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PRAME promotes *in vitro* leukemia cells death by regulating S100A4/p53 signaling

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Abstract. – OBJECTIVE: PRAME (Preferentially Expressed Antigen in Melanoma) is a tumor-associated antigen recognized by immunocytes, and it induces cytotoxic T cell-mediated responses in melanoma. PRAME is expressed in a wide variety of tumors, but in contrast with most other tumor-associated antigens, it is also expressed in leukemias. The physiologic role of PRAME remains elusive. Recently, it has found PRAME could be involved in the regulation of cell death in leukemias, but the mechanism of the function is unclear. Here, we confirm that PRAME induces leukemias cell death by regulation of S100A4/p53 signaling.

MATERIALS AND METHODS: The pCDNA3-PRAME plasmid and its control were transfected with the KG-1 cells. The pCDNA3-PRAME transfected KG-1 cells were then transiently transfected with S100A4 cDNA or wt-p53 siRNA. The PRAME siRNA and its control were transfected with the K562 cells. The PRAME siRNA transfected K562 cells were then transiently transfected with S100A4 siRNA or pGMp53-Lu. PRAME, S100A4 and P53 were detected by Western blot assay in different time point. Annexin V/propidium iodide and MTT methods were used to detect apoptosis and cell survival rate.

RESULTS: KG-1 cells overexpressing the PRAME gene significantly induces apoptosis and decreases proliferation *in vitro*, followed by down-regulation of S100A4 and up-regulation of p53. Up-regulation of S100A4 by S100A4 transfection inhibits PRAME-induced p53 up-regulation. Furthermore, up-regulation of S100A4 by S100A4 transfection or down-regulation of p53 by p53 siRNA transfection reduces apoptosis and increases proliferation *in vitro*. Knockdown of PRAME in K562 cells significantly increases proliferation *in vitro*, followed by up-regulation of S100A4 and down-regulation of p53. The downregulation of S100A4 by S100A4 siRNA transfection increased p53 expression. Furthermore, downregulation of S100A4 by S100A4 siRNA transfection or up-regulation of p53 by p53 transfection decreases proliferation *in vitro*.

CONCLUSIONS: Our results suggest that the leukemias expressing high levels of PRAME has a favorable prognosis. PRAME promotes *in vitro* leukemia cells death by regulating S100A4/p53 signaling.

Key Words:
Leukemia, PRAME, S100A4, P53, Proliferation, Apoptosis.

Introduction

The preferentially expressed antigen of melanoma (PRAME), a tumor-associated antigen, was originally identified as a gene which encodes an HLA-A24 restricted antigenic peptide presented to autologous tumour-specific cytotoxic T lymphocytes derived from a patient with melanoma and the expression profile of PRAME defines it as a cancer test antigen[1].

PRAME has found to be an absent or low expression in most normal tissues tested, such as bone marrow, CD34+sorted bone marrow cells, and sorted B and T lymphocytes[2-4]. However, high levels of PRAME are reported in malignant cells, such as primary and metastatic melanomas[5], acute and chronic leukaemias[6-8], Hodgkin's lymphoma[10], breast cancer[11], and head and neck squamous cell carcinomas[12]. In addition, in patients with AML, PRAME upregulation seems to be associated with higher rates of overall and disease-free survival and lower relapse rate compared with the AML patients having no or low PRAME expression[6,8,9].

Tajeddine et al[13] has reported that PRAME expression was associated with reduced proliferation of KG-1 leukemic cells. Furthermore, PRAME suppression could cause significantly decreased tumorigenicity of K562 cells in a xenograft model[13], suggesting that PRAME could be a target for leukemias therapy. However, a recent study found PRAME upregulation could also promote proliferation of various

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leukemic cell lines *in vitro*[14]. We, therefore, concluded that PRAME functions according to the different cell lineage or depending on the different genetic or epigenetic mechanisms.

The calcium-binding protein S100A4 promotes metastasis and is associated with patient outcome in some tumor types. Many studies in rodents *in vitro* and *in vivo* have provided evidence that S100A4 is directly involved in tumor cell survival, progression and metastasis[15]. In addition, S100A4 could also sequester and disable the tumor suppressor p53[16]. The down-regulation of S100A4 could, therefore, abrogate its inhibiting effect on p53, thus favoring apoptosis. Based on these biological evidence, the favorable prognostic effect of PRAME in AML and ALL could be explained by a down-regulation of S100A4 and a consecutive activation of *p*53, which results in the pro-apoptotic effects in the cells.

