Original Article

MiR-503 Contributes to Glucocorticoid Sensitivity in Acute Lymphoblastic Leukaemia via Targeting WNT3A

(miR-503 / glucocorticoids / sensitivity / acute lymphoblastic leukaemia)

C. TIAN[1], L. LIU[1], M. ZHENG[2], Z. YE[1], R. CHEN[3], X. LAN[1]

[1]Department of Paediatrics, Affiliated Hospital of Guangdong Medical University, Zhanjiang, Guangdong Province, China
[2]Department of Obstetrics, Affiliated Hospital of Guangdong Medical University, Zhanjiang, Guangdong Province, China
[3]Department of Paediatrics, Shunde Women's and Children's Hospital of Guangdong Medical University, Foshan, Guangdong Province, China

Abstract. Abnormal accumulation of lymphoblasts in the blood and bone marrow is the main characteristic of acute lymphoblastic leukaemia (ALL). Glucocorticoids are effective drugs for ALL, while glucocorticoid resistance is an obstacle to ALL therapy. MicroRNAs (miRNAs) are implicated in the drug resistance and modulate the response of ALL to glucocorticoids. The role of miR-503 in glucocorticoid sensitivity of ALL was investigated in this study. Firstly, T-leukaemic cells were isolated from patients with ALL. The human ALL cell line (CCRF/CEM) was incubated with dexamethasone to establish a glucocorticoid-resistant ALL cell line (CCRF/CEM-R). Data from MTT showed that IC_{50} (50% inhibitory concentration) of dexamethasone in T-leukaemic cells isolated from glucocorticoid-resistant ALL patients or CCRF/CEM-R was increased compared with IC_{50} in T-leukaemic cells isolated from glucocorticoid-sensitive ALL patients or CCRF/CEM. MiR-503 was down-regulated in glucocorticoid-resistant leukaemic cells and CCRF/CEM-R. Secondly, over-expression of miR-503 sensitized CCRF/CEM-R to dexamethasone. Moreover, over-expression of miR-503 also promoted the sensitivity of ALL cells to dexamethasone. Thirdly, miR-503 bound to WNT3A mRNA and negatively regulated the expression of WNT3A. Over-expression of miR-503 reduced protein expression of nuclear β-catenin, and over-expression of WNT3A attenuated the miR-503 over-expression-induced decrease in nuclear β-catenin. Lastly, the over-expression of miR-503-induced increased sensitivity of ALL-resistant cells and CCRF/CEM-R to dexamethasone was attenuated by over-expression of WNT3A. In conclusion, miR-503 targeted WNT3A mRNA to sensitize ALL cells to glucocorticoids through inactivation of the Wnt/β-catenin pathway.

Introduction

Acute lymphoblastic leukaemia (ALL) is a highly aggressive malignant haematological tumour with infection, fever, anaemia, and abnormal bleeding as the main clinical manifestations (Jain et al., 2014). Abnormal accumulation of lymphoblasts in the blood and bone marrow is the main characteristic of ALL (Imai, 2017). During the progression of ALL, multiple genetic alterations suppress cell apoptosis and promote cell proliferation and drug resistance, leading to leukaemogenesis (Iijima and Kiyokawa, 2016). ALL is one of the most common types of childhood cancers (Tissing et al., 2003), and glucocorticoids with the abilities of regulating cell proliferation and apoptosis are efficient drugs for ALL (Tissing et al., 2003). However, some patients are not sensitive enough or develop resistance to glucocorticoids in the clinical application, and glucocorticoid resistance is the leading cause of death in patients with ALL (Hunger and Mullighan, 2015). Therefore, it is of great significance to explore the mechanisms involved in glucocorticoid resistance in ALL, which would promote the efficacy of glucocorticoids and improve prognosis in ALL.

MicroRNAs (miRNAs) have been shown to be involved in various diseases, especially in tumour pro-

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Corresponding author: Xiang Lan, Department of Paediatrics, Affiliated Hospital of Guangdong Medical University, No. 57 South Renmin Avenue, Xiashan District, Zhanjiang City, Guangdong Province, China. Phone: (+86) 0759-2387454; e-mail: lanxiang_555@163.com

Abbreviations: ALL – acute lymphoblastic leukaemia, miRNA(s) – microRNA(s), NC – negative control.

