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NET1 Enhances Proliferation and Chemoresistance in Acute Lymphoblastic Leukemia Cells

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Acute lymphoblastic leukemia (ALL) is the most prevalent of pediatric cancers. Neuroepithelial cell-transforming 1 (NET1) has been associated with malignancy in a number of cancers, but the role of NET1 in ALL development is unclear. In the present study, we investigated the effect of NET1 gene in ALL cell proliferation and chemoresistance. We analyzed GEO microarray data comparing bone marrow expression profiles of pediatric B-cell ALL samples and those of age-matched controls. MTT and colony formation assays were performed to analyze cell proliferation. ELISA assays, Western blot analyses, and TUNEL staining were used to detect chemoresistance. We confirmed that NET1 was targeted by miR-206 using Western blot and luciferase reporter assays. We identified NET1 gene as one of the most significantly elevated genes in pediatric B-ALL. MTT and colony formation assays demonstrated that NET1 overexpression increases B-ALL cell proliferation in Nalm-6 cells. ELISA assays, Western blot analyses, and TUNEL staining showed that NET1 contributes to ALL cell doxorubicin resistance, whereas NET1 inhibition reduces resistance. Using the TargetScan database, we found that several microRNAs (miRNAs) were predicted to target NET1, including microRNA-206 (miR-206), which has been shown to regulate cancer development. To determine whether miR-206 targets NET1 in vitro, we transfected Nalm-6 cells with miR-206 or its inhibitor miR-206-in. Western blot assays showed that miR-206 inhibits NET1 expression and miR-206-in increases NET1 expression. Luciferase assays using wild-type or mutant 3 -untranslated region (3 -UTR) of NET1 confirmed these findings. We ultimately found that miR-206 inhibits B-ALL cell proliferation and chemoresistance induced by NET1. Taken together, our results provide the first evidence that NET1 enhances proliferation and chemoresistance in B-ALL cells and that miR-206 regulates these effects by targeting NET1. This study therefore not only contributes to a greater understanding of the molecular mechanisms underlying B-ALL progression but also opens the possibility for developing curative interventions.

Key words: Neuroepithelial cell-transforming 1 (NET1); Proliferation; Chemoresistance; Acute lymphoblastic leukemia (ALL); miR-206

INTRODUCTION

Even though acute lymphoblastic leukemia (ALL) can occur in people of all ages, its peak incidence is from 1 to 4 years of age[1]. With an approximately 1 in 2,000 risk of disease in children, ALL is the most prevalent of pediatric cancers[2]. Since the 1960s, there have been significant diagnostic and treatment advances that have helped achieve a 5-year event-free survival rate of around 85% in children[3]. However, the rate of 5-year event-free survival in adults is significantly lower at around 40%[4]. Although the reasons for this disparity are not entirely clear, there have been notable advancements in our understanding of ALL's genetic bases in children, more so than in adults[5]. Other barriers that must be overcome in both patient populations are disease recurrence and treatment resistance[6]. Despite the high rates of curative treatment in children, relapsed ALL is the fourth most prevalent pediatric malignancy and has a staggeringly low overall survival rate of 30%[7]. In the present study, we have identified the neuroepithelial cell-transforming 1 (NET1) gene as a significant enhancer of ALL cell proliferation and chemoresistance. Furthermore, we have determined that miR-206 is a negative regulator of NET1 and its oncogenic effects.

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The current treatment approach for ALL involves the use of a multidrug regimen divided into several phases (induction, consolidation, and maintenance), though the specific regimen depends on the patient's risk category and immunophenotype[8]. The main goal of induction therapy is complete remission, which is defined as eradication of all detectable leukemia cells from the blood and bone marrow as well as restoration of typical hematopoiesis[9]. In children, the induction phase is successful in more than 90% of cases, regardless of disease genetics, though presence of the t(9;22) translocation (Philadelphia chromosome) portends even better outcomes[10]. Consolidation therapy is begun soon after complete remission. While its main purpose is to eradicate any remaining leukemic lymphoblasts that have escaped detection or are undergoing clonal evolution, it is also crucial in preventing the emergence of drug resistance and relies on a multidrug regimen that often includes the anthracycline doxorubicin[11]. Finally, maintenance therapy entails the use of maintenance chemotherapeutics and is important for preventing disease relapse[12]. Given the risks of treatment nonresponse and relapse throughout the treatment process, understanding the genetic milieu of ALL is crucial to properly treating the patient's disease.

