MicroRNA-223 overexpression suppresses protein kinase C ε expression in human leukemia stem cell-like KG-1a cells

MALLIKA OSIRIPHAN[1,2], CHARAPAT INSUKHIN[1], SONGYOT ANUCHAPREEDA[1,3,4], PINYAPHAT KHAMPHIKHAM[1,4] and SUWIT DUANGMANO[1,3,4]

[1]Division of Clinical Microscopy, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Mueang Chiang Mai, Chiang Mai 50200, Thailand; [2]Master's Degree Program in Medical Technology (under the Chiang Mai University Presidential Scholarship), Faculty of Associated Medical Sciences, Chiang Mai University, Mueang Chiang Mai, Chiang Mai 50200, Thailand; [3]Cancer Research Unit of Associated Medical Sciences, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Mueang Chiang Mai, Chiang Mai 50200, Thailand; [4]Hematology and Health Technology Research Center, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Mueang Chiang Mai, Chiang Mai 50200, Thailand

Received February 1, 2024; Accepted May 1, 2024

DOI: 10.3892/mco.2024.2746

Abstract. MicroRNA-223 (miR-223) is dysregulated in various cancer types, including acute myeloid leukemia (AML). Despite this, there has been a lack of studies exploring the role of miR-223 in leukemic stem cells, particularly those involved in drug resistance, a major cause of chemotherapy failure in AML. The present study aimed to elucidate the impact of miR-223 on drug resistance in the leukemic stem-cell line, KG-1a. Two AML cell lines, KG-1 and KG-1a, differing in the proportion of $CD34^+CD38^-$ cells, were assessed for doxorubicin (DOX) sensitivity using the Cell Counting Kit-8 assay. The expression levels of miR-223 and protein kinase C ε (PKCε) were evaluated via reverse transcription-quantitative PCR and western blot analysis. The association between miR-223 and its target, PKCε, was confirmed by luciferase activity assay. The effects of miR-223 overexpression and PKCε inhibition were also evaluated in KG-1a cells using miR-223 mimic and small interfering (si)RNA transfection, respectively. Daunorubicin was then used to assess drug sensitivity in the siRNA-transfected KG-1a cells. Compared with KG-1 cells, KG-1a cells displayed greater resistance to DOX, and had increased PKCε levels and decreased miR-223 expression. Overexpression of miR-223 led to PKCε protein downregulation in KG-1a cells, which was further confirmed by a luciferase assay demonstrating miR-223 targeting of PKCε. However, despite these effects, miR-223 overexpression and PKCε inhibition did not change drug sensitivity in KG-1a cells compared with negative control cells. In summary, the present study demonstrated that miR-223 could target and silence PKCε expression in KG-1a cells; however, the chemoresistance of KG-1a cells to anthracycline drugs may not be directly associated with the low expression of miR-223.

Correspondence to: Dr Suwit Duangmano, Division of Clinical Microscopy, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, 110 Intawaroros Road, Sripoom, Mueang Chiang Mai, Chiang Mai 50200, Thailand
E-mail: suwit.du@cmu.ac.th

Key words: acute myeloid leukemia, leukemic stem cell, microRNA-223, protein kinase C ε, drug resistance

Introduction

Acute myeloid leukemia (AML) is one of the most prevalent hematological malignancies globally, constituting one-third of all adult leukemias (1-4). Despite advances in treatments leading to complete remission for most patients with AML, drug resistance remains a major factor in therapy failure and contributes to the short-term survival of these patients (4). Recently, accumulating evidence has implicated leukemic stem cells (LSCs), a dormant subset of leukemic cells, as pivotal in drug resistance and cancer relapse (5). These cells express the same markers as normal adult hematopoietic stem cells (HSCs) ($CD34^+$ and $CD38^-$) and exhibit stemness characteristics, such as self-renewal, proliferation and differentiation (5-8). In the past few years, several microRNAs (miRNAs/miRs), which represent small non-coding RNAs that act as post-transcriptional regulators, have been identified as participants in drug resistance mechanisms and LSC regulation, such as miR-22 and miR-126 (8,9). Therefore, investigations into miRNA-associated cancer pathogenesis are crucial for overcoming drug resistance events and regulating LSCs in AML.

Dysregulation of miR-223 has been documented in connection with various cancer types, including hepatocellular carcinoma, breast cancer and leukemia. Furthermore, numerous targets of miR-223, including insulin-like growth factor-1 receptor, monocytic enhancer factor 2C, microtubule destabilizer stathmin 1 and forkhead box protein

O1A, have been associated with cancer-related traits, such as cell proliferation, carcinogenesis and metastasis (10). However, few studies have explored the role of miR-223 in AML. Previous studies demonstrated low miR-223 expression in patients with AML, especially in those with a poor prognosis (11,12). Moreover, a marked increase in miR-223 expression was reported in patients with AML after treatment, regardless of whether complete remission was achieved or not (12). Another study demonstrated that miR-223 suppressed cell proliferation and enhanced cell apoptosis in HL-60 and K-562 cell lines by specifically targeting the expression of F-box/WD repeat-containing protein 7 (*FBXW7*) (13).

Protein kinase C ε (PKCε), an isoform of PKC, is encoded by the *PRKCE* gene. It phosphorylates a variety of protein targets and is known to participate in diverse cellular signaling pathways, including MAPK, ERK and PI3K/AKT pathways, which regulate various biological functions, such as proliferation, differentiation and apoptosis (14,15). PKCε has been implicated in drug resistance in several cancer types, including gallbladder cancer, lung cancer, renal cell carcinoma and prostate cancer (14,16-19). Additionally, a recent study identified high levels of *PRKCE* mRNA in gallbladder cancer stem cells (19). Another recent study demonstrated that PKCε overexpression has been shown to selectively confer resistance to daunorubicin (DNR) in the AML U937 and HEL cell lines. Furthermore, patients with high levels of PKCε protein exhibit a lower rate of complete remission compared with those with lower PKCε protein levels. Additionally, elevated PKCε expression has been associated with decreased disease-free survival, indicating a consistent pattern of treatment resistance (20).

The present study aimed to elucidate the roles of miR-223 and PKCε in the regulation of drug resistance mechanisms in LSCs to bridge current gaps in knowledge and offer valuable insights for the advancement of targeted therapies in AML.

