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Knockdown of PRAME enhances adriamycin-induced apoptosis in chronic myeloid leukemia cells

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Abstract. – OBJECTIVE: Leukemia is resistant to currently available chemotherapy, and new strategies have been proposed to improve its efficacy. Such an approach requires know of the mechanisms involved in the resistance and survival of leukemia cells. Previous studied has found that Preferentially Expressed Antigen of Melanoma (PRAME) is overexpressed in the leukemia cells, and knockdown of PRAME promoted apoptosis in leukemia K562 cells. In the present study, we investigated whether inhibition of PRAME could sensitize K562 cells to chemotherapy.

MATERIALS AND METHODS: K562 cells were treated with PRAME siRNA, and/or adriamycin (ADR), and cell viability and apoptosis, mRNA and protein expression levels were, then, evaluated. Furthermore, the efficacy of PRAME siRNA combined with ADR was further examined in established xenograft models.

RESULTS: PRAME suppression was sufficient to induce spontaneous apoptosis of K562 cells. PRAME knockdown showed antiproliferative effects and induced tumor regression in established K562 xenograft models. ADR showed antitumor activity against K562 cells, co-treatment with PRAME siRNA induced an increased apoptosis rate than the sum of the single-treatment rates and promoted tumor regression without enhanced body weight loss in the K562 xenograft models.

CONCLUSIONS: PRAME is responsible for the inherent low levels of spontaneous apoptosis in K562 cells. The combination of PRAME siRNA with ADR induced more intense apoptosis compared with each single treatment. PRAME siRNA in combination with ADR is well tolerated and shows greater efficacy than either agent alone in mouse xenograft models.

Key Words:
Chronic myeloid leukemia, Chemotherapy, PRAME.

Introduction

Myeloid leukemia is caused by aberrations in cellular function, in which deregulation of differentiation, growth and apoptosis results in progression of an oncogenic phenotype[1-2]. Myeloid leukemias are characterized by the immature myeloid progenitors accumulation[2]. Chronic myeloid leukemia (CML) is one of the commonest types of myeloid leukemia.

The broad-spectrum cytotoxic agents against fast-proliferating cells and molecular therapies targeting specific signal transduction pathways are the front-line therapy in leukemia[3-5]. Imatinib has been as the representation to treat CML, inducing an overall survival never seen in previous therapies[6]. However, up to 33% of the patients treated with imatinib does not reach the criteria associated with an optimal outcome[6]. Most of the treated patients with CML will relapse if treatment is withdrawn and numerous treated patients die due to imatinib drug resistance and blast crisis. These effects highlight the needs to approach mechanisms of CML imatinib drug resistance.

Preferentially Expressed Antigen of Melanoma (PRAME) gene, a tumor antigen recognized by HLA-24 and afterward presented to cytotoxic lymphocytes (CTL) against a melanoma surface antigen, was discovered in a melanoma patient in 1997[7]. It has recently been found overexpressed in hematologic tumors[8,9]. Overexpression of PRAME is associated with hypomethylation in its regulatory regions[9] and in pediatric acute leukemias confers a favorable prognosis, possibly by inhibiting tumorigenicity and enhancing apoptosis[9].

Recently, it has reported[10] that knockdown of PRAME by siRNA suppressed proliferation and

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promoted apoptosis in a K562 cell line. Bullinger et al[11] has found PRAME could impair differentiation and increase proliferation by blocking retinoic acid receptor (RAR) signaling and these might be reversed by all-trans retinoic acid (ATRA). Otherwise, combining retinoic acid with chemotherapy improves survival of patients with AML. This effect is more pronounced in leukemias that express high levels of PRAME. PRAME is an inhibitor of retinoic acid signaling, which may prove to be an important marker for retinoic acid response[12].