gression, through regulation of target genes (Zhang et al., 2020; Roy et al., 2021). MiRNAs have been regarded as diagnostic and prognostic biomarkers, as well as therapeutic targets for ALL (Drobna et al., 2018). Moreover, increasing evidence has also revealed that miRNAs play an important role in the regulation of glucocorticoid resistance in leukaemia (Autry et al., 2020). For example, miR-145 promoted the sensitivity of ALL cells to glucocorticoids through decreasing the drug resistance gene and increasing cell apoptosis (Long et al., 2020). MiR-142 promoted glucocorticoid resistance of ALL through inhibition of cell apoptosis and promoting cell proliferation (Liang et al., 2017). MiR-503 has been reported to be a tumour suppressor through inhibition of angiogenesis (Zhou et al., 2013). The G1/S transition (Xiao et al., 2013) and proliferation (Yang et al., 2017) of hepatocellular carcinoma were suppressed by miR-503, and miR-503 also repressed glycolysis of breast cancer cells (Huang et al., 2021). Previous study has shown that miR-503 promoted partial differentiation and cell-cycle arrest of acute myeloid leukaemia cells (Forrest et al., 2010). Moreover, Cobll1 was a binding target of miR-503 in the regulation of chronic myeloid leukaemia (Han et al., 2017). However, the role of miR-503 in progression of ALL has not been reported.

In this study, miR-503 was hypothesized to regulate the sensitivity of ALL cells to glucocorticoids. The expression level of miR-503 was decreased in the ALL cells from glucocorticoid-resistant patients. The manipulation of miR-503 expression and exploration of its interaction with the canonical WNT signalling pathway revealed a mechanism by which miR-503 can influence the sensitivity of T-leukaemic cells to glucocorticoids.

Material and Methods

Isolation of T-leukaemic cells

A total of 46 patients with confirmed ALL were recruited at the Affiliated Hospital of Guangdong Medical University. The clinical characteristics of the patients are shown in Table 1. Written informed consents were acquired from all the patients with the approval of the Institutional Review Ethics Committee of the Affiliated Hospital of Guangdong Medical University, and in accordance with the World Medical Association Declaration of Helsinki on ethical principles for medical research involving human subjects. Peripheral blood samples were obtained from these patients sensitive (N = 20) or resistant (N = 26) to glucocorticoids. Ficoll gradient centrifugation was used to separate mononuclear cells from the peripheral blood samples. T-leukaemic cells were then purified from the mononuclear cells via negative magnetic sorting with biotinylated anti-human antibodies, including anti-CD235a, anti-CD123, anti-CD56, anti-CD36, anti-CD19, anti-CD16, anti-CD14 (Pan T Cell Isolation Kit, Human, Miltenyi Biotec, Bergisch Gladbach, Germany).

Cell culture, treatment and cell transfection

Human ALL cell lines (CEM-C7, MOLT4, Jurkat, and CCRF/CEM) were purchased from ATCC (Manassas, VA). Cells were cultured in RPMI 1640 medium containing 10% foetal bovine serum and 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA). To establish glucocorticoid-resistant CCRF/CEM (CCRF/CEM-R) cells, CCRF/CEM cells were incubated with 0.1 μM dexametha-

Table 1. Clinical characteristics of patients with ALL.

Clinicopathological factor	Number of cases	miR-503 expression (high)	miR-503 expression (low)	P value
Number	46	19	27	
Gender				0.765
Male	23	10	13	
Female	23	9	14	
Age				0.925
<10	36	15	21	
≥10	10	4	6	
WBC				0.251
<50000	22	11	11	
≥50000	24	8	16	
CNS status				0.271
1	21	6	15	
2	15	8	7	
3	10	5	5	
Glucocorticoid resistance				0.014*
Yes	29	8	21	
No	17	11	6	

*P < 0.05, miR-503 expression (high)

sone (Sigma-Aldrich, Shanghai, China) for 10 days. To determine the effect of miR-503 on WNT signalling, Jurkat cells were transfected with miR-503-5p mimic (5'-UGGCUCUUAGUGAGCUGGUUG-3') miR-503-5p inhibitor (5'-GAGCAUUUCGGUCUGGAA-3') or the negative controls (NC mimic, NC inhibitor) using Lipofectamine 2000 (Invitrogen).