Materials and methods

Cell culture. The human acute myeloblastic leukemia KG-1 cell line (cat. no. CCL-246) and its quiescent variant KG-1a (cat. no. CCL-246.1), obtained from the American Type Culture Collection, served as LSC models in the present study. KG-1a cells display less mature morphological, cytochemical and functional characteristics compared with KG-1 cells (21). Furthermore, the unresponsiveness of KG-1a cells to colony-stimulating factor and the lack of expression of human leukocyte antigen contributes to their increased resistance to differentiation-inducing drugs (22). Both cell lines were cultured in Iscove's modified Dulbecco's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 20% FBS (Capricorn Scientific), 1 mM L-glutamine (Cytiva), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.). 293T cells, used for the luciferase activity assay, were kindly provided by Dr Pinyaphat Khamphikham (an author of the present study). The 293T cells were cultured in DMEM (Nacalai Tesque, Inc.) containing 10% FBS, 1 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. All cell lines were cultured at 37˚C in a humidified atmosphere with 5% CO_2 and were passaged every 2-3 days or upon reaching 70-80% confluence.

Drug sensitivity assay. KG-1 and KG-1a cells were seeded in 96-well plates at a density of $1.5x10^4$ cells per well and were exposed to doxorubicin (DOX) (Fresenius Kabi Oncology, Ltd.) at concentrations of 0.03125, 0.0625, 0.125, 0.25, 0.5 and 1.0 μg/ml. For the DNR sensitivity test, small interfering (si)RNA-transfected KG-1a cells were seeded at the same density and treated with DNR (APeXBIO Technology LLC) at concentrations of 0.03125, 0.0625, 0.125 and 0.25 μg/ml. Blank wells containing only growth medium were utilized to subtract background signals. Following 48 h of incubation at 37˚C with 5% CO_2, the Cell Counting Kit (CCK)-8 assay (Abbkine Scientific, Co., Ltd.) was conducted following the manufacturer's protocol. After incubation with the CCK-8 reagent for 2 h at 37˚C, the absorbance at 450 nm was determined using a microplate reader (Metertech, Inc.). The experiment was repeated three times, and cell survival was determined using the following formula: % Cell viability=[mean optical density (OD) of the treated group-blank/mean OD of the untreated control group-blank] x100. The half-maximal inhibitory concentration (IC_{50}) value was established by plotting the cell viability rate (y-axis) against DOX concentration (x-axis) to generate a linear equation: y=ax + b. The IC_{50} value was calculated as follows: IC_{50}=(50-b)/a.

RNA isolation and reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from KG-1 and KG-1a cells using TRI Reagent® (Molecular Research Center, Inc.). The concentration of isolated total RNA was measured using a Qubit 4 fluorometer (Invitrogen; Thermo Fisher Scientific, Inc.) with the Qubit RNA High Sensitivity Assay kit (Invitrogen; Thermo Fisher Scientific, Inc.). To reverse transcribe mature miR-223, the RNA samples underwent conversion into *U6* and miR-223-specific cDNA using SuperScript III Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) with RT primers, according to the protocol of Varkonyi-Gasic and Hellens (23). For the conversion of mRNA into cDNA, total RNA (1 μg) was reverse transcribed into cDNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo Life Science) following the manufacturer's protocol. The RT reactions were performed on an MJ Mini thermocycler (Bio-rad Laboratories, Inc.). qPCR was performed using SensiFAST SYBR No-ROX reagent kit (Meridian Bioscience, Inc.). The primers used in the present study are listed in Table I. The qPCR was performed on a CFX Opus 96 Real-time PCR system (Bio-Rad Laboratories, Inc.) with the following cycling conditions: 95˚C for 2 min, followed by 40 cycles at 95˚C for 5 sec and 60˚C for 15 sec. The samples were independently analyzed three times, and relative expression was assessed using the $2^{-\Delta\Delta Cq}$ method (24), with *U6* and *GAPDH* as reference genes for miR-223 and *PRKCE*, respectively. As each replicated experiment was conducted individually at different time points, the expression levels of miR-223 or *PRKCE* of the control groups were standardized to 1, and the relative expression of the other groups were normalized to the expression of the control groups within each replicate.

Western blot analysis. Cells were lysed, and proteins were extracted using RIPA buffer composed of 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5 mM EDTA, 0.1% SDS and a protease inhibitor cocktail (HiMEdia Laboratories, LLC). The

Table I. Primer sequences used for RT-quantitative PCR.

Gene	Direction	Sequence (5'-3')
miR-223 RTp[a]	N/A	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGGGGTA
U6 RTp[a]	N/A	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAAAAATATG
miR-223[a]	Forward	AACACGCTGTCAGTTTGTCAA
	Reverse	GTCGTATCCAGTGCAGGGT
U6[a]	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCACGAATTTGCGT
GAPDH[b]	Forward	AACGGGAAGCTTGTCATCAATGGAAA
	Reverse	GCATCAGCAGAGGGGGCAGAG
PRKCE[a]	Forward	AGCCTCGTTCACGGTTCT
	Reverse	TGTCCAGCCATCATCTCG

[a]Synthesized by Macrogen, Inc.; [b]synthesized by Bio Basic, Inc. RTp, reverse transcription primer; N/A, not applicable; miR, microRNA; *PRKCE*, protein kinase C ε.

Table II. miRNA mimics and siRNAs.

Molecule	Strand	Sequence (5'-3')
mimic-NC	Sense	UUCUCCGAACGUGUCACGUTT
	Antisense	ACGUGACACGUUCGGAGAATT
miR-223 mimic	Sense	UGUCAGUUUGUCAAAUACCCCA
	Antisense	GGGUAUUUGACAAACUGACAUU
si-NC	Sense	GGCAAGACCAGCUGCGACAUU
	Antisense	AAUGUCGCAGCUGGUCUUGCC
si-PRKCE#1	Sense	CCAGUCUGAAUACAGGUAGAUAUTA
	Antisense	UAAUAUCUACCUGUAUUCAGACUGGAA
si-PRKCE#2	Sense	GUCAAUAAUUUUGAGCAAGACUUTA
	Antisense	UAAAGUCUUGGUCAAAAUUAUUGACGU
si-PRKCE#3	Sense	UGAAAGCUUUCAUGACGAAGAAUCC
	Antisense	GGAUUCUUCGUCAUGAAAGCUUUCAAG

NC, negative control; miRNA/miR, microRNA; si, siRNA; *PRKCE*, protein kinase C ε.

quantification of the extracted proteins was determined using Qubit 4 fluorometer and a Qubit protein assay kit (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, protein samples (50 μg per lane) were combined with loading buffer, boiled at 95˚C for 5 min, separated on 7.5% SDS-PAGE gels, and then transferred to PVDF membranes. The membranes were blocked with 5% skimmed milk in PBS at room temperature for 3 h, followed by overnight incubation at 4˚C with primary rabbit antibody targeting PKCε (1:1,000 dilution; cat. no. 2683; Cell Signaling Technology, Inc.) or for 1 h at room temperature with primary rabbit antibody targeting GAPDH (1:16,000 dilution; cat. no. ABS16; MilliporeSigma). The membranes were rinsed six times (5 min each time) with 0.1% Tween-PBS solution and subsequently incubated with HRP-conjugated anti-rabbit IgG secondary antibody (1:20,000 dilution; cat. no. W401B; Promega Corporation) for 1 h at room temperature. Immobilon Forte Western HRP substrate (MilliporeSigma) was used for signal development, and the density of protein bands was analyzed using Quantity One software version 4.6.8 (Bio-Rad Laboratories, Inc.). Each replicated experiment was performed separately at a different time point. The levels of the control groups were standardized to 100%, and the relative expression of the other groups was normalized against the expression of the control group within each replicate.