It is well known that Adriamycin (ADR) chemoresistance is a major cause of treatment failure in CML. Therefore, how to treat patients with CML who are resistant ADR is an important and urgent issue for clinical hematology. Synthetic small interfering RNAs (siRNAs) are promising gene-targeting agents that have shown great potential, particularly for development as specific anti-leukemia treatment[13]. In the present study, we investigated whether suppression of PRAME could sensitize K562 human CML cell line to chemotherapeutic agent cytarabine. We also demonstrate that RNAi is a functional pathway in myeloid leukemia cell lines and suggest the potential utility of the RNAi phenomenon as a novel therapeutic approach to myeloid leukemia.

Materials and Methods

Cell Line

K562 cells were purchased from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were maintained in Roswell Park Memorial Institute 1640 (RPMI l640) containing 10% fetal bovine serum (FBS) at 37°C in an environment with 5% CO_2.

PRAME siRNA Transfection

PRAME-short interfering RNA [PRAME siRNA (h)] was purchased by Invivogen (Guangzhou, China). Three different double strand siRNA oligonucleotides (Invitrogen, Shanghai, China) were utilized. A validated medium GC scramble (SCR) double strand siRNA oligonucleotide (Invitrogen, Shanghai, China) was used as control for transfection. The siRNA oligonucleotide showing the highest efficiency of PRAME mRNA knocking-down in the three cell lines was utilized for the experiments reported in the manuscript. To perform transfection, cells were seeded the day before the experiment in 25-cm^2 flasks at a density of 1.8×10^5 cells (60% confluence). Transfections were carried out using Lipofectamine 2000 and Opti-MEM GlutaMax medium (Invitrogen, Beijing, China) medium without antibiotics. The incubation time for oligonucleotide/Lipofectamine 2000 complexes was 5 h. The total incubation time before drug treatment was 72 h at 37°C. Cells were selected by G418 (400 μg/Ml) during at least 2 weeks.

Quantitative Real-Time PCR

cDNA synthesis from k562 cells was conducted using a Taqman Cells-to-cDNA Kit (Ambion, Hangzhou, China) following the manufacturer's instructions. Quantitative real-time PCR was done by using an ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA). The human PRAME and 18s rRNA primers, and probe reagents used in the reactions were Pre-Developed TaqMan Assay Reagents (Applied Biosystems).

Western Blot Assay

Cells were lysed in a buffer composed of 150 mM NaCl, 50 mM Tris (pH 8.0), 5 mM EDTA, 1% (v/v) nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 25 μg/ml leupeptin for 30 min at 4°C. After clarification, equal amounts of protein extracts were resolved by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose filter. After blocking with buffer containing 20 mM Tris-HCl (pH 7.5) and 500 mM NaCl, 5% nonfat milk for 1 h at room temperature, the filter was incubated with specific antibodies to PRAME for 1 h at room temperature followed by horseradish peroxidase (HRP)-labeled secondary antibody. Blots were developed using a chemiluminescent detection system (ECL, Amersham Biosciences Ltd., Little Chalfont, Buckinghamshire, UK).

Cell Viability

After 72 hs of siRNA transfection or, K562 cells were treated with ADR (1.5 μM) for 72 hs, cells were harvested by trypsinization (trypsin 0.25% w/v, 1 mmol/L ethylenediaminetetraacetic acid), washed in phosphate buffered saline (PBS), and diluted 1:10 in trypan blue (Life Technologies, Carlsbad, CA, USA). Each

sample underwent four separate analyses of cell number. For each sample, total cell number was counted and nonviable cells scored by trypan blue uptake. Each experiment was repeated on three separate occasions; representative data are shown.

