and 72 h after transfection. All experiments were conducted in triplicate. Statistical analysis was conducted using Student t-test for comparisons between two groups and analysis of variance for comparisons among three groups. *P<0.05 and **P<0.01 vs. control/NC group; #P<0.05 and ##P<0.01. miR, microRNA; BTK, Bruton's tyrosine kinase; PLCγ2, phospholipase Cγ2; PCNA, proliferating cell nuclear antigen; NC, negative control.

development and the mechanisms of these effects remained unclear. In the present study, the data demonstrated that miR-425 inhibits the proliferation of CLL cells through regulation of the BTK/PLCγ2 signalling pathway. It was observed that miR-425 was significantly downregulated in the B lymphocytes of CLL patients as compared with its expression in normal B lymphocytes. Furthermore, overexpression of miR-425 significantly inhibited the proliferation of MEC-1 cells and altered the cell cycle distribution.

miR-425 is involved in the regulation of cell proliferation in various diseases. For instance, Ma *et al* (25) demonstrated that upregulation of miR-425 promotes the cell growth of gastric cancer cells by targeting phosphatase and tensin homolog (PTEN). Feng *et al* (26) reported that cell invasion and metastasis of hepatocellular carcinoma cells was promoted by miR-425-5p through suppressor of cancer cell invasion (SCAI)-mediated dysregulation of multiple signalling pathways. Di Leva *et al* (27) also demonstrated that *in vitro* and *in vivo* miR-425 expression reduced proliferation, and impaired tumourigenesis and metastasis in aggressive breast cancer cells. A recent study also revealed that miR-425-5p inhibits differentiation and proliferation in porcine intramuscular preadipocytes (28). In the present study, it was demonstrated that miR-425 inhibits the proliferation of MEC-1 cells, indicating that miR-425 serves an important role in the development of CLL, which is consistent with the results published by Bottoni *et al* (23).

To further investigate the mechanisms underlying the inhibitory effect of miR-425 on MEC-1 cells, a luciferase reporter assay was used to confirm that miR-425 targeted BTK mRNA. In addition, the expression levels of BTK, PLCγ2, and the proliferation-associated proteins Ki-67 and PCNA were assessed by RT-qPCR and western blotting. The results displayed that miR-425 upregulation significantly inhibited the expression of all the aforementioned proteins. BTK is considered crucial for CLL cell survival and functions as an activator of PLCγ2; therefore, it is proposed that miR-425 transfection inhibits the expression of PLCγ2 as a secondary effect of BTK loss. A study by Byrd *et al* (1) demonstrated that the BTK inhibitor ibrutinib was effective in relapsed CLL. Singh *et al* (3) also reported that inhibition of BTK or phosphoinositide 3-kinase resulted in reduced viability, proliferation and fibronectin-dependent cell adhesion in CLL *in vitro* and *in vivo*. In the present study, miR-425 was found to inhibit the proliferation of MEC-1 cells, and it is propose that this effect was mediated through the inhibition of BTK/PLCγ2 signalling, and of Ki-67 and PCNA expression levels. Furthermore, the proliferation of MEC-1 cells was only partially rescued when BTK was overexpressed in cells transfected with miR-425 mimics, which indicated that other factors may be involved. Taken together, more targets of miR-425 may exist, including PTEN and SCAI, which may potentially affect CLL cell proliferation, warranting further investigation in future studies.

In conclusion, the present study investigated the effect of miR-425 on the proliferation of CLL cells and the possible mechanisms responsible for this regulation. The results demonstrated that miR-425 upregulation inhibited the proliferation of MEC-1 cells, and this effect appeared to be mediated through the inhibition of BTK/PLCγ2 signalling, and of Ki-67 and PCNA expression levels. These results provide a deeper insight for understanding the development of CLL and reveal a potential novel target for the treatment of CLL patients.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors' contributions

CY designed the current study, performed clinical experiments and edited and reviewed the manuscript. LH performed the experiments and acquired the data. XL analyzed the data and reviewed the manuscript. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Hunan Provincial People's Hospital (Changsha, China).

Patient consent for publication

Informed consent was obtained from participants.

Competing interests

The authors declare that they have no competing interests.

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