Cell transfection. Mimic-negative control (NC) was obtained from Shanghai GenePharma Co., Ltd., while miR-223 mimic was purchased from Ambion (Thermo Fisher Scientific, Inc.). For the knockdown of PKCε, a TriFECTa RNAi kit, comprising siRNAs si-*PRKCE*#1, si-*PRKCE*#2 and si-*PRKCE*#3, was purchased from Integrated DNA Technologies, Inc. si-NC was purchased from Ambion; Thermo Fisher Scientific, Inc. The sequences of miRNA mimics and siRNAs utilized in the present study are listed in Table II. The transfection of miRNA mimics or siRNAs into the cells was performed using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol.

For miRNA mimic transfection, KG-1a cells were transfected with either 25 nM miR-223 mimic or mimic-NC for 24 h at 37˚C. Regarding siRNA transfection, KG-1a cells were transfected with 25 nM of either si-NC, si-*PRKCE*#1, si-*PRKCE*#2 or si-*PRKCE*#3 for 48 h at 37˚C. Subsequent experiments were conducted instantly following transfection.

Dual-luciferase reporter assay. To investigate whether PKCε serves as a direct target of miR-223, TargetScan software (https://www.targetscan.org/vert_80/) was used to predict the binding targets of miR-223, one of which was identified to be *PRKCE* mRNA. The pmiRGLO plasmid (Promega Corporation) was used to generate constructs with the 3' untranslated region (UTR) sequence of *PRKCE*, encompassing the miR-223 binding site. These constructs included pmiRGLO-*PRKCE*-3'UTR-wild-type (WT) and pmiRGLO-*PRKCE*-3'UTR-mutant (Mut). Co-transfection of these constructed plasmids (200 ng each) with miR-223 mimic or mimic-NC (10 pmol each) was performed in 293T cells using Lipofectamine 2000 reagent in accordance with the manufacturer's protocol. After 48 h of transfection at 37˚C, firefly and *Renilla* luciferase activities were promptly quantified using the Dual-Glo Luciferase Assay System kit (Promega Corporation) and a CLARIOstar Plus microplate reader (BMG Labtech GmbH). The firefly luciferase activity was normalized to the *Renilla* luciferase activity of each sample. This experiment was independently repeated three times.

Statistical analysis. Data are presented as the mean ± standard deviation. Data analysis was conducted using SPSS version 20 (IBM Corp.). An unpaired Student's t-test was used to make comparisons between two groups. Differences among multiple groups were analyzed using one-way ANOVA followed by Dunnett's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-223 is downregulated and PKCε is upregulated in the drug-resistant KG-1a cell line. The assessment of drug resistance in LSC cell lines involved treating both KG-1 and KG-1a cells with varying concentrations of DOX. The findings indicated a significantly higher IC_{50} in KG-1a cells (0.650±0.004 μg/ml) compared with that in KG-1 cells (0.435±0.02 μg/ml) (P<0.001), revealing that KG-1a cells exhibited higher resistance to DOX (Fig. 1A).

Using the RT-qPCR method, miR-223 levels were examined in KG-1 and KG-1a cells. There was a significantly higher expression level of miR-223 in KG-1 cells compared with that in KG-1a cells (P<0.001), indicating that miR-223 was expressed at a lower level in the KG-1a cell line (Fig. 1B). To further examine PKCε expression in KG-1 and KG-1a cells, both RT-qPCR and western blot analysis were employed. The results demonstrated that PKCε expression was significantly higher in KG-1a cells at both the mRNA (P<0.001) and protein levels (P<0.01), compared with that in the KG-1 cell line (Fig. 1C and D).

miR-223 targets and inhibits the expression of PKCε. To investigate the association between miR-223 and PKCε, KG-1a cells were transfected with miR-223 mimic. After 24 h of transfection, RT-qPCR analysis revealed a significant increase in miR-223 expression in the miR-223 mimic group compared with that in the NC group (P<0.01; Fig. 2A), indicating successful transfection.

Subsequent evaluation of PKCε expression at both the mRNA and protein levels post-transfection showed no significant difference in *PRKCE* mRNA levels between the miR-223 mimic group and mimic-NC group (P=0.455; Fig. 2B). However, there was a significant decrease in PKCε protein level in the miR-223 mimic group compared with that in the mimic-NC group (P<0.01; Fig. 2C), suggesting that the miR-223 mimic inhibited the protein expression of PKCε.

Considering the predicted binding site of miR-223 on *PRKCE* mRNA, as obtained through the bioinformatics software TargetScan, a dual-luciferase reporter assay was conducted to validate this interaction. There was a significant reduction in luciferase activity in cells co-transfected with *PRKCE*-3'UTR-WT and miR-223 mimic (P<0.05), while no changes were observed in the luciferase activity of cells co-transfected with *PRKCE*-3'UTR-Mut and miR-223 mimic, compared with that in the corresponding mimic-NC group (P=0.132) (Fig. 2D). These findings indicate that miR-223 can target and suppress PKCε expression.

Overexpression of miR-223 and inhibition of PKCε are not associated with the drug sensitivity of KG-1a cells. To explore the impact of miR-223 overexpression on drug sensitivity in KG-1a cells, miR-223 mimic-transfected cells were subjected to varying concentrations of DOX. The results showed no significant difference in DOX sensitivity between the miR-223 mimic group and the mimic-NC group, with IC_{50} values of 0.614±0.15 and 0.558±0.08 μg/ml, respectively (P=0.598; Fig. 3A). This suggests that the overexpression of miR-223 did not affect the DOX sensitivity of the KG-1a cell line.