Soft Agar Colony Formation Assay

Anchorage-independent growth was assessed by monitoring colony formation in soft agar according to the method of Odate et al[14] and make slight change. First, 0.5% agarose in growth medium was added to a six-well plate and allowed to solidify. Then, the experiment was classified into several groups as below: (1) K562 cells (2) stable PRAME siRNA transfected K562 cells (3) K562 cells treated with ADR (1.5 μM) for 4 hs (4) stable PRAME siRNA transfected K562 cells treated with ADR (1.5 μM) for 4 hs (5) control siRNA transfected K562 cells (6) stable control siRNA transfected K562 cells treated with ADR (1.5 μM) for 4 hs. Then, 1×10^4 cells above per well were plated in triplicate in 0.3% agarose onto the bottom agarose. The cells were incubated at 37°C in a 5% CO_2 atmosphere for 15 days. Fresh growth medium (0.5 mL/well) was added after 1 week of incubation. At the end of incubation, colonies were stained with 0.005% crystal violet for 1 h and photographed. The stained colonies were counted using the AccuCount 1000 automated colony counter system developed at NIH (National Institutes of Health, Bethesda, MD, USA).

In vitro Apoptosis Assays

Apoptosis analysis was performed using Cell Death Detection ELISA^{PLUS} (Boehringer, Mannheim, Germany) to measure DNA fragmentation, following the manufacturer's instruction. Briely, cells were seeded at a density of 1×10^4 cells per well in a 96-well format. Cells were lysed in 200 ml lysis buer and incubated at room temperature for 30 min. Twenty ml of supernatant was transferred into an ELISA microtiter plate for analysis. Absorbance was measured at 405 nm and 490 nm on a Thermo Max Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Orthotopic Xenograft of K562 Cells

Tumors were induced by injecting 1.5×10^7 cells (K562 cells,K562 /PRAME siRNA or K562/control siRNA cells) s.c. into the right flanks of 5-week-old male nude mice. Body weight and tumor size was monitored weekly by caliper measurements of the length, width and height, and volume was calculated using the formula for a semi-ellipsoid (length × width^2 × 0.5). When tumor volume reached approximately 50-100 mm^3 (3-4 weeks), ADR (5 mg/kg) was administered i.v. once every week for 3 weeks. The first day of drug treatment was designated as day 0, and observation continued until the day 21. Tumor-bearing mice were sacrificed after 3 weeks and tumor masses were excised. All animal experiments were done in accordance with institutional guidelines.

In vivo TUNEL Assay

TUNEL (dUTP nick end labelling) assay were performed on the paraffin sections of nude mouse tumours of orthotopic xenograft of K562 cells according to the method of Wang et al[15]. TUNEL positive cells were counted as a percentage of total cells.

Statistical Analysis

Statistical comparisons were performed using Student's unpaired *t*-tests (for two groups) or one-way non-parametric ANOVA for more than two groups. Data are expressed as the mean ± SD, depending on the context as indicated in the figure legends, and differences between means were considered significant at $p < 0.05$.

Results

Effect of PRAME siRNA on PRAME Expression in K562 Cells

siRNA was transciently transfected into the K562 cells for 72 h. As expected, expressions of PRAME both mRNA and protein in PRAME siRNA transfected K562 cells were significantly lower than those in Control siRNA or non-transfected cells analyzed by quantitative PCR (Figure 1A) and Western blot (Figure 1B), respectively. After 72 hours post transfection, PRAME mRNA was reduced by more than 80% compared with cells transfected with negative control siRNA, suggesting that RNAi could effectively inhibit PRAME expression in K562 cells. In the PRAME siRNA stably transfected K562 cells, PRAME mRNA and protein was also completed inhibited by quantitative PCR and Western blot (data not shown).