Cell proliferation and chemoresistance are two major barriers to achieving complete remission that likely arise at least in part from a patient's genetics. Because B-cell ALL (B-ALL) accounts for around 85% of pediatric ALL cases, in this study we decided to focus on this ALL subtype[13]. In the past decades, genetic studies using oligonucleotide arrays and high-throughput sequencing have determined that B-ALL is highly heterogeneous and can exist as genetically distinct clones in the same person[14]. Dysregulated cell proliferation in ALL results not only from hallmark chromosomal aberrations (e.g., rearrangements of *BCR/ABL* and *TCF3*) but also from deletion, amplification, and point mutations in genes that regulate B lymphocyte development[15]. For example, Mullighan et al. discovered in 2008 that myriad genomic copy number abnormalities (CNAs) contribute to B-cell hyperproliferation and disease relapse[16]. The development of chemoresistance following induction therapy may also be largely due to a patient's genetics. For example, Irving et al. found in 2016 that *NRAS/KRAS* mutations confer chemoresistance to relapse clones[17]. Given the highly individualized genetic milieus that induce relapse and chemoresistance, it is crucial to develop treatments based on an individual patient's predisposition for these properties.

In the present study, we identified the cancer-associated gene NET1 as one of the most significantly elevated genes in human pediatric B-ALL samples and in human B-cell leukemia Nalm-6 cells. From there, we demonstrated that NET1 overexpression significantly increases B-ALL cell proliferation and resistance to doxorubicin. Ultimately, we discovered for the first time that miR-206 inhibits B-ALL cell proliferation and chemoresistance by negatively regulating NET1 expression.

MATERIALS AND METHODS

Patient Samples

The pediatric B-cell ALL samples ($n = 20$) and age-matched control samples ($n = 20$) were obtained from Shenzhen Longhua People's Hospital. Only cases with bone marrow (BM) samples containing 70% leukemic cells were included in this study. Age-matched subjects with no manifestations of any hematological malignancy were used as controls. Bone marrow specimens and peripheral blood were collected prior to the initial treatment. All patients were diagnosed B-cell ALL with age ranging from 0 to 12 years, and the median age of the patients was 7 years; 7 were males and 6 females, and 18 patients with the t(4;11) and 2 with the t(9;11) abnormalities. All consents were provided by guardians of the participants, with ethical approval from Shenzhen Longhua People's Hospital.

GSE Database Analysis

NET1 gene expression was analyzed using online microarray database GSE34670[18]. Twenty-five pediatric cALL samples were included, and expression patterns were analyzed using high-density DNA microarrays HG-U133A. Principal component analysis clearly distinguished between leukemia samples and normal controls (nine highly purified fetal early pre-B-cell samples). Significance analysis of microarrays revealed 487 genes significantly upregulated and 572 downregulated genes in leukemic cells.

Real-Time PCR

Total RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. First-strand cDNAs were synthesized using a mixture of oligo(dT) 12–18 primers with Superscript reverse transcriptase (Invitrogen). 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) according to the manufacturer's instructions. The relative levels of miR-206 mimic expression were calculated by the $2^{-\ Ct}$ 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 following thermal cycling was conducted: 95°C for 5 min, followed by 35 cycles of amplification (94°C for

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20 s, 58°C for 25 s, and 72°C for 30 s). The relative levels of gene expression were represented using the $2^{-\ Ct}$ method. The primer sequences are as follows: *NET1*, 5 -GTTCAGCTTCTGGAGGATGC-3 (forward) and 5 -CTTGTGGAACACGTCATTGG-3 (reverse); *GAPDH*, 5 -GGGTGTGAACCATGAGAAGT-3 (forward) and 5 -TGAGTCCTTCCACGATACCAA-3 (reverse); miR-206, 5 -CAAGATGGCGACTTACGGATG-3 (forward) and 5 -CTGCAGGTAGGACAAACGTG-3 (reverse); U6, 5 -CTCGCTTCGGCAGCACA-3 (forward) and 5 -AACGCTTCACGAATTTGCGT-3 (reverse).

Cell Culture

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% CO_2.

Cell Transfection

When Nalm-6 cells reached 70%–80% confluence, miR-206, miR-206 inhibitor, negative control (NC), and/or NET1 siRNA were transfected using Lipofectamine® RNAiMAX Reagent. Cells were cotransfected 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.

MTT Assay

MTT assays were used to measure Nalm-6 cell proliferation. A total of 5×10^3 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 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% CO_2 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, Tokyo, Japan).

Western Blot

Western blot was performed as previously reported[19]. Briefly, 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, P.R. 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 against GAPDH. Antibody complexes were detected with the ECL Western blot kit (Pierce). The following primary antibodies were used: anti-cleaved caspase 3, anti-BCL2, anti-NET1, and anti-GAPDH. All primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

ELISA Assay

Apoptosis of Nalm-6 cells was assayed by the Cell Death Detection Elisa Plus kit (Boehringer 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.

TUNEL Staining Assay

TUNEL staining was performed to assess the apoptotic Nalm-6 cells according to the manufacturer's protocol (Promega, Madison, WI, USA).

Luciferase Reporter Assay

Wild-type NET1 3 -UTR fragments were amplified and cloned into the psiCHECK2 luciferase reporter vector. The mutated NET1 3 -UTR was generated utilizing the NET1 3 -UTR plasmid as a template and mutating the miR-206 seed-binding site using the QuikChange® Multi Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). Wild-type or mutated NET1 3 -UTR luciferase reporter plasmid was cotransfected into Nalm-6 cells with miR-206 or NC. Transfected cells were incubated for 48 h, and relative luciferase activity was measured using a dual-luciferase reporter system (Promega).