Cell viability

The isolated T-leukaemic cells from patients were seeded in a 96-well plate containing 0.001, 0.005, 0.025, 0.125, 0.625, 3.125, 15.625, 78.125 μM dexamethasone for 24 h. CCK8 solution (Dojindo, Tokyo, Japan) was then added into each well for another incubation for 2 h. Absorbance at 450 nm was measured by Thermo Multiskan MK3 (Thermo Fisher Scientific Inc, Waltham, MA). CEM-C7, MOLT4, Jurkat, CCRF/CEM, and CCRF/CEM-R cells were also seeded in a 96-well plate containing 0.002, 0.006, 0.018, 0.054, 0.162, 0.486, 1.458, 4.374, 13.122, 39.366, 118.098, μM dexamethasone for 24 h. The cells were then subjected to CCK8 analysis. CEM-C7, MOLT4, Jurkat, and CCRF/CEM-R cells were transfected with miR-503 mimic, negative control (NC) mimic or cotransfected with miR-503 mimic and pcDNA-WNT3A (Genepharma, Suzhou, China) using Lipofectamine 2000 (Invitrogen). Two days later, cells were treated with different concentrations of dexamethasone and then subjected to CCK8 analysis. IC_{50} values were calculated using the Graph Pad Prism 5 software.

qRT-PCR

RNAs were isolated from T-leukaemic cells and ALL cells using Trizol (Invitrogen) and then reverse-transcribed into cDNAs. The cDNAs were contacted with SYBR Green Master mix (Roche, Mannheim, Germany) for qRT-PCR analysis of miR-503 and *WNT3A*. *GAPDH* and *U6* were used as endogenous controls, and the relative expression of the target genes was calculated by the $2^{-\Delta\Delta t}$ method. The primer sequences:

miR-503: forward: 5'-CGCGGGATCGGGTCAGA-3'
reverse: 5'-GGGAACATGTTGATCTCAG-3'
WNT3A: forward: 5'-CTCCTCTCGGATACCTCTTAGTG-3'
reverse: 5'-ACCACAGTCCATGCCATCAC-3'
GAPDH: forward: 5'-CATGAGAAGTATGACAACAGCCT-3'
reverse: 5'-CCTTCCACGATACCAAAGT-3'
U6: forward: 5'-CTCGCTTCGGCAGCACA-3'
reverse: 5'-AACGCTTCACGAATTTGCGT-3'

were used in this study.

Dual luciferase reporter assay

The sequence of wild-type 3'UTR WNT3A (Fig. 4A) was subcloned into the pmirGLO luciferase reporter vector (Promega, Madison, WI) and named as pmirGLO-WNT3A-wt. The sequence of mutant 3'UTR WNT3A that lost the binding ability with miR-503 (Fig. 4A) was also subcloned into the pmirGLO luciferase reporter vector and named as pmirGLO-WNT3A-mut. Jurkat cells were co-transfected with pmirGLO-WNT3A-wt or pmirGLO-WNT3A-mut and miR-503 mimic or NC mimic. Two days later, the luciferase activities were determined by the Lucifer Reporter Assay System (Promega).

Western blot

A CelLytic NuCLEAR Extraction Kit (Sigma-Aldrich) was used to isolate nuclear and cytoplasmic cell lysates from ALL cells. Lysates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked by 5% bovine serum albumin and probed with primary antibodies: anti-WNT3A and anti-β-actin (1 : 2000; Abcam, Cambridge, MA), anti-β-tubulin and anti-histone H3 (1 : 3000; Abcam). The membranes were then incubated with corresponding secondary antibodies (1 : 4000; Abcam), and the protein strips were visualized by a Colorimetric Western Blotting Kit (Sigma-Aldrich).

Statistical analysis

All the data were expressed as mean ± S.D. and analysed by Student's *t*-test or one-way analysis of variance. A P value of < 0.05 was considered as statistically significant.

Results

MiR-503 was down-regulated in glucocorticoid-resistant ALL

To investigate the role of miR-503 in glucocorticoid-resistant ALL, 46 patients with ALL (N = 20 for patients with glucocorticoid-sensitive ALL and N = 26 for patients with glucocorticoid-resistant ALL) were recruited (Table 1). The expression of miR-503 was significantly associated with glucocorticoid resistance of the patients (Fig. 1B). T-leukaemic cells were isolated from patients with glucocorticoid-resistant or sensitive ALL. IC_{50} of dexamethasone in glucocorticoid-resistant leukaemic cells was higher than in glucocorticoid-sensitive leukaemic cells (Fig. 1A), confirming the resistance of glucocorticoid-resistant leukaemic cells to dexamethasone. The expression of miR-503 was down-regulated in glucocorticoid-resistant leukaemic cells compared with glucocorticoid-sensitive leukaemic cells (Fig. 1B), suggesting that miR-503 might be involved in the glucocorticoid resistance of ALL.