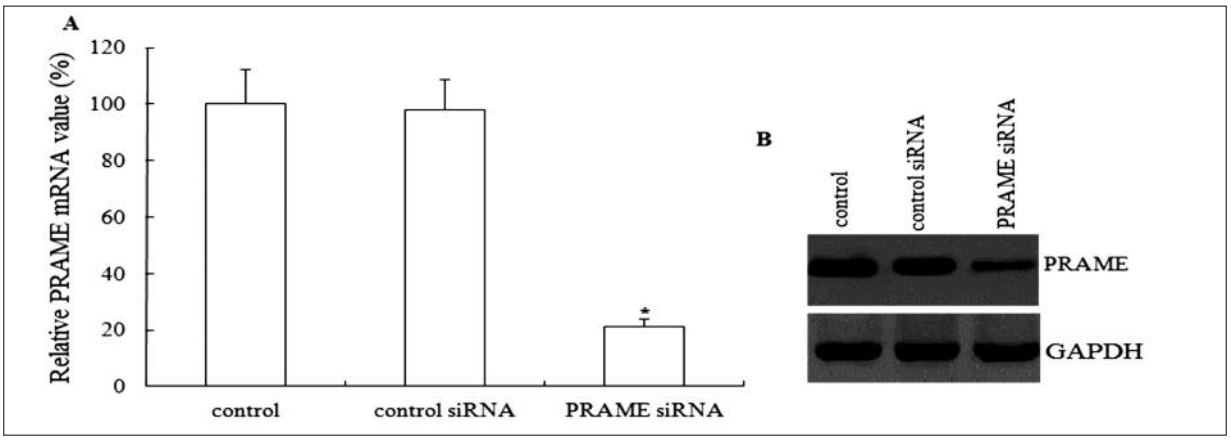

Figure 1. Effects of PRAME siRNA on PRAME mRNA and protein assessed by quantitative RT-PCR and western blot assays. siRNA was transciently transfected into the HL-60 cells for 72 h. PRAME mRNA ***(A)*** and protein ***(B)*** in PRAME siRNA cells were significantly inhibited (*vs*. control, *$p < 0.01$).

Effects of PRAME Gene Knockdown on Cell Growth and Spontaneous Apoptosis

To assess the validity of targeting PRAME for CML therapy, we first examined the effect of siRNA-mediated PRAME knockdown on K562 cell growth and apoptosis. The PRAME down-regulation resulted in reduced viable cell counts (31%) at 72 hours posttransfection (Figure 2A). ELISA assay showed that PRAME gene knockdown was accompanied spontaneous apoptosis (11.6%) (Figure 2B). To determine whether PRAME knockdown suppresses long-term sur-