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

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were expressed as mean ± SD of at least three independent experiments.

RESULTS

Upregulation of NET1 in B-ALL Patients

To identify B-ALL-associated gene expression and function, we analyzed GEO microarray data comparing bone marrow expression profiles of B-ALL patients with those of healthy donors (GSE34670)[18]. In this dataset, we compared the gene expression between 25 primary early pre-B-cell ALL samples and 9 highly purified fetal early pre-B-cell samples, and identified *NET1* as one of the most significantly elevated genes in B-ALL patients ($p < 0.001$) (Fig. 1A). To confirm that *NET1* is dysregulated in B-ALL patients, we performed real-time PCR to compare clinical B-ALL bone marrow with healthy control samples ($n = 20$). Consistent with the microarray analysis, *NET1* expression was significantly increased in clinical ALL samples ($p < 0.001$) (Fig. 1B). We then randomly chose four B-ALL samples and two healthy control samples and compared NET1 expression by Western blot. As shown in Figure 1C, NET1 was overexpressed in the B-ALL samples. Based on these results, we hypothesized that NET1 enhances oncogenesis in B-ALL.

NET1 Promotes B-ALL Cell Proliferation

To investigate the biological function of NET1 in B-ALL, we decreased or increased NET1 expression in Nalm-6 B cells using NET1 siRNA or NET1 plasmid, respectively (Fig. 2A and B). MTT assays showed that NET1 upregulation promoted the proliferation of Nalm-6 cells, whereas NET1 downregulation suppressed cell proliferation (Fig. 2C). Furthermore, colony formation assays showed that knockdown of NET1 significantly decreased the number of Nalm-6 cell colonies, whereas overexpression of NET1 increased colony numbers (Fig. 2D). Overall, these results indicate that NET1 increases B-ALL cell proliferation, while the absence of NET1 decreases B-ALL cell proliferation.

NET1 Promotes B-ALL Cell Chemoresistance

To determine the effect of NET1 on Nalm-6 cell chemoresistance, we treated Nalm-6 B transfected cells with 200 nM doxorubicin for 1 h. Following 12 h of growth in culturing medium, we then used ELISA assays to assess cell apoptosis. As shown in Figure 3A, NET1 knockdown increased cell apoptosis, while NET1 overexpression reduced apoptosis after doxorubicin treatment. Western blot analyses then demonstrated that NET1 overexpression inhibited caspase 3 and promoted Bcl-2 protein expression, whereas NET1 inhibition increased cleaved caspase 3 and reduced Bcl-2 protein expression (Fig. 3B). TUNEL staining assays further confirmed these findings, showing that NET1 overexpression resisted doxorubicin-induced Nalm-6 cell apoptosis, while NET1 inhibition enhances apoptosis (Fig. 3C). Taken together, these results indicate that NET1 contributes to B-ALL cell chemoresistance.

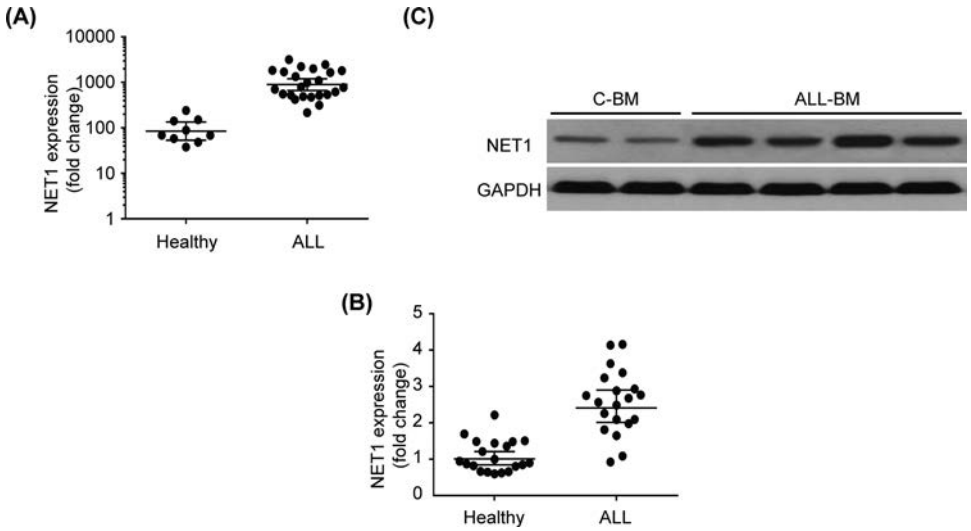

Figure 1. Upregulation of neuroepithelial cell-transforming 1 (NET1) in acute lymphoblastic leukemia (ALL) patients. (A) *NET1* expression in 25 ALL bone marrow samples compared with healthy donor B cells analyzed by microarray (GEO DataSets GSE34670, $p < 0.001$). (B) Real-time PCR analysis of *NET1* mRNA expression in ALL patient bone marrow samples compared with healthy donor B cells ($n = 20$, $p < 0.001$). (C) Western blot of NET1 protein expression in bone marrow samples of four ALL patients and two healthy controls.