Over-expression of miR-503 promoted glucocorticoid sensitivity in glucocorticoid-resistant ALL

Human ALL cells, CCRF/CEM, were incubated with dexamethasone to establish glucocorticoid-resistant ALL cells (CCRF/CEM-R). IC_{50} of dexamethasone was significantly increased in CCRF/CEM-R (9.97 μM) compared to CCRF/CEM (0.12 μM) (Fig. 2A), confirming the resistance of CCRF/CEM-R to dexamethasone. MiR-503 was also down-regulated in CCRF/CEM-R (Fig. 2A). CCRF/CEM-R was then transfected with the

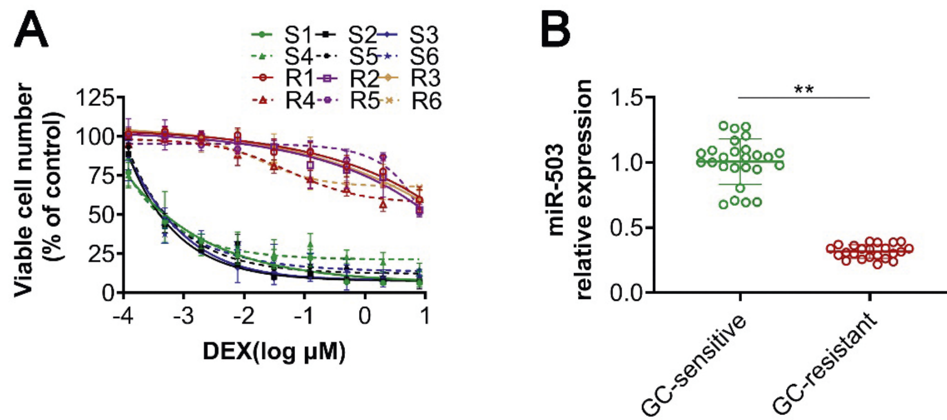

Fig. 1. miR-503 was down-regulated in glucocorticoid-resistant ALL cells
(**A**) IC_{50} of dexamethasone (DEX) in glucocorticoid-resistant leukaemic cells (R1-R6) was higher than in glucocorticoid-sensitive (S1-S6) leukaemic cells. (**B**) Expression of miR-503 was down-regulated in glucocorticoid (GC)-resistant leukaemic cells compared with GC-sensitive leukaemic cells. ** $P < 0.01$.

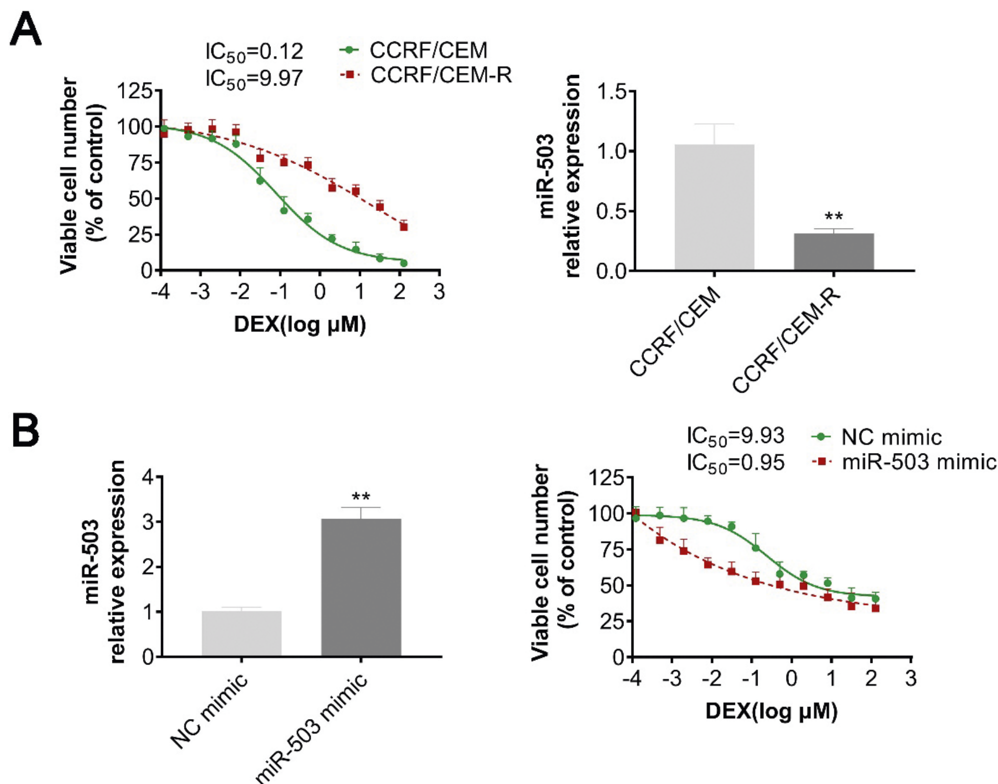

Fig. 2. Over-expression of miR-503 promoted glucocorticoid sensitivity in glucocorticoid-resistant ALL cells
(**A**) IC_{50} was significantly increased in CCRF/CEM-R (9.97 μM) compared to CCRF/CEM (0.12 μM) cells. Expression of miR-503 was down-regulated in CCRF/CEM-R compared to CCRF/CEM cells. (**B**) Transfection with miR-503 mimic increased miR-503 expression in CCRF/CEM-R cells compared with the negative control (NC mimic). Over-expression of miR-503 reduced IC_{50} (0.95 μM) compared to transfection with NC mimic (9.93 μM). ** vs. CCRF/CEM or NC mimic, $P < 0.01$.