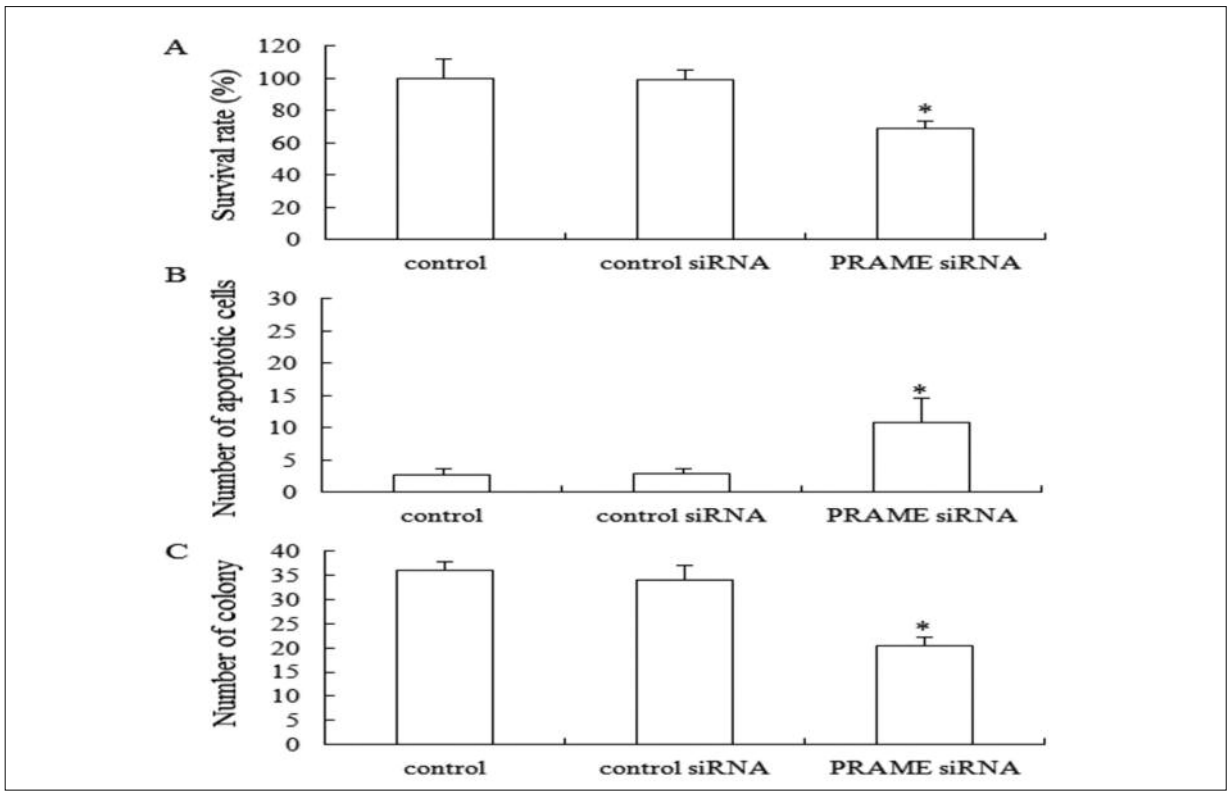

Figure 2. The effect of PRAME knockdown on K562 cells death. ***A,*** Cell proliferation was performed using MTT assay. ***B,*** The apoptosis assay was performed using ELISA. ***C,*** Clonogenic assay of K562 cells infected with PRAME siRNA or control siRNA. *vs*. control, *$p < 0.05$.

vival of K562 cells, clonogenic assays were done. We found that infection with PRAME siRNA significantly impaired the abilities of K562 cells to form colonies over a 14-day period (Figure 2C). PRAME siRNA infection inhibited colony formation by 44% compared with a control siRNA.

Knockdown of PRAME Sensitized K562 Cells to ADR

K562 cells treated with ADR (1.5 μM) for 72 hs decreased cell number to 29% of the untreated cells. When chemotherapy was combined with PRAME inhibition, there was a significant decrease in cell number to 89% compared with chemotherapy alone (Figure 3A). We monitored the apoptotic response of K562 cells following ADR treatment by ELISA assay. There was a little increase in cell apoptosis number (9.4%) treated with ADR (1.5 μM) alone. When chemotherapy was combined with PRAME inhibition, there was a significant increase in cell apoptosis number (28.4%) (Figure 3B).

To determine whether PRAME knockdown combined with ADR (1.5 μM) treatment suppresses long-term survival of K562 cells, clonogenic assays were done. We found that treated with ADR combined with PRAME siRNA significantly impaired the abilities of K562 cells to form colonies over a 14-day period compared with ADR alone (Figure 2C). ADR combined with PRAME siRNA inhibited colony formation by 90% compared with an ADR alone.

PRAME Knockdown Combined with ADR in K562 Xenograft Models

The efficacy of PRAME knockdown in combination with ADR was examined in K562 cell xenografts. Tumors were induced by injecting 1.5×10^7 cells (K562 cells, K562/PRAME siRNA or K562/control siRNA cells) s.c. into the right flanks of five-week-old male nude mice. When tumor volume reached approximately 50-100 mm^3 (3-4 weeks), ADR (5 mg/kg) was administered i.v. once every week for 3 weeks. PRAME siRNA in concomitant combination