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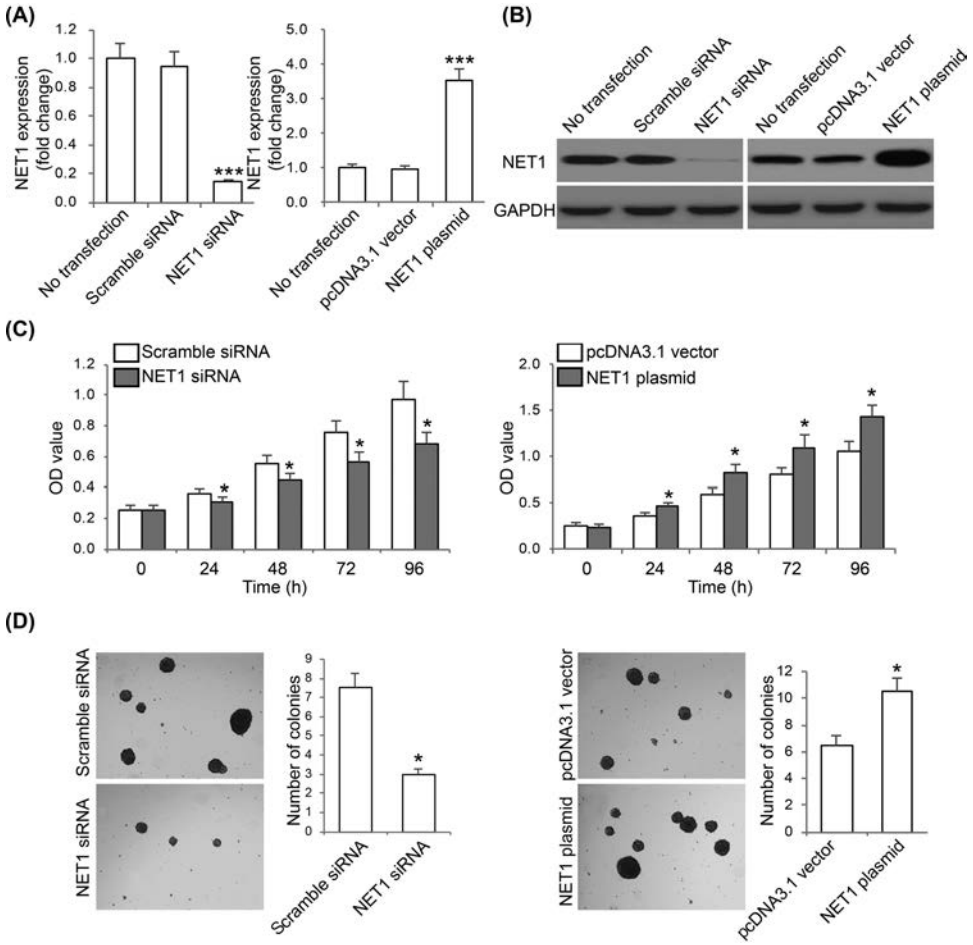

Figure 2. NET1 promotes ALL cell proliferation. (A) qRT-PCR of *NET1* expression in Nalm-6 cells transfected with NET1 siRNA or scramble siRNA, and NET1 overexpression plasmid or pcDNA3.1 vector. (B) Western blot of NET1 expression in Nalm-6 cells transfected with NET1 siRNA or scramble siRNA, and NET1 overexpression plasmid or pcDNA3.1 vector. (C) MTT assay of indicated Nalm-6 cells tested at different time points (0, 24, 48, 72, and 96 h). (D) Representative micrographs of colony formation assay of indicated Nalm-6 cells. $*p < 0.05$, $***p < 0.001$.

NET1 Is Targeted by miR-206

To determine whether the oncogenic effects of NET1 could be prevented, we next investigated whether NET1 is regulated by miRNA. Using the TargetScan biological target database, we found that several miRNAs were predicted to target NET1. These miRNAs included miR-206, which has been shown to play pivotal roles in cancer development (Fig. 4A). To determine whether miR-206 does in fact target NET1, we transfected Nalm-6 cells with miR-206 or its inhibitor miR-206-in. qRT-PCR and Western blot assays showed that miR-206 inhibited NET1 expression, while miR-206-in increased NET1 expression (Fig. 4B and C). We then generated luciferase reporters of wild-type NET1 3 -UTR and mutated NET1 3 -UTR, and performed luciferase assays by cotransfecting these reporters with miR-206 or negative control into Nalm-6 cells (Fig. 4D). As shown in Figure 4E, ectopic expression of miR-206 significantly decreased the luciferase activity of the wild-type NET1 3 -UTR luciferase reporter. We further observed a negative correlation between miR-206 expression and *NET1* mRNA expression in 20 ALL samples (Fig. 4F). Overall, these findings suggest that miR-206 binds the 3 -UTR of NET1, thereby reducing NET1 expression.