To investigate the role of PKCε in the drug sensitivity of KG-1a cells, the cells were transfected with si-NC, si-*PRKCE*#1, si-*PRKCE*#2 and si-*PRKCE*#3 to assess the effects of PKCε knockdown. The results indicated that si-*PRKCE*#3 was the most effective siRNA for suppressing PKCε expression, resulting in significantly lower *PRKCE* mRNA expression compared with the si-NC group (P<0.05). Conversely, the si-*PRKCE*#1 (P=0.917) and si-*PRKCE*#2 (P=0.291) groups exhibited no significant differences in *PRKCE* mRNA expression compared with the si-NC group (Fig. 3B). PKCε protein levels were evaluated in the si-*PRKCE*#3 group, confirming a reduction in PKCε levels in si-*PRKCE*#3-transfected cells compared with those in the si-NC group (P<0.05; Fig. 3C). Consequently, KG-1a cells transfected with si-*PRKCE*#3 were treated with various concentrations of DNR, as previous findings have suggested that PKCε overexpression confers selective resistance to DNR in AML (20). However, the results demonstrated that DNR sensitivity was not changed after PKCε knockdown compared with the si-NC group, with IC_{50} values of 0.157±0.01 and 0.172±0.01 μg/ml, respectively (P=0.076; Fig. 3D). These findings suggest that neither miR-223 overexpression nor PKCε knockdown appears to be directly associated with drug sensitivity in KG-1a cells.

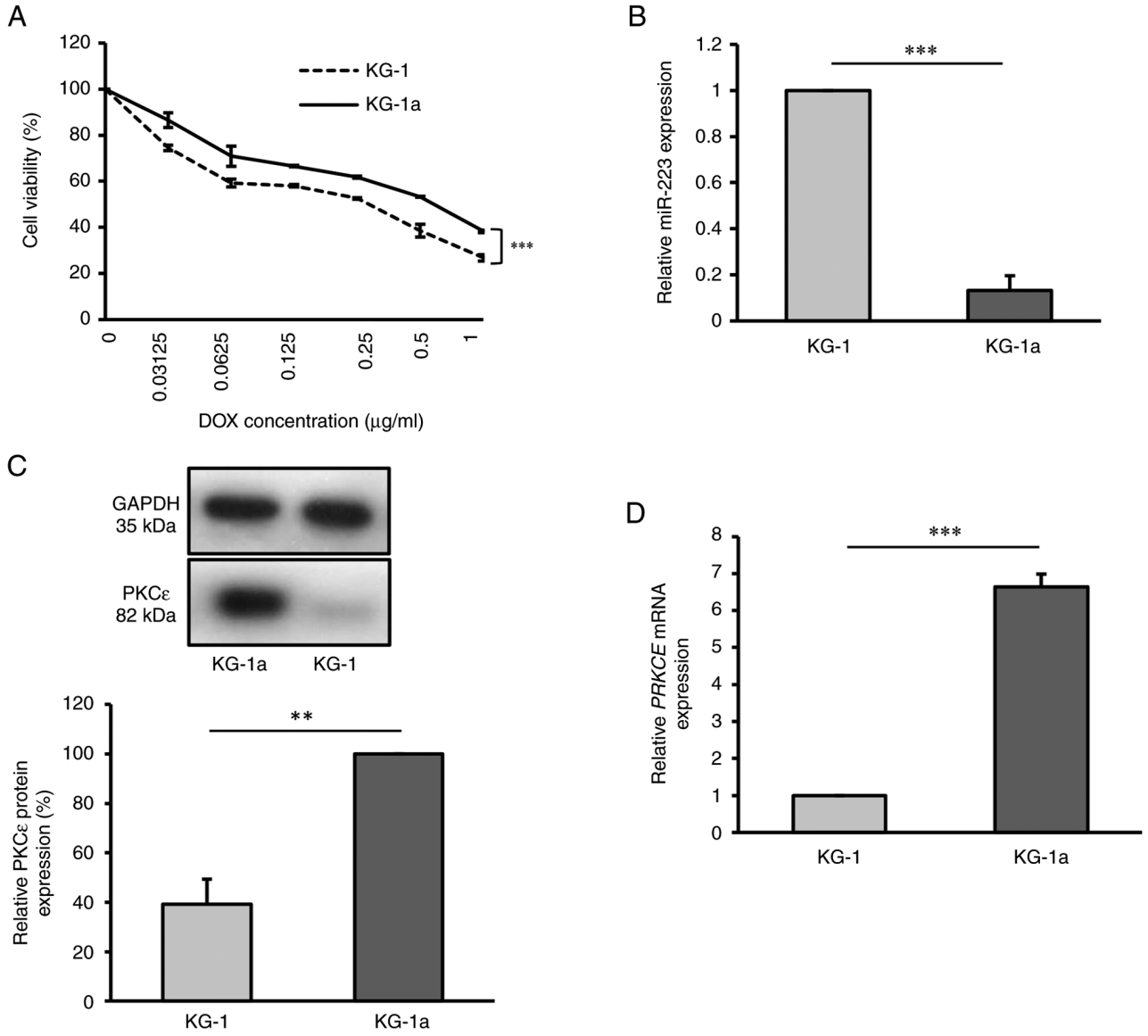

Figure 1. Drug resistant KG-1a cell line exhibits downregulation of miR-223 and upregulation of PKCε. (A) Cell viability of KG-1 and KG-1a cell lines after DOX treatment for 48 h, as detected by Cell Counting Kit-8 assay. (B) Relative miR-223 expression in KG-1 and KG-1a cells, as detected by RT-qPCR. (C) Relative PKCε protein level in KG-1 and KG-1a cells, as detected by western blot analysis. (D) Relative *PRKCE* mRNA expression in KG-1 and KG-1a cells, as detected by RT-qPCR. The values of KG-1 group in (B), (C) and (D) were normalized to 1 or 100% without accounting for standard error, due to the use of different batches for each replicate. **P<0.01; ***P<0.001 (n=3 replicates/group). The data were analyzed using unpaired Student's t-test. DOX, doxorubicin; miR, microRNA; PKCε/*PRKCE*, protein kinase C ε; RT-qPCR, reverse transcription-quantitative PCR.

Discussion

AML is a prevalent global hematological malignancy (1-4), with significant advancements in molecular targeted therapy leading to complete remission in a number of patients. However, affordability remains a considerable challenge in developing countries, driven by high treatment expenses, limited accessibility, inadequate insurance coverage, significant out-of-pocket costs and income disparities. Furthermore, the lack of treatment options in certain countries poses a challenge, considering that targeted therapies may not be provided according to the standard of care or may not be available for specific cancer types (25-29). Additionally, drug resistance remains the primary cause of chemotherapy failure, impacting patient survival rates (4). Therefore, studies into traditional chemotherapy remain crucial to provide effective treatment options. Given the association of miR-223 with various cancer types (10) and bioinformatics predictions identifying the *PRKCE* gene as one of the targets of miR-223, the present study aimed to elucidate the roles of miR-223 and PKCε in regulating drug resistance mechanisms in LSCs.