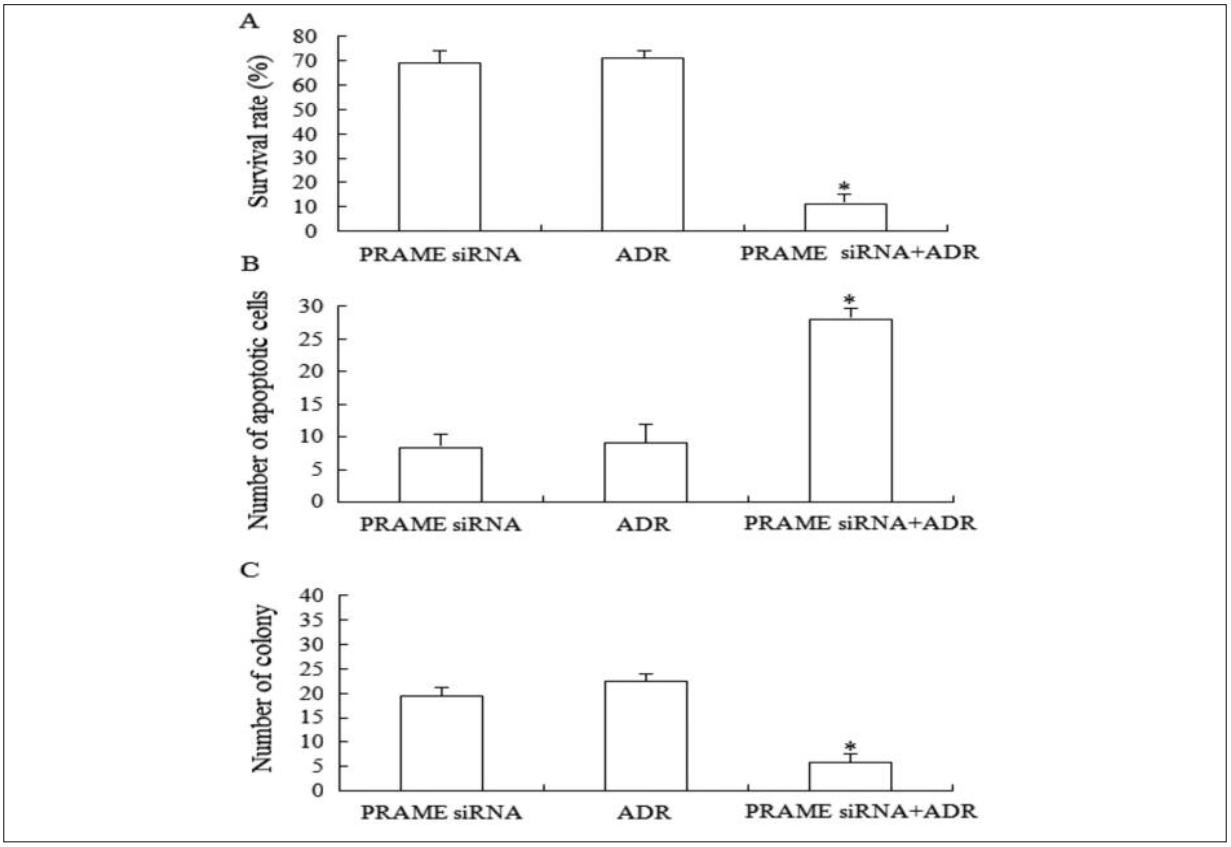

Figure 3. The effect of ADR and PRAME knockdown on K562 cells death. ***A,*** Cell proliferation was performed using MTT assay. ***B,*** The apoptosis assay was performed using ELISA. ***C,*** Clonogenic assay of K562 cells infected with PRAME siRNA or combined with ADR. *$p < 0.01$.