miR-503 mimic (Fig. 2B). Transfection with the miR-503 mimic reduced IC_{50} (0.95 μM) compared to cells transfected with the NC mimic (9.93 μM) (Fig. 2B), demonstrating that over-expression of miR-503 sensitized glucocorticoid-resistant ALL to dexamethasone.

Over-expression of miR-503 promoted glucocorticoid sensitivity in ALL

Human ALL cell lines (CEM-C7, MOLT4, Jurkat) were also transfected with the miR-503 mimic (Fig. 3A).

Over-expression of miR-503 also reduced IC_{50} of dexamethasone in Jurkat (Fig. 3B), MOLT4 (Fig. 3C) and CEM-C7 cells (Fig. 3D) compared to cells transfected with the NC mimic, indicating that over-expression of miR-503 sensitized ALL to dexamethasone.

MiR-503 bound to WNT3A and mediated activation of WNT/β-catenin

WNT3A mRNA was predicted to be a potential binding target of miR-503 (Fig. 4A). The luciferase activity assay showed a suppressive effect of miR-503 over-expression on the activity of pmirGLO-WNT3A-wt (Fig. 4B), suggesting that miR-503 binds to 3'UTR of WNT3A. Jurkat cells were then transfected with the miR-503 mimic or inhibitor (Fig. 4C). Data from qRT-PCR (Fig. 4D) and Western blot (Fig. 4E) indicated that miR-503 negatively modulated expression of WNT3A in Jurkat cells. Moreover, over-expression of miR-503 promoted cytoplasmic β-catenin expression while reducing nuclear β-catenin expression in Jurkat cells (Fig. 4F and 4G). However, the cytoplasmic expression of β-catenin was reduced and the nuclear β-catenin expression was enhanced by inhibition of miR-503 (Fig. 4F and 4G). Over-expression of WNT3A attenuated the miR-503 over-expression-induced increase in cytoplasmic β-catenin and decrease in nuclear β-catenin (Fig. 4H), revealing that miR-503 targeted WNT3A to suppress activation of the WNT/β-catenin pathway.

MiR-503/WNT3A mediated glucocorticoid sensitivity in ALL

Human ALL cell lines (CEM-C7, MOLT4, Jurkat) and CCRF/CEM-R were co-transfected with the miR-503 mimic and pcDNA-WNT3A to investigate the role of miR-503/WNT3A axis in glucocorticoid sensitivity of ALL. Over-expression of WNT3A restored the reduced IC_{50} of dexamethasone in Jurkat cells induced by miR-503 over-expression (Fig. 5A). The miR-503 over-expression-induced decrease in IC_{50} of dexamethasone in MOLT4 (Fig. 5B) and CEM-C7 cells (Fig. 5C) was also reversed by over-expression of WNT3A. Over-expression of WNT3A attenuated the miR-503 over-expression-induced decrease in IC_{50} of dexamethasone in CCRF/CEM-R cells (Fig. 5D), indicating that miR-503 promoted glucocorticoid sensitivity in ALL and glucocorticoid-resistant ALL via targeting WNT3A.