miR-206 Inhibits B-ALL Cell Proliferation

To study the biological function of miR-206 in ALL development, we transiently expressed miR-206, miR-206 inhibitor, or negative controls in Nalm-6 B cells (Fig. 5A). MTT assays demonstrated that ectopic miR-206 expression suppressed Nalm-6 cell proliferation, whereas miR-206 inhibitor promoted cell proliferation (Fig. 5B). Furthermore, ectopic expression of miR-206 significantly decreased the colony number of Nalm-6 cells in colony formation assays, whereas miR-206 inhibitor increased

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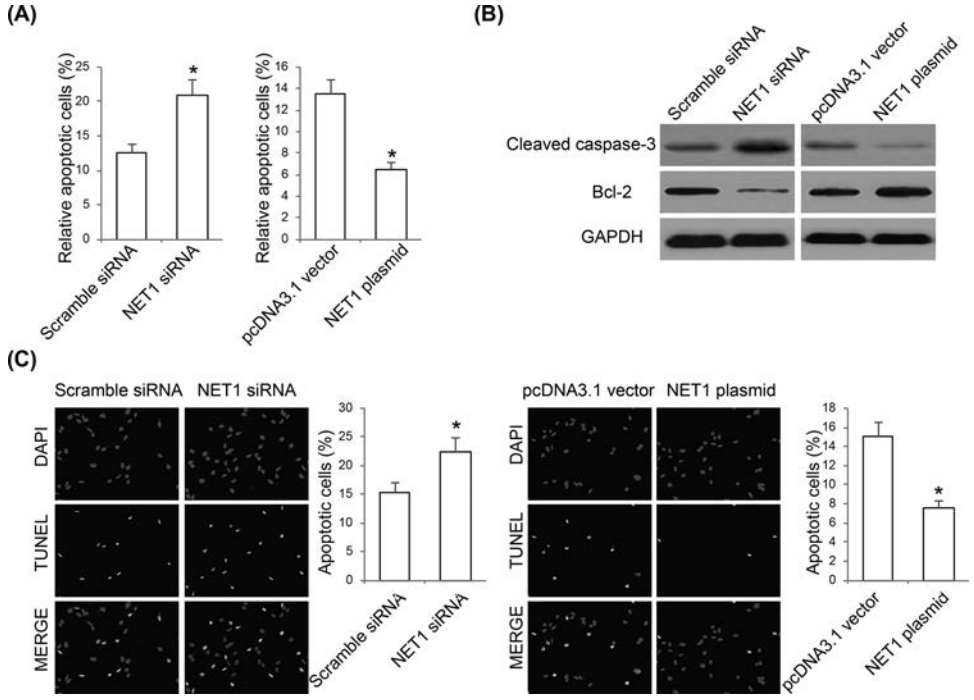

Figure 3. NET1 promotes ALL cell chemoresistance. Nalm-6 cells transfected with siRNA or scramble siRNA were treated with doxorubicin, and then (A) cell death detection ELISA, (B) Western blot analysis for caspase 3 and Bcl-2, and (C) TUNEL staining assays were performed. *$p < 0.05$.

colony numbers (Fig. 5C). These results indicate that miR-206 inhibits the B-ALL cell proliferation that NET1 otherwise promotes.

miR-206 Inhibits B-ALL Cell Chemoresistance

We next studied the effect of miR-206 on Nalm-6 cell chemoresistance. Transfected Nalm-6 cells were treated with 200 nM doxorubicin for 1 h and cultured in growing medium for 12 h. Apoptotic cells were then detected using ELISA assays. As shown in Figure 6A, miR-206 overexpression causes increased cell death in response to chemotherapy-induced DNA damage, whereas miR-206 inhibitor reduced cell death. Western blot analysis revealed that miR-206 overexpression increased cleaved caspase 3 and reduced Bcl-2 protein levels, while miR-206 inhibitor decreased caspase 3 expression and increased Bcl-2 protein levels (Fig. 6B). TUNEL staining confirmed these results by showing that miR-206 significantly increased Nalm-6 cell apoptosis following doxorubicin treatment, while the miR-260 inhibitor had the opposite effect (Fig. 6C). Overall, these findings strongly suggest that miR-260 inhibits NET1-induced B-ALL cell chemoresistance.

NET1 Is Required for miR-206 Function

To examine whether miR-206 affects B-ALL cells through NET1, we knocked down NET1 expression in Nalm-6 cells and inhibited miR-206 expression. Inhibition of miR-206 could not promote NET1 expression in NET1 knockdown Nalm-6 cells (Fig. 7A). We then performed a series of experiments and found that miR-206 inhibition failed to rescue B-ALL cell fate after NET1 knockdown (Fig. 7B–F). These results suggest that miR-206 functions ALL cells through NET1.