The current study showed that KG-1a cells exhibit a higher IC_{50} for DOX compared with KG-1 cells, which is consistent with the findings of a previous study that reported that KG-1 cells were more responsive to DOX than KG-1a cells (30). The difference in the percentage of $CD34^+$ $CD38^-$ LSCs between the two cell lines (75.95% in KG-1 and 92.82% in KG-1a), as revealed in our previous study (31), indicates that the number of $CD34^+$ $CD38^-$ LSCs may impact the chemosensitivity of these cell lines. It is well-established that LSCs, characterized by dynamic origins, derive from various cell types within leukemia, demonstrating their complex adaptability in

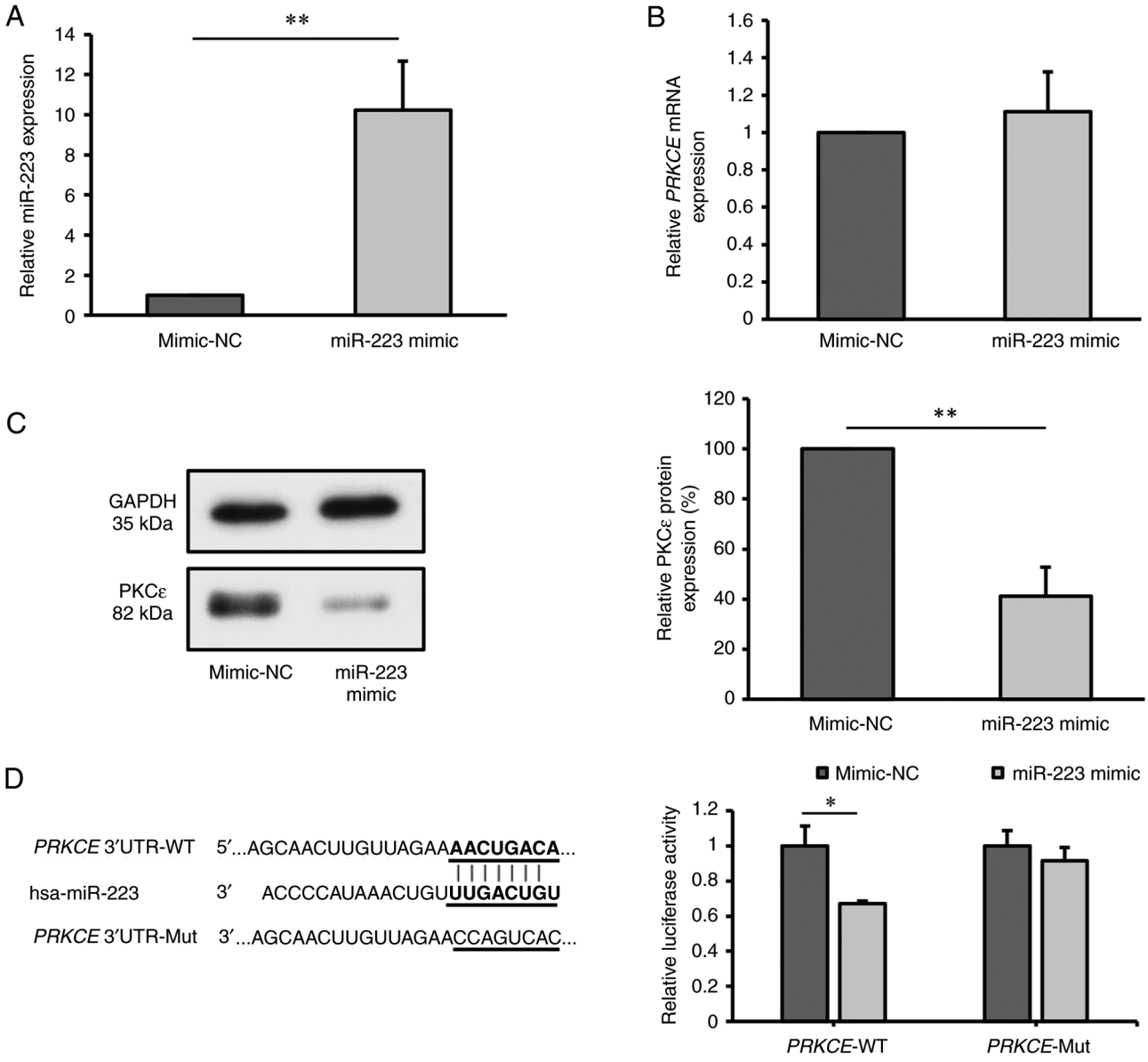

Figure 2. miR-223 targets and inhibits the expression of PKCε. (A) Relative miR-223 expression in KG-1a cells after transfection with miR-223 mimic, detected by RT-qPCR; mimic-NC values were normalized to 1. (B) Relative *PRKCE* mRNA expression in KG-1a cells after transfection with miR-223 mimic, as detected by RT-qPCR. (C) Relative PKCε protein level in KG-1a cells after transfection with miR-223 mimic, as detected by western blot analysis. (D) Binding sites between miR-223 and *PRKCE* mRNA predicted by TargetScan, and relative PKCε luciferase activity measured by dual-luciferase assay. The values of mimic-NC group in (B) and (C) were normalized to 1 or 100% without considering standard error, as each replicate was conducted using different batches. *P<0.05; **P<0.01 (n=3 replicates/group). The data were analyzed using unpaired Student's t-test. miR, microRNA; NC, negative control; PKCε/*PRKCE*, protein kinase C ε; WT, wild-type; Mut, mutant; UTR, untranslated region; RT-qPCR, reverse transcription-quantitative PCR.

disease progression and treatment. These cells harbor various elusive resistance mechanisms, such as inherent dormancy, overexpression of ATP-binding cassette transporters, defects in apoptotic signals, resistance to apoptosis and senescent signals, metabolic reprogramming and epigenetic alternations, which contribute to differences in chemosensitivity (32).

In the present study, lower miR-223 expression was observed in KG-1a cells compared with KG-1 cells. Similarly, a previous study reported lower expression of miR-223 in blast cells from AML patients within fractions containing $CD34^{+}CD38^{-}$ LSCs compared with fractions of more committed leukemic cells lacking the CD34 marker (11). Downregulation of miR-223 has been observed in patients with AML, particularly those with intermediate and unfavorable prognoses (11-13). It is therefore likely that miR-223 functions as a tumor-suppressing miRNA in AML.

