miR-187-5p Regulates Cell Growth and Apoptosis in Acute Lymphoblastic Leukemia via DKK2

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Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy and causes a high rate of mortality in affected adults. Many subtypes of ALL exist with disruptions in distinct genetic pathways, including those regulated by miRNAs. Here we identify miR-187-5p as being highly upregulated in B-cell ALL and a driver of cellular proliferation and suppressor of apoptosis. We show that miR-187-5p directly targets the 3′-UTR of DKK2 to mediate these effects. We further determine that inhibition of DKK2 by miR-187-5p in Nalm-6 B cells leads to inappropriate activation of Wnt/β-catenin signaling. Together, these findings reveal that the miR-187-5p–DKK2 pathway regulates Wnt/β-catenin signaling, cell growth, and apoptosis. Our findings provide the first evidence of a role for miR-187-5p in promotion of B-cell ALL.

Key words: Acute lymphoblastic leukemia (ALL); miR-187-5p; DKK2; Cell growth; Cell apoptosis

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most prevalent childhood cancer and the leading cause of cancer-related death in children and young adults (1,2). Although ALL occurs less frequently in adults, the mortality rate is significantly higher. Genomic profiling and sequencing studies have identified many subtypes of ALL with varying degrees of malignancy, indicating numerous distinct pathways underlie ALL (3). Identification of these pathways will lead to enhanced diagnosis and targeted therapies that will improve upon adult ALL outcomes and toxic side effects of current chemotherapeutics (2).

Wnt proteins regulate key developmental processes, including proliferation and cell fate decisions (4,5). Recent studies have implicated the Wnt signaling pathway as a potential driver of leukemia including ALL (2,4,6–13). In the presence of Wnt proteins, β-catenin migrates into the nucleus where it modulates the expression of many genes (9). Abnormal Wnt signaling has been identified in subsets of childhood ALL, independent of other known ALL abnormalities such as Notch activation (2,4,6,7).

MicroRNAs (miRNAs) are also critical regulators of gene expression with the potential to influence large gene regulatory networks (14–16). Over the past decade a body of literature points to a major role for miRNAs in cancer initiation, progression, and metastasis (15,17). It has been shown that miRNAs may function as oncogenes in gain-of-function models, or alternatively, as tumor suppressors in loss-of-function models. These results have been substantiated in human cancers in which altered miRNA expression has been repeatedly reported. For example, a majority of patients with B-cell chronic lymphocytic leukemia exhibit deletions of miR-15 and miR-16, suggesting a loss of tumor-suppressing function (3).

We hypothesized that miRNA dysregulation likely contributes to B-cell ALL. Through transcriptional profiling of ALL patients we identified MiR-187-5p as being highly expressed in ALL patients. We further prove that miR-187-5p promotes cellular proliferation and inhibits apoptosis in Nalm-6 B cells. Finally, we determine that miR-187-5p enacts these actions mechanistically through downregulation of DKK2 leading to activation of Wnt/β-catenin signaling.

MATERIALS AND METHODS

Patient Samples

The pediatric B-cell ALL samples and age-matched control samples (n = 20) were obtained from Daqing Oilfield...
Only cases with bone marrow (BM) samples containing ≥70% leukemic cells were enrolled in this study. Age-matched subjects with no manifestations of any hematological malignancy were used as control. All patients were under 12 years of age. Diagnoses of pediatric samples into CD10+/CD19+ B-cell lineage were determined by immunophenotyping. All consents were provided by guardians of participants, with ethical approval from Daqing Oilfield General Hospital.

**Real-Time PCR**

Total RNA was extracted from fresh tissues and cells using TRIZol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. MicroRNA quantification from extracted RNA was performed using the SYBR Green method. U6 snRNA was used as an internal control for PCR analysis on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer’s instructions. The relative levels of miR-187-5p expression were calculated by the $2^{-\Delta\Delta CT}$ method. For mRNA quantification, SYBR Green PCR master mix (Applied Biosystems) was used with the 7900HT system. GAPDH was used as a housekeeping control gene. The relative levels of gene expression were represented using the $2^{-\Delta\Delta CT}$ method.

**Cell and Culture Conditions**

Human B-cell leukemia Nalm-6 cells were grown in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin (Gibco), 10% fetal bovine serum (FBS; Gibco), and maintained at 37°C in a humidified atmosphere with 5% CO₂.