Materials and Methods

Cell Line

KG-1 and K562 cells were purchased from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). KG-1 and K562 cells were maintained in RPMI l640 containing 10% fetal bovine serum (FBS) at 37°C in an environment with 5% CO_2.

Stable pcDNA3.1-PRAME Plasmid Constructs and Transfection

To construct a plasmid expressing PRAME, the full-length human PRAME gene cDNA clone (Tzrd. Co., Beijing, China) was digested with SalI and BamHI, then cloned into pCDNA3 to generate pCDNA3-PRAME (pPRAME). All the constructions were verified by sequence analysis. For transfection studies, KG-1 cells were plated at a density of 1×10^6 cells per well in six-well plates and incubated for 24 h in complete medium. The cells were then transfected with 2 ∝g of the pPRAME by using a Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) for 48 h following the instructions of the manufacturer. For controls, the same amount of empty vector pcDNA3.1 was also transfected. For the selection of a stably-transfected KG-1 cells, G418 (Life Technologies) was added to the culture medium 48 hours after transfection at a concentration of 600 ug/mL. After 4 weeks of selection by G418, these stably transfected cells were screened by Western blot assay.

Transient pcDNA3.1-S100A4 Plasmid Transfection

The pcDNA3.1-S100A4 plasmid was kindly gifted by Doc.Ren[20]. For transfection studies, stably transfected KG-1 cells were plated at a density of 1×10^6 cells per well in six-well plates and incubated for 24 h in complete medium. The cells were then transiently transfected with 4 μg of the pcDNA3.1-S100A4 plasmid by using a Lipofectamine 2000 Reagent. For controls, the same amount of pcDNA3.1 was also transfected.

Transient wt-p53 siRNA Plasmid Transfection

Wt-p53 siRNA or non-targeting siRNA was obtained from Dharmacon, Lafayette, Shanghai, China. Dharma FECT4 transfection reagent was used to transfect siRNAs according to manufacturer's protocols. The pcDNA3.1-PRAME plasmid treated KG-1 cells were transfected at a density of 1×10^4 cells per milliliter with a siRNA concentration of 50 nM and incubated in antibiotic-free complete medium for 72 h before harvesting.

Stable PRAME siRNA Transfection

PRAME-short interfering RNA [PRAME siRNA (h)] was purchased from Invivogen (Guangzhou, China). It was cloned in a psiRNA-h7SK expression vector (Invivogen, Guangzhou, China). As control, we used a siRNA targeted to green fluorescent protein (GFP-siRNA). Control and PRAME-siRNA-encoding plasmids were transfected with K562 cells. Cells were selected by G418 (400 μg/ml) during at least 2 weeks.

Transient S100A4 siRNA Plasmid Transfection

S100A4 siRNA or non-targeting siRNA were obtained from Invivogen, Guangzhou, China. Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) was used to transfect siRNAs according to manufacturer's protocols. The PRAME-siRNA transfected K562 cells were transfected at a density of 1×10^4 cells per milliliter with a siRNA concentration of 50 nM and incubated in antibiotic-free complete medium for 72 h before harvesting.

Transient pGMp53-Lu Transfections

pGMp53-Lu and its control was obtained from Genomeditech, Shanghai, China. The stable pcDNA3.1-PRAME siRNA transfected K562 cells were transiently transfected with pGMp53-Lu

(50 nM) with Lipofectamine 2000 (Invitrogen, Shanghai, China) according to manufacturer's protocols and incubated in antibiotic-free complete medium for 72 h before harvesting.

Western Blot Assay

Total protein extracts were prepared from cells in different groups, dissolving SDS-PAGE buffer, and transferred onto polyvinylidenedifluoride membranes. Proteins were detected using antibodies against PRAME and S100A4 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and p53 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). and β-actin (1:8000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibodies were peroxidase-conjugated anti-rabbit IgG or peroxidase-conjugated anti-mouse IgG (all 1:10000). Blots were developed using enhanced chemiluminescence (ECL Plus) reagents from Amersham Biosciences (GE Healthcare, Buckinghamshire, UK).