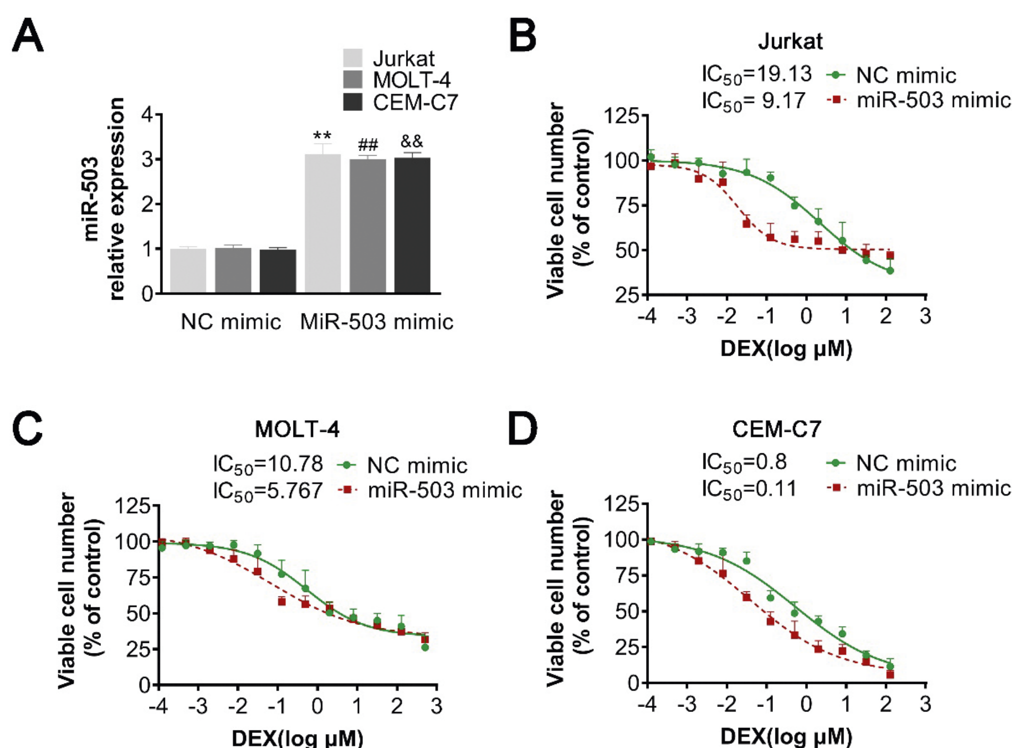

Fig. 3. Over-expression of miR-503 promoted glucocorticoid sensitivity in ALL
(**A**) Transfection with miR-503 mimic increased miR-503 expression in human ALL cell lines (CEM-C7, MOLT4, Jurkat). (**B**) Transfection with miR-503 mimic reduced IC_{50} of dexamethasone in Jurkat cells. (**C**)Transfection with miR-503 mimic reduced IC_{50} of dexamethasone in MOLT4 cells. (**D**) Transfection with miR-503 mimic reduced IC_{50} of dexamethasone in CEM-C7 cells. **, ##, && represent transfection with miR-503 mimic in Jurkat, MOLT4, and CEM-C7 cells vs. NC mimic, $P < 0.01$.