**Cell Transfection**

When Nalm-6 cells reached 70–80% confluence, miR-187-5p, miR-187-5p inhibitor, negative control (NC), and/or DKK2 siRNA was transfected using Lipofectamine® RNAiMAX Reagent. Cells were co-transfected with miRNA and luciferase reporter plasmid using Lipofectamine® 3000 Reagent. All transfections were performed according to the manufacturer’s instructions. At 6 h after transfection, the medium was replaced with fresh medium containing 10% fetal bovine serum and cells were cultured for an additional 48 h. The sequences of miRNA are as follows: NC, 5'-UUCUCCGAACGUGUCACGU-3'; miR-187-5p, 5'-GCUACAACAGCAGCCGAGC-3'; miR-187-5p-in, 5'-CGGGCCCAGAACAACGGC-3'.

**MTT Assay**

MTT assays were used to measure Nalm-6 cell proliferation. A total of $5 \times 10^5$ cells were seeded into each well of 96-well plates and transfected with miRNA and/or siRNA. MTT (0.5 mg/ml) was then added into

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**Figure 1.** miR-187-5p is upregulated in ALL. (A) miR-187-5p expression in 43 ALL bone marrow or peripheral blood samples compared with healthy donors analyzed by microarray (GEO DataSets GSE34670, $p<0.001$). (B) Real-time PCR analysis of miR-187-5p expression in ALL patient bone marrow samples compared with healthy donor cord blood ($n=20$, $p<0.001$).

**Axin2** forward 5'-TACCGGAGGATGCTGAAGGC-3' and reverse 5'-CACTGGCCGATTCTTCCTT-3'; **LEF1** forward 5'-AAATAAAGTGGCCCGTGGTGC-3' and reverse 5'-CTGGATCCCTTGGTAGTTC-3'; **DKK2** forward 5'-AGTGATGAAGTTGGGAGGTATTGCC-3' and reverse 5'-TGTCATTATGCAAGCGGTACTG-3'; **GAPDH** forward 5'-GGGTGTGAACCATGAGAAGT-3' and reverse 5'-TGGAGTCCTTCCACGATACCAA-3'.
fresh complete medium (100 μl). Plates were incubated at 37°C for 4 h. The medium was then replaced with 100 μl of DMSO (Sigma-Aldrich, St. Louis, MO, USA), and the plates were shaken at room temperature for 5 min. Absorbance was then measured at a wavelength of 570 nm.

**Colony Formation**

To assay colony formation, six-well plates were coated with 1 ml of 0.6% soft agar in RPMI-1640 culture media containing 10% FBS. Transfected Nalm-6 cells were then seeded onto coated six-well plates in 2 ml of 0.3% soft agar in RPMI-1640 culture media supplemented with 10% FBS at 37°C in a 5% CO2 incubator. On the next day, 1 ml of complete medium was added to the well. Cells were then grown for 10 days, and the culture medium was changed every 2 days. Images were taken under a microscope (Olympus, Japan).

**Western Blot**

Cells were lysed in radioimmunoprecipitation assay lysis buffer [Tris-buffered saline (TBS), 0.5% deoxycholic acid, 0.1% SDS, and 1% NP-40] with protease inhibitors (Boster, Wuhan, China). Protein concentration was determined by the bicinchoninic acid assay (Pierce, Rockford, IL, USA), and 30–40 μg of total protein from each sample was separated by SDS-PAGE gel. Protein was transferred to PVDF membranes (Millipore, Bedford, MA, USA) and incubated with a primary antibody followed by incubation with an HRP-conjugated secondary antibody (Boster). To verify equal loading of samples, membranes were incubated with a control primary antibody.

**Figure 2.** miR-187-5p promotes ALL cell proliferation. (A) Expression of miR-187-5p in Nalm-6 cells transfected with miR-187-5p or negative control (NC, upper), and miR-187-5p inhibitor (miR-187-5p in) or NC (lower). (B) MTT assay of indicated Nalm-6 cells tested at different time points (0, 24, 48, 72, and 96 h). (C) Representative micrographs of colony formation assay of indicated Nalm-6 cells. Each bar represents the mean ± SD of three independent experiments. *p < 0.05, ***p < 0.001.
against GAPDH. Antibody complexes were detected with the ECL Western blot kit (Pierce). The following primary antibodies were used: anti-claved caspase 3, anti-BCL2, anti-DKK2, anti-β-catenin, anti-histone H3, and anti-GAPDH. All primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

**Cell Death Detection ELISA Assay**

Apoptosis of Nalm-6 cells was assayed by the Cell Death Detection Elisa Plus Kit (Boheringer Mannheim, Indianapolis, IN, USA) according to manufacturer’s instructions. For this assay, Nalm-6 cells were cultured in FBS-free RPMI-1640 culture medium for 24 h, then collected and subjected to apoptotic cell quantification using monoclonal antibodies directed against DNA and histones. Absorbance was measured at 405 nm.