DISCUSSION

In this study, we have revealed a critical role for NET1 in B-ALL cell proliferation and chemoresistance. We have also identified miR-206 as a negative regulator of NET1 and its oncogenic effects. Using human pediatric B-ALL samples, age-matched control samples, and GEO microarray data analyses, we began by demonstrating that NET1 is significantly overexpressed in B-ALL specimens. After then finding that NET1 promotes B-ALL cell proliferation and doxorubicin resistance, we used the TargetScan biological target database to identify miR-206 as a possible binder of NET1's 3 -UTR. To confirm that miR-206 does actually target NET1, we transfected Nalm-6 cells with miR-206 or its inhibitor miR-206-in and measured the effects on NET1 expression by Western blot. We also generated luciferase reporters of wild-type NET1 3 -UTR and mutated NET1 3 -UTR and performed luciferase assays that showed that miR-206 specifically binds NET1's 3 -UTR. MTT, colony formation, and ELISA assays ultimately demonstrated that miR-206 inhibits the

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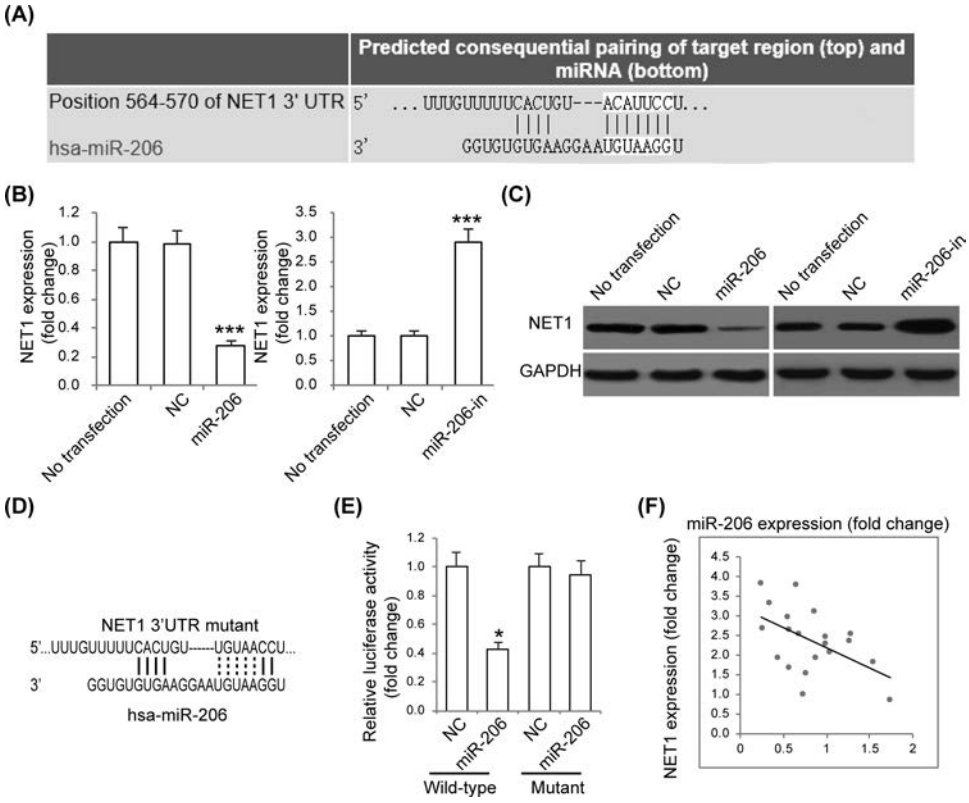

Figure 4. NET1 is targeted by microRNA-206 (miR-206). (A) Schematic representation of the human mature miR-206 sequence and the miR-206 target site in the 3 -untranslated region (3 -UTR) of *NET1* mRNA. (B) qRT-PCR of *NET1* in Nalm-6 cells transfected with miR-206 or miR-206 inhibitor (miR-206-in) compared with control cells 48 h after transfection. (C) Western blot of NET1 in Nalm-6 cells transfected with miR-206 or miR-206-in compared with control cells 48 h after transfection. GAPDH served as a loading control. (D) Schematic representation of the miR-206 sequence and the mutant of *NET1* mRNA 3 -UTR containing five altered nucleotides in the putative target site. (E) Luciferase assay of psiCHECK2-NET1 3 -UTR wild-type or mutant reporter cotransfected with miR-206 or NC. (F) Correlation analysis of miR-206 and NET1 mRNA in 20 ALL patients by quantitative PCR ($r = -0.518$, $p < 0.001$). *$p < 0.05$, ***$p < 0.001$.

B-ALL cell proliferation and chemoresistance promoted by NET1. Overall, these results contribute greatly to our current understanding of the roles that NET1 and miR-206 play in B-ALL treatment and progression.