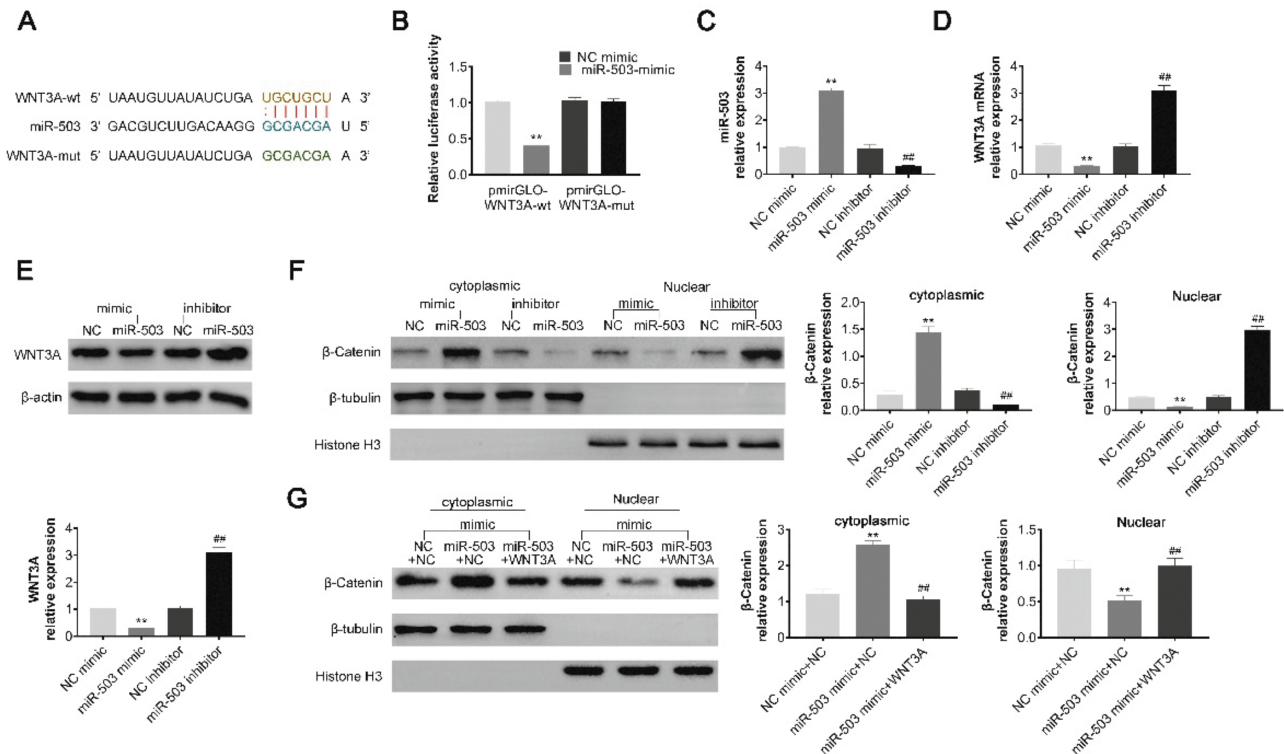

Fig. 4. miR-503 binds to WNT3A mRNA and activates the WNT/β-catenin signalling pathway
(**A**) Potential binding site between WNT3A mRNA and miR-503. (**B**) Transfection with miR-503 mimic reduced the activity of pmirGLO-WNT3A-wt compared with NC mimic, while both miR-503 mimic and NC mimic transfection had no significant effect on the activity of pmirGLO-WNT3A-mut. (**C**) Transfection with miR-503 mimic or inhibitor increased or decreased miR-503 expression in Jurkat cells compared with the negative controls (NC mimic or NC inhibitor). (**D**) Transfection with miR-503 mimic or inhibitor decreased or increased WNT3A mRNA expression in Jurkat cells. (**E**) Transfection with miR-503 mimic or inhibitor decreased or increased WNT3A protein expression in Jurkat cells. (**F**) Transfection with miR-503 mimic decreased nuclear β-catenin protein expression and increased cytoplasmic β-catenin expression in Jurkat cells. Transfection with miR-503 inhibitor increased nuclear β-catenin protein expression and decreased cytoplasmic β-catenin expression in Jurkat cells. (**G**) Over-expression of WNT3A attenuated the miR-503 over-expression-induced increase of cytoplasmic β-catenin and decrease of nuclear β-catenin. ** vs. NC mimic or NC mimic + NC, ## vs. NC inhibitor or miR-503 mimic + NC, $P < 0.01$.

Discussion

Previous study has shown that glucocorticoid induced alterations in miRNA expression in ALL, and miRNAs regulated glucocorticoid sensitivity (Rainer et al., 2009). Inhibition of miR-17~92 and miR-142-3p suppressed the acquired dexamethasone resistance in ALL (Sakurai et al., 2019). Since miR-503 was reported to be a tumour suppressor, the effect of miR-503 on glucocorticoid resistance of ALL was investigated in this study.

Firstly, miR-503 was found to be down-regulated in the leukaemic cells isolated from patients with glucocorticoid-resistant ALL compared with glucocorticoid-sensitive ALL. Moreover, miR-503 was also reduced in CCRF/CEM cells with acquired dexamethasone resistance. Furthermore, cell proliferation of human ALL cells and CCRF/CEM-R was suppressed by miR-503 over-expression. These results showed that miR-503 promoted the sensitivity of ALL cells and glucocorticoid-resistant ALL cells to dexamethasone. However, the effect of miR-503 on the cell apoptosis of ALL cells and glucocorticoid-resistant ALL cells should be investigated to identify the suppressive effect of miR-503 on the development of insensitive ALL.

In line with the previous study that miR-503 targeted WNT3A to regulate proliferation and apoptosis of mouse pre-osteoblast cells (Li, Q. et al., 2019), the results in this study showed that miR-503 binds to 3'UTR of WNT3A and negatively regulates WNT3A expression in ALL cells. WNT/β-catenin signalling was inactivated by miR-503 to suppress gastric cancer cell proliferation and invasion (Li, W. et al., 2019). Over-expression of miR-503 in this study reduced nuclear β-catenin expression and promoted cytoplasmic β-catenin expression to thus suppressed WNT/β-catenin signalling. Moreover, over-expression of WNT3A attenuated the miR-503 over-expression-induced increase in cytoplasmic β-catenin and decrease in nuclear β-catenin, revealing a tight