**Luciferase Reporter Assay**

Wild-type DKK2 3′-UTR fragments were amplified and cloned into the psiCHECK2 luciferase reporter vector via XhoI and NcoI sites. The mutated DKK2 3′-UTR was generated utilizing the DKK2 3′-UTR plasmid as a template and mutating the miR-187-5p seed binding site using the QuikChange® Multi Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). DKK2 3′-UTR luciferase and TOPflash luciferase activities were performed as described previously (18).

**Statistical Analysis**

Data were analyzed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Statistical differences were determined by paired t-test or ANOVA, with values of p < 0.05 considered statistically significant. Results were expressed as mean ± SD of at least three independent experiments.

**RESULTS**

**miR-187-5p Is Upregulated in ALL**

To identify ALL-associated miRNA species we analyzed GEO microarray data comparing bone marrow expression profiles from ALL patients with CD10+/CD19+ cord blood B cells from healthy donors (GSE34670) (19). In this dataset, we identified miR-187-5p as one of the most significantly elevated miRNAs in ALL patients (p < 0.001) (Fig. 1A). To confirm miR-187-5p is dysregulated in ALL patients, we performed real-time PCR to compare clinical ALL bone marrow samples with healthy controls (n=20). Consistent with the microarray analysis, miR-187-5p expression was significantly increased in clinical ALL samples (p<0.001) (Fig. 1B). Taken together, these data confirm that the expression of miR-187-5p is upregulated in ALL.

**miR-187-5p Promotes ALL Cell Proliferation and Inhibits Apoptosis**

To study the biological function of miR-187-5p in ALL we transiently expressed miR-187-5p, miR-187-5p inhibitor, or negative controls in Nalm-6 B cells (Fig. 2A). MTT assays demonstrated that ectopic miR-187-5p expression promoted the proliferation of Nalm-6 cells, whereas miR-187-5p inhibitor suppressed cell proliferation (Fig. 2B). Ectopic expression of miR-187-5p also significantly increased the colony number of Nalm-6 cells in colony formation assays, whereas miR-187-5p inhibitor decreased colony numbers (Fig. 2C). These results are consistent with a role for miR-187-5p in promotion of cell proliferation. We then determined the effect of miR-187-5p on Nalm-6 cell apoptosis. Western blot analysis reveals that overexpression of miR-187-5p inhibits caspase 3 and induces Bcl-2 protein (Fig. 3A). Reciprocally, miR-187-5p inhibitor

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**Figure 3.** miR-187-5p inhibits ALL cell apoptosis. Transfected Nalm-6 cells were cultured in FBS-free medium for 24 h, and then Western blot analysis for caspase 3 and Bcl-2 (A), and cell death detection ELISA (B) assays were performed. Each bar represents the mean ± SD of three independent experiments. *p<0.05.
increases caspase 3 expression and reduces Bcl-2 protein (Fig. 3A). To determine the effect of miR-187-5p on apoptosis we examined the effect of gain or loss of miR-187-5p function on DNA damage response. Overexpression of miR-187-5p versus control results in decreased cell death in response to DNA damage, whereas miR-187-5p inhibitor leads to increased cell death (Fig. 3B). Collectively, these data reveal that miR-187-5p promotes ALL cell proliferation and inhibits apoptosis.