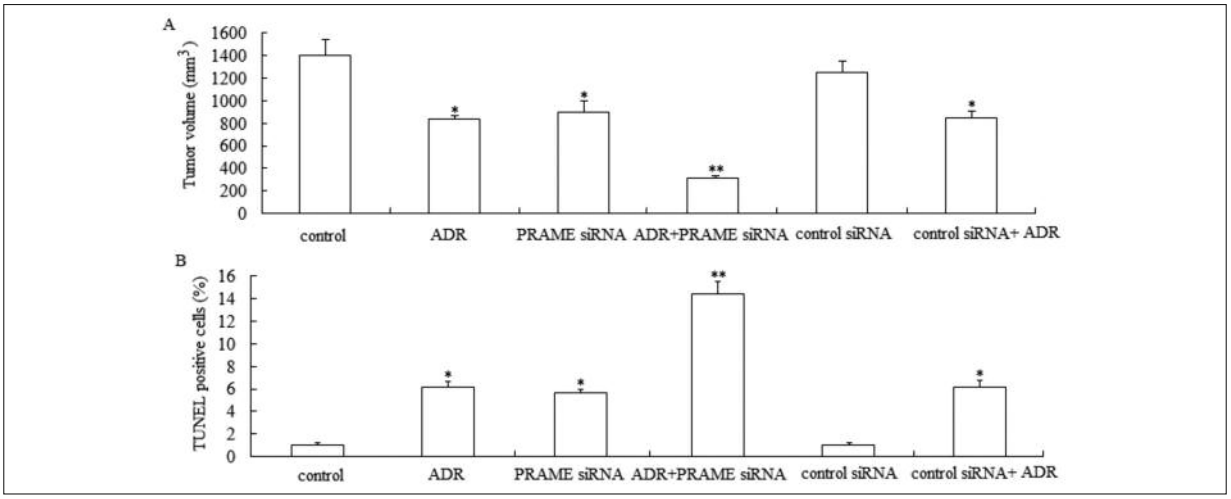

Figure 4. Response of K562 cells grown as orthotopic xenografts to ADR. ***A,*** Mice were inoculated with K562 or K562 siRNA cells and allowed to grow for 3-4 weeks until tumors measured 50-100 mm^3. ADR (5 mg/kg) was administered i.v. once every week for 3 weeks. Tumor volumes are quantified. Results are expressed as mean ± SD. *$p < 0.05$, **$p < 0.01$ (control vs. treated, n = 6). ***B,*** Formalin-fixed sections from ADR treated K562 or K562 siRNA tumors, excised after 3 weeks of treatment, were TUNEL labelled. *$p < 0.05$, **$p < 0.01$ control *vs*. treated.

with ADR significantly inhibited tumor growth compared with each single-compound group ($p < 0.01$) in K562 established tumors (Figure 4A-B). ADR significantly decreases the volume and weigh of the tumors derived from untransfected K562 or control siRNA cells, but has no statistically significant effect on the volume of K562 siRNA tumors (Figure 4A-B). No significant decrease in body weight was observed in the combination group as compared with the ADR group. These results indicated that PRAME siRNA in combination with ADR was tolerated in mice and enhanced the *in vivo* tumor response to ADR.

To further investigate the mechanism of the observed tumor-suppressive activities, we examined the effect of ADR alone and in combination with siRNA on tumor cell apoptosis by TUNEL (Figure 4B). The average number of apoptosis index measured in 5 randomly selected microscopic fields in the ADR group and PRAME siRNA was 6.2% and 7.2%. The PRAME siRNA in combination with ADR group showed a significant increase in the number of apoptosis index. The calculated average was 16.8% ($p < 0.01$, Student's *t*-test *vs*. control group). No significant difference was observed between the ADR-treated group and the control siRNA group in combination with ADR group ($p > 0.05$, Student's *t*-test).

Discussion

The goal of RNA interference (RNAi) is to effectively reduce protein levels in targeted cells and to study the functional consequences of its removal. To produce greater efficacy and less toxicity in cancer cells is what we needed with RNAi. RNAi represents a new alternative for CML treatment, which could overcome drug acquired resistance and sensitize CML cell to chemotherapy[5].