In contrast to miR-223, PKCε was observed to be upregulated in KG-1a cells at both the mRNA and protein levels, compared with the KG-1 cell line. The bioinformatics prediction software TargetScan, along with a luciferase assay, confirmed that miR-223 binds to the 3'UTR of *PRKCE* mRNA, resulting in the inhibition of the luciferase activity. Additionally, overexpression of miR-223 suppressed PKCε protein expression in KG-1a cells, although there was no notable impact on *PRKCE* mRNA levels. A previous study similarly reported that, in non-small cell lung cancer cell lines, miR-143 mimic transfection resulted in the reduction of PKCε protein levels, while mRNA levels remained unchanged (33). Typically, miRNAs function by

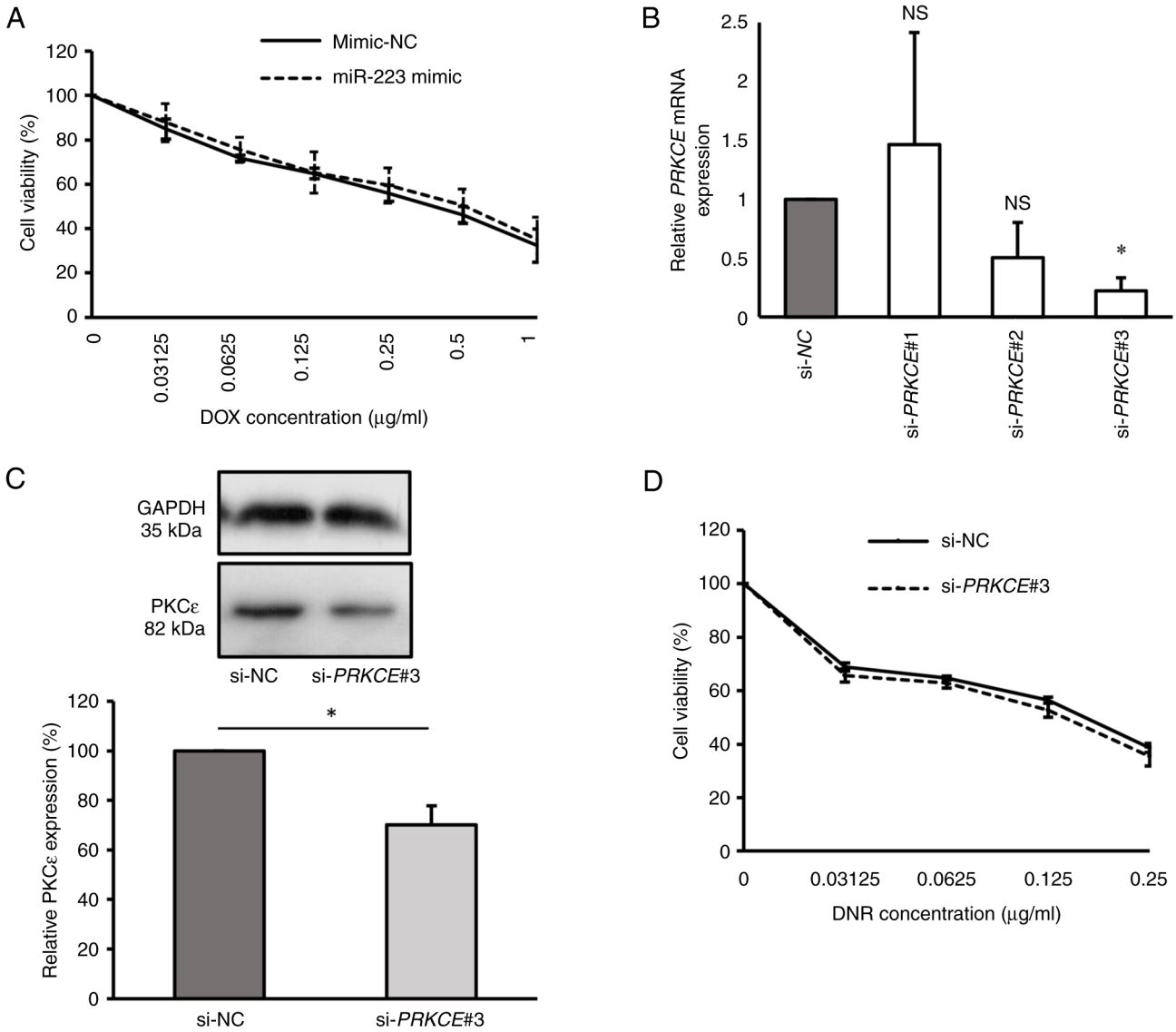

Figure 3. Overexpression of miR-223 and inhibition of PKCε are not associated with the drug sensitivity of KG-1a cells. (A) Cell viability of transfected KG-1a cells after DOX treatment for 48 h, as detected by CCK-8 assay. (B) Relative *PRKCE* mRNA expression in KG-1a cells after transfection with *PRKCE* siRNAs, as detected by reverse transcription-quantitative PCR. (C) Relative PKCε protein level in KG-1a cells after transfection with si-*PRKCE*#3, as detected by western blot analysis. (D) Cell viability of PKCε-knocked down KG-1a cells after DNR treatment for 48 h, as detected by CCK-8 assay. The values of si-NC group in (B) and (C) were normalized to 1 or 100% without considering standard error, as each replicate was conducted using different batches. *P<0.05 vs. si-NC (n=3 replicates/group). The data were analyzed using unpaired Student's t-test or one-way ANOVA followed by Dunnett's post hoc test. DOX, doxorubicin; miR, microRNA; NC, negative control; si, siRNA; PKCε/*PRKCE*; protein kinase C epsilon; DNR, daunorubicin; CCK-8, Cell Counting Kit-8; NS, non-significant.

inhibiting translation when they only partially match the 3'UTR of target genes. However, if there is a perfect match, miRNAs induce mRNA cleavage of the target genes (34,35). In animal cells, most interactions between miRNAs and their binding sequences are not completely complementary, and mismatches usually occur, especially in the central region of the target sequence (35). Based on the findings of the present study, miR-223 likely inhibits *PRKCE* mRNA translation. However, a previous study found that when miRNAs are temporarily overexpressed using mimics, the mRNA levels of target genes decrease 30 min after transfection, but start to recover after 12 h, while protein levels begin to recover after 24 h (36). In the present study, changes in *PRKCE* mRNA and protein levels were examined 24 h after transfection. It cannot therefore be definitively determined whether miR-223 functions as a translation suppressor for *PRKCE* mRNA or if the observed effect is due to the recovery of *PRKCE* mRNA following transfection. Previous studies have also demonstrated the contrasting expression of miR-223 and PKCε. Reduced miR-223 expression and the accompanying elevation of PKCε levels were associated with the formation of Gottron's papules in dermatomyositis (37). Conversely, in ovarian cancer, increased miR-223 expression was observed alongside decreased levels of PKCε (38). Together with the results of the present study, this indicates that miR-223 can target and suppress PKCε protein expression.

Despite the aforementioned findings, the overexpression of miR-223 did not affect DOX sensitivity in the KG-1a cell line. As miRNAs have the capacity to regulate numerous genes by binding to the 3'UTR of target mRNAs, it is plausible that miR-223 may target multiple genes apart from PKCε.