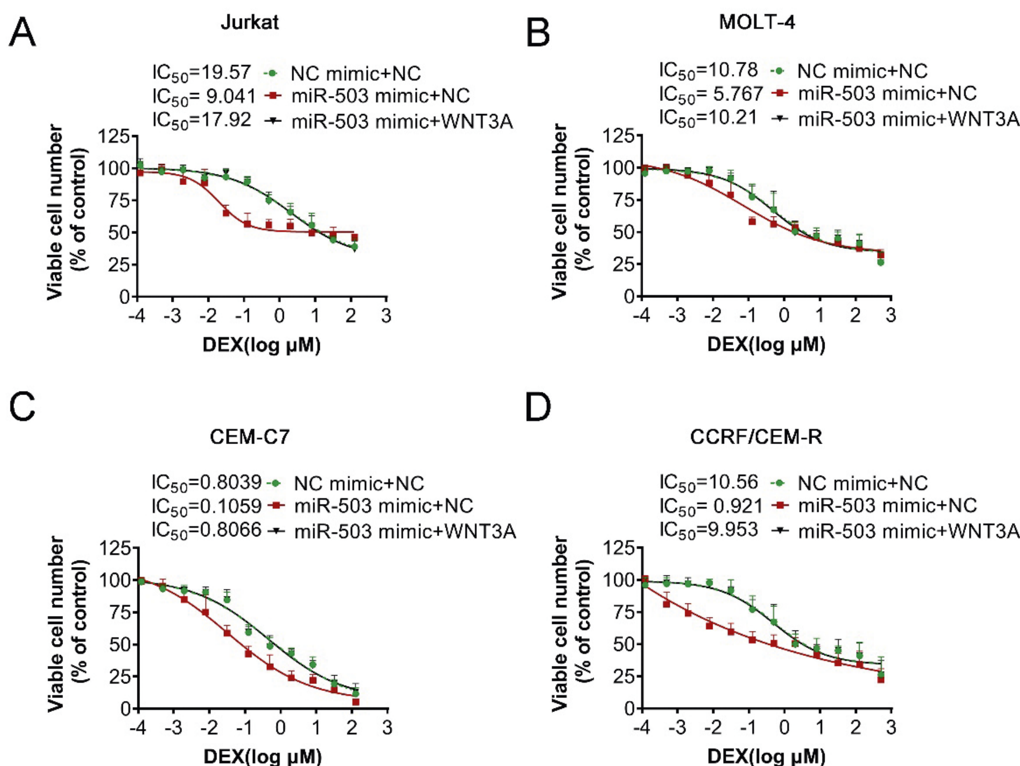

Fig. 5. miR-503/WNT3A mediated glucocorticoid sensitivity in ALL
(**A**) Over-expression of WNT3A attenuated the miR-503 over-expression-induced decrease of IC_{50} in Jurkat cells. (**B**) Over-expression of WNT3A attenuated the miR-503 over-expression-induced decrease of IC_{50} in MOLT4 cells. (**C**) Over-expression of WNT3A attenuated the miR-503 over-expression-induced decrease of IC_{50} in CEM-C7 cells. (**D**) Over-expression of WNT3A attenuated the miR-503 over-expression-induced decrease of IC_{50} in CCRF/CEM-R cells.

link between miR-503 and the WNT/β-catenin pathway in ALL.

The Wnt/β-catenin pathway is essential for cell differentiation and development, and activation of Wnt/β-catenin is implicated in the progression and initiation of haematological malignancies, including ALL (Chiarini et al., 2020). Inhibition of Wnt/β-catenin is considered to be a potential strategy for the treatment of ALL (Evangelisti et al., 2020). Increasing evidence has shown that inhibition of Wnt/β-catenin improved chemosensitivity and suppressed multidrug resistance in ALL (Dandekar et al., 2014; Fu et al., 2019). The results in this study showed that over-expression of WNT3A attenuated the miR-503 over-expression-induced decrease in cell viability in ALL and glucocorticoid-resistant ALL cells, revealing that miR-503 might contribute to the glucocorticoid sensitivity in ALL through suppression of the Wnt/β-catenin pathway.

In summary, miR-503 was down-regulated in glucocorticoid-resistant leukaemic T cells and ALL cell lines. Over-expression of miR-503 promoted glucocorticoid sensitivity in glucocorticoid-resistant ALL cells through binding with WNT3A mRNA and inhibiting the Wnt/β-catenin pathway. Therefore, miR-503 might be regarded as a therapeutic target for glucocorticoid-resistant ALL.

Competing interests

The authors state that there are no conflicts of interest to disclose.

Ethics approval

Ethical approval was obtained from the Institutional Review Ethics Committee of the Affiliated Hospital of Guangdong Medical University.

Statement of informed consent

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

Contribution of authors

C. T. and L. L. designed the study, supervised the data collection, M. Z. analysed the data, interpreted the data, Z. Y., R. C. and X. L. prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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