**miR-187-5p Directly Targets DKK2**

We next investigated putative miR-187-5p targets to elucidate the mechanism by which miR-187-5p regulates proliferation and apoptosis of ALL cells. Using the TargetScan database we analyzed predicted potential targets of miR-187-5p and identified DKK2 as having an ideal target sequence in the 3'-UTR (Fig. 4A) (20). We then performed real-time PCR analysis comparing miR-187-5p expression with DKK2 expression in clinical ALL bone marrow samples. We observed a negative correlation between miR-187-5p and DKK2 mRNA expression in 20 ALL samples, suggesting DKK2 might be a direct target of miR-187-5p (r = −0.61, p < 0.05) (Fig. 4B). Indeed, Western blotting revealed that the expression of DKK2 decreased in Nalm-6 cells overexpressing miR-187-5p and increased in cells treated with miR-187-5p inhibitor (Fig. 4C). To determine if DKK2 is a direct target of miR-187-5p, we constructed a luciferase reporter of DKK2...
3’-UTR containing miR-187-5p binding sites. Our results show that ectopic expression of miR-187-5p decreased, while miR-187-5p inhibitor increased, the luciferase activity of the DKK2 3’-UTR luciferase reporter (Fig. 4D). By contrast, the DKK2 3’-UTR luciferase reporter with a mutant miR-187-5p binding site was not altered by ectopic miR-187-5p or miR-187-5p inhibitor (miR-187-5p-in) (Fig. 4E and F). Collectively, these data prove that miR-187-5p directly targets and downregulates DKK2.

**miR-187-5p Modulates the Wnt/β-Catenin Signaling Pathway Through DKK2 to Control Cell Proliferation and Apoptosis**

DKK2 has been reported as either an agonist or antagonist of the Wnt/β-catenin signaling pathway depending on the cellular context (21,22). To explore the effect of miR-187-5p on Wnt/β-catenin signaling, we extracted nuclear protein from Nalm-6 cells overexpressing miR-187-5p or treated with miR-187-5p inhibitor. Compared to control treatment, miR-187-5p promoted nuclear β-catenin, while miR-187-7p inhibitor reduced nuclear β-catenin (Fig. 5A). We next tested if miR-187-5p promotes activation of the Wnt/β-catenin transcriptional cascade. Nalm-6 B cells were treated with Wnt/β-catenin luciferase reporter (TOPflash) and cotreated with miR-187-5p or miR-187-5p inhibitor. As expected, overexpression of miR-187-5p in Nalm-6 cells results in enhanced TOPflash activity, and miR-187-5p inhibitor impairs TOPflash activity compared to the control (Fig. 5B). Consistent with this data, downstream targets of the Wnt/β-catenin signaling pathway, Axin2 and LEF1, are also transcriptionally activated with miR-187-5p and repressed with miR-187-5p inhibitor (Fig. 5C and D).

We next examined whether the effect of miR-187-7p on cell growth and apoptosis is DKK2 dependent. DKK2 expression was silenced by DKK2-specific siRNA in the presence or absence of miR-187-5p inhibitor as confirmed by Western blot in Nalm-6 cells (Fig. 6A). We next tested whether growth was affected using the MTT assay. Treatment of Nalm-6 cells with DKK2 siRNA resulted in increased proliferation. Interestingly, miR-187-5p inhibitor no longer
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suppressed the growth of Nalm-6 cells in the presence of DKK2 siRNA, indicating that miR-187-5p acts through DKK2 to regulate growth (Figs. 2B and 6B). Likewise, miR-187-5p inhibitor does not increase apoptosis of Nalm-6 cells in the presence of DKK2 siRNA (Figs. 3B and 6C), indicating that miR-187-5p acts through DKK2 to regulate apoptosis. To test whether miR-187-5p also regulates Wnt/β-catenin signaling through DKK2 we used the Wnt/β-catenin luciferase reporter (TOPflash). As expected, knockdown of DKK2 expression results in enhanced TOPflash activity. In the presence of DKK2 siRNA, miR-187-5p inhibitor no longer suppresses TOPflash activity (Figs. 5B and 6D). Therefore, DKK2 plays an essential role in miR-187-5p-mediated activation of Wnt/β-catenin signaling, cellular proliferation, and suppression of apoptosis in ALL.