For example, a previous study in colorectal cancer cells demonstrated that miR-223 promoted DOX resistance by regulating epithelial-mesenchymal transition via targeting of *FBXW7* (39). Therefore, it is possible that miR-223 may regulate other genes that have a more significant impact on drug resistance than PKCε in the KG-1a LSC line.

It was also observed that downregulation of PKCε using siRNA did not improve the sensitivity to DNR in KG-1a cells. Despite a recent study by Nicholson *et al* (20), which demonstrated that inducing PKCε overexpression in AML cell lines resulted in specific resistance to DNR through an increase in P-glycoprotein levels, it was also observed that PKCε knockdown in the AML U937 and MV4-11 cell lines did not impact chemosensitivity. This indicates that PKCε inhibition alone may be insufficient to restore drug resistance in AML cells, possibly due to the redundancy among PKC isoforms. Drug resistance in LSCs can arise from various mechanisms beyond drug efflux transporters. LSCs employ crucial signaling pathways, such as Wnt/β-catenin, Hedgehog, NOTCH and PI3K/AKT, to manage their stemness traits. This regulation induces a state of dormancy (G_0 state), which protects them from cell cycle-specific factors that target actively proliferating cells. Dysfunctions of apoptotic signals and senescence mechanisms in LSCs also contribute to chemotherapy failure. Metabolic reprogramming enables LSCs to adapt to energy level fluctuations, while epigenetic modifications and reprogramming further enhance their stemness properties (32). Some studies have discussed the role of PKCε in the regulation of stemness features, such as differentiation and self-renewal. For example, downregulation of PKCε was indicated to preferentially promote the differentiation of colorectal cancer cells (40). The phosphorylation of ERK-1/2 and AKT, which typically promotes differentiation, was reduced through short hairpin RNA-mediated knockdown of PKCε in human pluripotent stem cells, resulting in a metastable undifferentiated state (15). In another study, PKCε exhibited no notable influence on the efficiency of colony formation, but eliminated the formation of cobblestone area-forming cells, indicating that PKCε selectively promotes the quiescence of HSCs (41). Based on this evidence, PKCε may preferably function in the regulation of differentiation or self-renewal signaling pathways rather than by directly influencing drug sensitivity in AML.

The present study is constrained by certain limitations. Firstly, the experiments were conducted *in vitro*, specifically focusing on a single cell line, KG-1a cells, which may not fully represent the diversity of LSCs. The second limitation is the absence of clinical studies to confirm the results of the *in vitro* experiments, particularly the evaluation of miRNA-223 and PKCε levels in newly diagnosed patients with AML before and after treatment, as well as the therapeutic response of these patients. Therefore, addressing these limitations should be a priority for future research endeavors.

Overall, the present study unveiled the downregulation of miR-223 in human LSC-like KG-1a cells. The biological function of miRNAs is to regulate target genes. In the present study, a luciferase reporter assay system and bioinformatics analysis were utilized to validate PKCε as one of the target genes of miR-223 in KG-1a LSCs. However, both the overexpression of miR-223 and PKCε knockdown failed to improve the chemosensitivity of KG-1a cells, suggesting that the miR-223/PKCε axis may not be associated with the sensitivity of KG-1a cells to anthracycline drugs, such as DOX and DNR. Consequently, further investigations are warranted to elucidate the roles of miR-223 and PKCε in the drug resistance of LSCs. This understanding is crucial for unraveling drug resistance mechanisms and achieving objectives related to using miRNAs as biomarkers for diagnosis and prognosis, as well as developing targeted therapies in AML.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Master's degree program in Medical Technology, Faculty of Associated Medical Science, Chiang Mai University (through the Chiang Mai University Presidential Scholarship; grant no. 2564-070) and the Fundamental Fund 2020 from Chiang Mai University (grant no. R000030108).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MO performed the experiments, and contributed to data acquisition, statistical analysis and the preparation of the manuscript. SD contributed to the study conception and design and the manuscript review. CI performed the experiments regarding the PKCε knockdown. SA advised on the design of the experiments regarding cell culture. PK provided the 293T cell line and advised on the use of molecular techniques during the experiments. SD and MO confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Deschler B and Lübbert M: Acute myeloid leukemia: Epidemiology and etiology. Cancer 107: 2099-2107, 2006.
2. Pelcovits A and Niroula R: Acute myeloid leukemia: A review. R I Med J (2013) 103: 38-40, 2020.
3. Vakiti A and Mewawalla P: Acute myeloid leukemia. In: StatPearls [Internet], StatPearls Publishing, Treasure Island, FL, 2022.
4. Zhang J, Gu Y and Chen B: Mechanisms of drug resistance in acute myeloid leukemia. Onco Targets Ther 12: 1937-1945, 2019.