NET1 is a protein-coding gene and guanine nucleotide exchange factor (GEF) for RhoA that was first isolated in 1996 by Chan et al. from neuroepithelioma cells[20]. In this originating study, NET1 was shown to regulate cell growth and induce tumorigenesis. Since then, NET1 has been associated with a number of cancers, including breast cancer[21], hepatocellular carcinoma[22], and non-small cell lung cancer[23]. In these studies, NET1 was shown to contribute to more aggressive cancer phenotypes with heightened proliferation, metastasis, and clinical stage. In some cancers, NET1 has even been shown to be a viable therapeutic target. For example, Ahmad et al. found in 2014 that miR-22 targeted and suppressed the oncogenic effects of NET1 in chronic myeloid leukemia (CML)[24]. Interestingly, Net1 has also been shown to facilitate RhoB-mediated cell death following ionizing radiation, which, like chemotherapy, functions by damaging the DNA of hyperproliferating cells[25]. However, prior to the present study, the effects of NET1 overexpression had never been assessed in the context of B-ALL. Our findings that NET1 significantly enhances B-ALL cell proliferation and chemoresistance build upon the body of research that suggests it has a crucial role in the regulation of oncogenesis and chemotherapeutic effects.

miRNAs are short (20–24 nucleotides) noncoding, evolutionarily conserved RNAs that bind the 3 -UTRs of target mRNAs, thereby regulating gene expression post-transcriptionally. miRNAs were initially largely appreciated for their roles in fundamental cellular functions, such as the regulation of growth, differentiation, apoptosis, and stress responses[26]. More recently, however, they have been implicated in a variety of cancers, including ALL, and are increasingly recognized as potential crucial biomarkers and treatment targets[27,28]. In 2017, Ramani et al.

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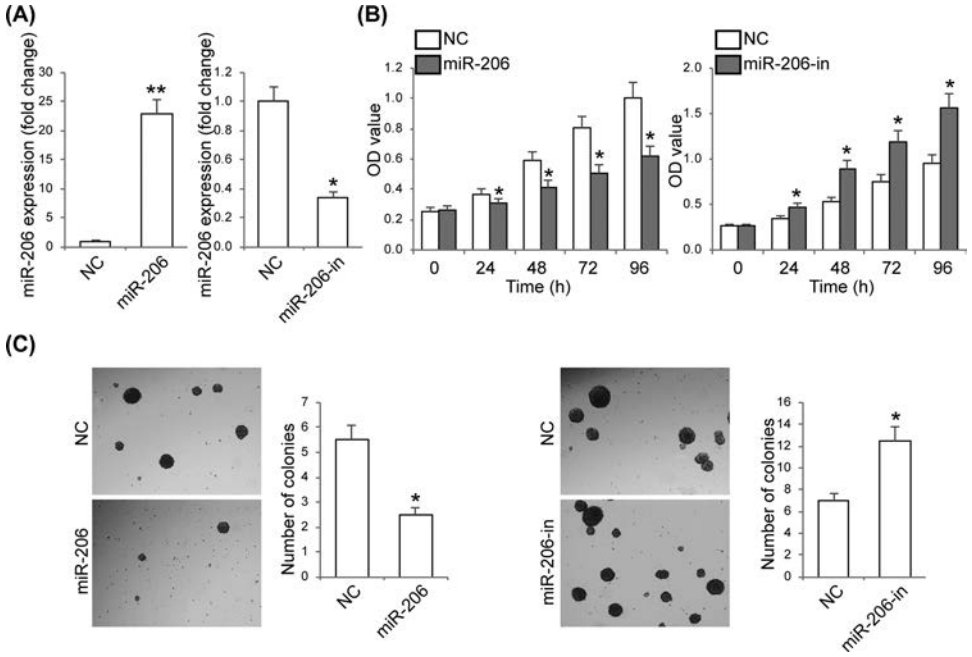

Figure 5. miR-206 inhibits ALL cell proliferation. (A) Real-time PCR analysis of miR-206 expression in Nalm-6 cells transfected with miR-206 or NC, and miR-206-in or NC. (B) MTT assay of indicated Nalm-6 cells tested at different time points (0, 24, 48, 72, and 96 h). (C) Representative micrographs of colony formation assay of indicated Nalm-6 cells. *$p < 0.05$, **$p < 0.01$.