The results presented in this study show that RNAi is a functional pathway with biological impact in myeloid leukemia cell lines and that this phenomenon has potential applicability as a therapeutic approach to myeloid leukemia. The RNAi using PRAME siRNA duplexes succeeded in significantly decreasing the endogenous levels of the target PRAME proteins and PRAME mRNA genes expression. In K562 cells, PRAME knockdown resulted in significant growth retardation, higher rates of endogenous apoptosis and decreased tumor growth in established xenograft[10]. Our data also indicated that ADR, alone and in combination with PRAME siRNA, strongly inhibited K562 cells growth *in vitro* and tumor growth *in vivo*. Furthermore, we could show that the combination therapy inhibited primary tumor volume of mice more assertively than treatment with a single agent.

To explain the potential antitumor effect of ADR alone and in combination with PRAME siRNA, we considered two basic theories.

First, ADR may directly inhibit tumor cell proliferation. Our results *in vitro* provided supportive evidences for above hypotheses. We found an inhibition of tumor cell proliferation of 29% after single ADR treatment, and 31% after single PRAME siRNA treatment, respectively. Combination treatment with ADR and PRAME siRNA results in a significant inhibition of tumor cell proliferation of 89% in K562 cells *in vitro*. Otherwise, combination treatment also significantly inhibited colony formation. Furthermore, combination treatment with ADR and PRAME siRNA also results in a significant inhibition of tumor xenograft models. In Hodgkin lymphoma cells, targeting PRAME increased sensitivity for cisplatin, etoposide and retinoic acid by upregulation of some anti-apoptotic factors[16]. In acute myelogenous leukaemia cells, PRAME overexpression is associated with the decreased expression of apoptotic proteins and an overexpression of genes encoding ABC transporters[17]. However, the exact mechanisms of why combined treatment significantly inhibited cell proliferation compared to treatment alone in K562 cells is not very clear.

Second, the observed antitumor effect of ADR alone and in combination with PRAME siRNA may be a result of drug-induced apoptosis. Tanaka et al[10] has recently found that targeting PRAME suppressed proliferation and blocked cell cycle G(0)/G(1), followed by increased cell apoptosis. Tajeddine et al[9] reported that PRAME overexpression inhibited the expression of the heat-shock protein Hsp27, the cyclin-dependent kinase inhibitor p21, and the calcium-binding protein S100A4, followed the decreased cell apoptosis and increased tumorigenicity of K562 leukemic cells in nude mice. Our studies showed that ADR might have an effect on regulation of programed cell death, supported by the fact that a nearly 9.2% induction of tumor cell apoptosis was observed *in vitro* in K562 cells treated by single-agent ADR treatment, and 6.2% TUNEL positive cells in mice treated by single-agent ADR treatment. We also found an increase of apoptotic cells after single PRAME siRNA treatment. A nearly 9.2% induction of tumor cell apoptosis was observed *in vitro* in K562 cells after single PRAME siRNA treatment, and 7.2% TUNEL positive cells in mice treated by single PRAME siRNA treatment. However, combination treatment with ADR and PRAME siRNA results in 28.4% increase of apoptotic cells *in vitro*, and 16.8% increase of apoptotic cells *in vivo*.

Accordingly, the combination therapy has significant advantages compared with the single-agent treatment. Both ADR and PRAME siRNA are important for inhibiting tumor cell proliferation and promotes apoptosis. On the other hand, PRAME siRNA plays a major role in regulating programmed cell death in the combination therapy-treated group.

Conclusions

Our data demonstrated that PRAME has a critical role in the proliferation and drug resistance of K562 cells. Specific knockdown of PRAME expression by siRNA induced apoptosis and synergistically enhanced sensitivity of leukemic cells to ADR. The combination therapy of ADR and PRAME knockdown is an attractive approach for combined therapeutic strategies to the treatment of leukemia. These warrants further investigation in clinical trials.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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