5. Marchand T and Pinho S: Leukemic stem cells: From leukemic niche biology to treatment opportunities. Front Immunol 12: 775128, 2021.
6. Hanekamp D, Cloos J and Schuurhuis GJ: Leukemic stem cells: Identification and clinical application. Int J Hematol 105: 549-557, 2017.
7. Jordan CT: The leukemic stem cell. Best Pract Res Clin Haematol 20: 13-18, 2007.
8. Wang X, Huang S and Chen JL: Understanding of leukemic stem cells and their clinical implications. Mol Cancer 16: 2, 2017.
9. Phi LTH, Sari IN, Yang YG, Lee SH, Jun N, Kim KS, Lee YK and Kwon HY: Cancer stem cells (CSCs) in drug resistance and their therapeutic implications in cancer treatment. Stem Cells Int 2018: 5416923, 2018.
10. Aziz F, Chakraborty A, Khan I and Monts J: Relevance of miR-223 as potential diagnostic and prognostic markers in cancer. Biology (Basel) 11: 249, 2022.
11. Gentner B, Pochert N, Rouhi A, Boccalatte F, Plati T, Berg T, Sun SM, Mah SM, Mirkovic-Hösle M, Ruschmann J, *et al*: MicroRNA-223 dose levels fine tune proliferation and differentiation in human cord blood progenitors and acute myeloid leukemia. Exp Hematol 43: 858-868.e7, 2015.
12. Yu G, Yin Z, He H, Zheng Z, Chai Y, Xuan L, Lin R, Wang Q, Li J and Xu D: Low serum miR-223 expression predicts poor outcome in patients with acute myeloid leukemia. J Clin Lab Anal 34: e23096, 2020.
13. Xiao Y, Su C and Deng T: miR-223 decreases cell proliferation and enhances cell apoptosis in acute myeloid leukemia via targeting FBXW7. Oncol Lett 12: 3531-3536, 2016.
14. Ding L, Wang H, Lang W and Xiao L: Protein kinase C-epsilon promotes survival of lung cancer cells by suppressing apoptosis through dysregulation of the mitochondrial caspase pathway. J Biol Chem 277: 35305-35313, 2002.
15. Kinehara M, Kawamura S, Tateyama D, Suga M, Matsumura H, Mimura S, Hirayama N, Hirata M, Uchio-Yamada K, Kohara A, *et al*: Protein kinase C regulates human pluripotent stem cell self-renewal. PLoS One 8: e54122, 2013.
16. Flescher E and Rotem R: Protein kinase C epsilon mediates the induction of P-glycoprotein in LNCaP prostate carcinoma cells. Cell Signal 14: 37-43, 2002.
17. Huang B, Cao K, Li X, Guo S, Mao X, Wang Z, Zhuang J, Pan J, Mo C, Chen J and Qiu S: The expression and role of protein kinase C (PKC) epsilon in clear cell renal cell carcinoma. J Exp Clin Cancer Res 30: 88, 2011.
18. Wang H, Zhan M, Xu SW, Chen W, Long MM, Shi YH, Liu Q, Mohan M and Wang J: miR-218-5p restores sensitivity to gemcitabine through PRKCE/MDR1 axis in gallbladder cancer. Cell Death Dis 8: e2770, 2017.
19. Zhang GF, Wu JC, Wang HY, Jiang WD and Qiu L: Overexpression of microRNA-205-5p exerts suppressive effects on stem cell drug resistance in gallbladder cancer by down-regulating PRKCE. Biosci Rep 40: BSR20194509, 2020.
20. Nicholson R, Menezes AC, Azevedo A, Leckenby A, Davies S, Seedhouse C, Gilkes A, Knapper S, Tonks A and Darley RL: Protein kinase C epsilon overexpression is associated with poor patient outcomes in aml and promotes daunorubicin resistance through p-glycoprotein-mediated drug efflux. Front Oncol 12: 840046, 2022.
21. Skopek R, Palusińska M, Kaczor-Keller K, Pingwara R, Papierniak-Wyglądała A, Schenk T, Lewicki S, Zelent A and Szymański Ł: Choosing the right cell line for acute myeloid leukemia (AML) research. Int J Mol Sci 24: 5377, 2023.
22. Koeffler HP, Billing R, Lusis AJ, Sparkes R and Golde DW: An undifferentiated variant derived from the human acute myelogenous leukemia cell line (KG-1). Blood 56: 265-273, 1980.
23. Varkonyi-Gasic E and Hellens RP: Quantitative stem-loop RT-PCR for detection of microRNAs. In: RNAi and plant gene function analysis: Methods and protocols. Kodama H and Komamine A (eds). Humana Press, Totowa, NJ, pp145-157, 2011.
24. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
25. Lopes Gde L Jr, de Souza JA and Barrios C: Access to cancer medications in low- and middle-income countries. Nat Rev Clin Oncol 10: 314-322, 2013.
26. Cherny N, Sullivan R, Torode J, Saar M and Eniu A: ESMO European consortium study on the availability, out-of-pocket costs and accessibility of antineoplastic medicines in Europe. Ann Oncol 27: 1423-1443, 2016.
27. Jain M and Mukherjee K: Economic burden of breast cancer to the households in Punjab, India. Int J Med Public Health 6: 13, 2016.
28. Ruff P, Al-Sukhun S, Blanchard C and Shulman LN: Access to cancer therapeutics in low- and middle-income countries. Am Soc Clin Oncol Educ Book 35: 58-65, 2016.
29. Kaiser AH, Rotigliano N, Flessa S, Ekman B and Sundewall J: Extending universal health coverage to informal workers: A systematic review of health financing schemes in low- and middle-income countries in Southeast Asia. PLoS One 18: e0288269, 2023.
30. Chueahongthong F, Tima S, Chiampanichayakul S, Berkland C and Anuchapreeda S: Co-treatments of edible curcumin from turmeric rhizomes and chemotherapeutic drugs on cytotoxicity and FLT3 protein expression in leukemic stem cells. Molecules 26: 5785, 2021.
31. Panyajai P, Amnajphook N, Keawsangthongcharoen S, Chiampanichayakul S, Tima S and Anuchapreeda S: Study of leukemic stem cell population (CD34+/CD38-) and WT1 protein expression in human leukemic cell lines. J Assoc Med Sci 51: 38-44, 2018.
32. Niu J, Peng D and Liu L: Drug resistance mechanisms of acute myeloid leukemia stem cells. Front Oncol 12: 896426, 2022.
33. Zhang N, Su Y and Xu L: Targeting PKCε by miR-143 regulates cell apoptosis in lung cancer. FEBS Lett 587: 3661-3667, 2013.
34. Oliveto S, Mancino M, Manfrini N and Biffo S: Role of microRNAs in translation regulation and cancer. World J Biol Chem 8: 45, 2017.
35. O'Brien J, Hayder H, Zayed Y and Peng C: Overview of MicroRNA biogenesis, mechanisms of actions, and circulation. Front Endocrinol (Lausanne) 9: 402, 2018.
36. Jin HY, Gonzalez-Martin A, Miletic AV, Lai M, Knight S, Sabouri-Ghomi M, Head SR, Macauley MS, Rickert RC and Xiao C: Transfection of microRNA mimics should be used with caution. Front Genet 6: 340, 2015.
37. Inoue K, Jinnin M, Yamane K, Makino T, Kajihara I, Makino K, Honda N, Nakayama W, Fukushima S and Ihn H: Down-regulation of miR-223 contributes to the formation of Gottron's papules in dermatomyositis via the induction of PKCε. Eur J Dermatol 23: 160-167, 2013.
38. Khan K, Zafar S, Badshah Y, Ashraf NM, Rafiq M, Danish L, Shabbir M, Trembley JH, Afsar T, Almajwal A and Razak S: Cross talk of tumor protein D52 (TPD52) with KLF9, PKCε, and MicroRNA 223 in ovarian cancer. J Ovarian Res 16: 202, 2023.
39. Ding J, Zhao Z, Song J, Luo B and Huang L: MiR-223 promotes the doxorubicin resistance of colorectal cancer cells via regulating epithelial-mesenchymal transition by targeting FBXW7. Acta Biochim Biophys Sin (Shanghai) 50: 597-604, 2018.
40. Gobbi G, Di Marcantonio D, Micheloni C, Carubbi C, Galli D, Vaccarezza M, Bucci G, Vitale M and Mirandola P: TRAIL up-regulation must be accompanied by a reciprocal PKCε down-regulation during differentiation of colonic epithelial cell: Implications for colorectal cancer cell differentiation. J Cell Physiol 227: 630-638, 2012.
41. Nicholson RL, Knapper S, Tonks A and Darley RL: PKC-Epsilon overexpression is associated with poor outcomes in AML and promotes chemoresistance and hematopoietic stem cell quiescence. Blood 134 (Suppl 1): S2704, 2019.

Copyright © 2024 Osiriphan et al. This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.