**DISCUSSION**

In our study, we build from genome-wide efforts to profile transcriptional dysregulation in leukemia and identify miR-187-5p as a highly upregulated miRNA in B-cell ALL (19). We independently confirmed miR-187-5p to be highly expressed in patients with B-cell ALL compared to control. Using Nalm-6 B cells as a cancer model, we uncovered miR-187-5p’s role in promoting cellular proliferation and inhibiting apoptosis. Our data demonstrate that miR-187-5p directly inhibits DKK2 via targeting of the DKK2 3'-UTR. We prove that inhibition of DKK2 by miR-187-5p in Nalm-6 B cells leads to inappropriate activation of Wnt/β-catenin signaling. We further determine the effect of miR-187-5p on cell growth and apoptosis to be mechanistically dependent on the Wnt signaling pathway. This study provides the first evidence of a role for miR-187-5p in promotion of B-cell ALL malignancy. Further studies are needed to determine if inhibition of miR-187-5p may be a viable treatment of ALL in vivo.

miRNAs control critical biological processes including development and hematopoiesis through posttranscriptional regulation of target genes (3,14–16). Thousands of publications suggest the involvement of miRNAs in the development of nearly all types of human cancer (23). Aberrant miRNA expression profiles are characteristic of different subtypes in acute leukemia (3). Dysregulated expression of miRNAs may contribute to leukemogenesis.

**Figure 6.** miR-187-5p regulates ALL cell proliferation and apoptosis via DKK2. (A) Western blot of DKK2 in Nalm-6 cells treated with DKK2 siRNA with or without miR-187-5p inhibitor and negative controls. GAPDH served as a loading control. (B) MTT assay in Nalm-6 cells treated as indicated. (C) Cell death detection assay in Nalm-6 cells treated as indicated. (D) TOPflash luciferase assay in Nalm-6 cells treated as indicated. Each bar represents the mean ± SD of three independent experiments. *p<0.05.
by disrupting control of hematopoietic processes (3). Profiling miRNAs in ALL may be beneficial for predicting the risk of drug resistance, treatment failure, and disease relapse. Indeed, a recent study used association data from 14 highly dysregulated miRNA genes to improve the predictive value for long-term clinical outcome in children with ALL (3). Our data support the importance of miRNA regulation of the Wnt/β-catenin signaling cascade in at least a subset of ALL.

Wnt/β-catenin signaling is critical to cell growth and regulates cell cycle, cell adhesion, and cellular development (5,6). Wnt proteins have been extensively studied in connection with malignancies and are causatively involved in development of several types of leukemias, including acute myeloid leukemia and ALL (4,7). In B-cell progenitor ALL cell lines and in primary B-cell ALL cells, the Wnt/β-catenin pathway is activated by the overexpression of Wnt genes including WNT2B, WNT5A, WNT10B, and WNT16B as well as Wnt receptors FZD7 and FZD8 (6). Furthermore, overexpression of LEF-1 miRNAs are predictors of poor prognosis in patients with adult B-precursor ALL (6). These observations indicate that canonical Wnt signaling pathway plays a role in pathogenesis of B-cell ALL. Our results demonstrate that overactivation of the Wnt pathway through the action of miR-187-5p/DKK2 may contribute to ALL and that Wnt-inhibition may be a promising therapeutic approach.

DKK2 is a member of a small family of conserved secreted glycoproteins known as the Dickkopf proteins (21). Dkks specifically inhibit the Wnt/β-catenin signaling cascade. Dkk1 and Dkk2 bind to low-density lipoprotein receptor-related protein (LRP) 5/6 with high affinity. Unlike Dkk1, which is a pure inhibitor of Wnt/β-catenin signaling, Dkk2 is able to act either as inhibitor or activator of the pathway, depending on the cellular context (21). In particular, alterations of Dkk expression have been observed in a number of cancer models, supporting its role as a target of miR-187-5p in ALL. DKK2 has been previously implicated in cancer cells supporting a critical role of Wnt signaling in a broad range of cancers (24,25).

The discovery of miRNAs as powerful regulators of gene expression and the prevalence of alterations in miRNA expression in hematological malignancies sheds new light on our understanding of leukemia. In our study we demonstrate that miR-187-5p is highly upregulated in ALL. Together, results of this study suggest that miR-187-5p may be targeted and highlight the clinical relevance of miRNAs in hematological malignancy and support a major role for dysregulated miRNAs in cancer. Future studies will determine if miR-187-5p can be used to aid in the diagnosis, prognosis, and determination of optimal treatment options for ALL. In addition, future studies should address whether inhibitors directed against miR-187-5p or the Wnt/β-catenin pathway can improve ALL outcomes in vivo. Together, this study highlights the clinical relevance of miR-187-5p in Wnt-mediated activation of B-cell growth and malignancy.

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