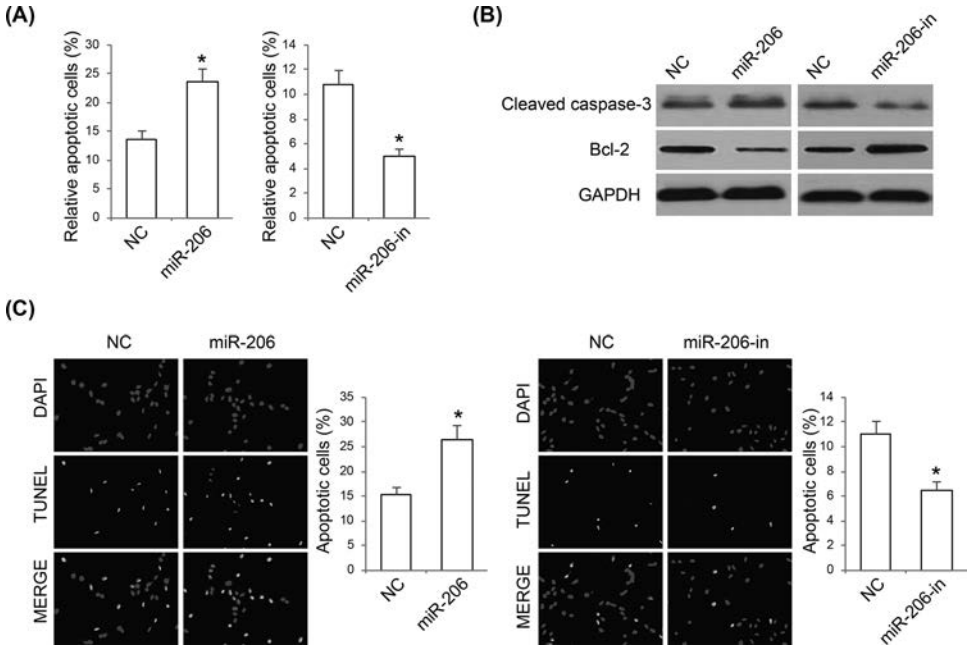

Figure 6. miR-206 inhibits ALL cell chemoresistance. Transfected Nalm-6 cells (as indicated in Fig. 5) were treated with doxorubicin, and then (A) cell death detection ELISA, (B) Western blot analysis for caspase 3 and Bcl-2, and (C) TUNEL staining assays were performed. *$p < 0.05$.

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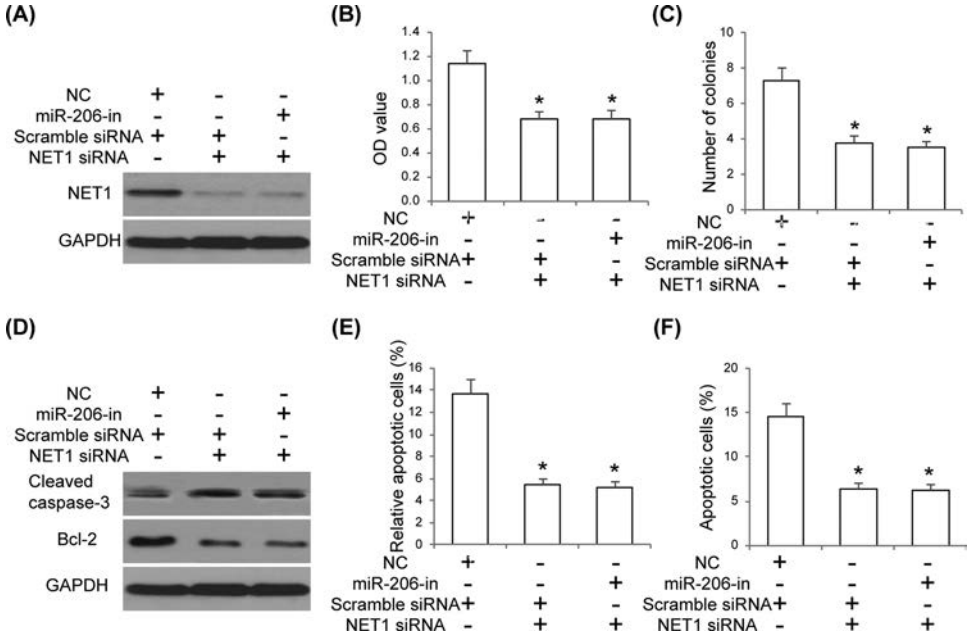

Figure 7. NET1 is required for miR-206 function. (A) Western blot of NET1 expression in NET1 knockdown Nalm-6 cells with or without miR-206 inhibition. (B) MTT, (C) colony formation, (D) Western blot of cleaved caspase 3 and Bcl-2 expression, (E) cell death detection ELISA, and (F) TUNEL staining assays in (A) cell groups. $*p < 0.05$.

discovered signatures of differentially expressed miRNAs in ALL to better risk stratify pediatric patients and identified several dysregulated miRNA-mediated molecular networks, including VEGF, IGF-1, and JAK/STAT[29]. Additionally, Correia et al. found in 2016 that downregulation of miR-146b by oncogene TAL1 promotes aggressive phenotypes of T-cell ALL (T-ALL), suggesting that exogenous miR-146b can potentially contribute to treatment strategies[30]. miR-206, which we have demonstrated in the present study to negatively regulate NET1, has not previously been associated with ALL. However, it has been shown to regulate various oncogenic properties in other cancers, including breast cancer[31], chondrosarcoma[32], and lung adenocarcinoma[33]. The present study is the first to establish the role of miR-206 as a negative regulator of B-ALL cell proliferation and chemoresistance.

CONCLUSIONS

Overall, this study provides the first evidence that NET1 enhances proliferation and chemoresistance in B-ALL cells and that miR-206 regulates these effects by targeting NET1. It is important for future studies to investigate the mechanisms by which NET1 exerts its oncogenic effects and how miR-206 acts as a tumor suppressor. Crucially, this study creates the foundation for improving the diagnosis, treatment, and prognosis of B-ALL through the incorporation of NET1 and miR-206 into clinical decision making.

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