Apoptosis Assay

The apoptosis assay was performed using annexin V/propidium iodide according to the manufacturer's protocols[17]. Briefly, cells were harvested and the pellets were washed twice with PBS. After 48 h, the cells were diluted to 1×10^6 cells/ml in annexin binding buffer, and incubated for 15 min with the Alexa Fluor 647 annexin V conjugate. The necrotic cells were identified by propidium iodide staining, and the cells were analyzed on a Coulter Epics XL flow cytometry (Beckman Coulter, Fullerton, CA, USA). The data were collected from 10000 cells per experimental group. All experiments were repeated at least 4 times to ensure reproducibility.

Cell Proliferation assay

Cells transfected with the pPRAME plasmid or with a control plasmid were seeded at a density of 1×10^3/well in 96-well culture dishes. After 24 h, the cells were incubated with MTT (0.5 mg/ml; Sigma) at 37°C for 4 h and then with DMSO at room temperature for 1 h. At 72 h, the spectrophotometric absorbance of the samples was determined by using Ultra Multifunctional Microplate Reader at 495 nm.

Statistical Analysis

Student's *t*-test was used to determine whether the difference between control and experimental samples was significant ($p<0.05$).

Results

PRAME Overexpression Suppresses S100A4, Followed by P53 Up-Regulation in KG-1 cells

To investigate whether PRAME-mediated P53 expression was subject to transcriptional regulation by S100A4 downregulation, we first transfected KG-1 cells (low PRAME expression) with pcDNA3-PRAME plasmid. We found that PRAME expression inhibited the induction of S100A4 expression and upregulated the p53 expression (Figure 1A).

To investigate whether P53 expression was negatively regulated by S100A4, pCDNA3-PRAME/KG-1 cells was transfected with S100A4 cDNA plasmid. The results showed that S100A4 was overexpressed in the pcDNA3-PRAME/KG-1 cells, P53 expression was significantly reduced in the pcDNA3-PRAME/KG-1 cells in the presence of PRAME (Figure 1B).

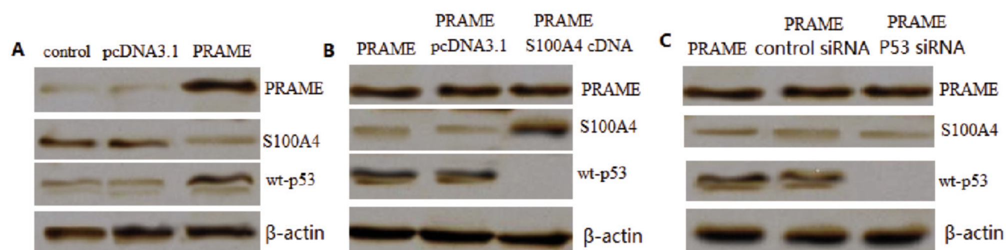

Figure 1. The effect of PRAME overexpression on S100A4 and p53 expression. ***A,*** KG-1 cells was transfected with pcDNA3-PRAME plasmid, PRAME, S100A4, wt-P53 was detected by Western blot assay. ***B,*** pcDNA3-PRAME/KG-1 cells was transfected with S100A4 cDNA plasmid, PRAME, S100A4,wt-P53 was detected by Western blot assay. ***C,*** pcDNA3-PRAME/KG-1 cells was transfected with p53 siRNA, PRAME, S100A4, wt-P53 was detected by Western blot assay.

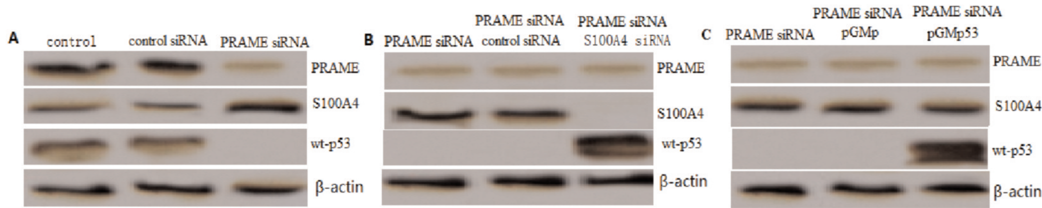

Figure 2. The effect of PRAME inhibition on S100A4 and p53 expression. ***A,*** K562 cells was transfected with PRAME siRNA, PRAME, S100A4 and wt-P53 expression was detected by Western blot assay. ***B,*** PRAME siRNA/K562 cells was transfected with S100A4 siRNA, PRAME, S100A4, wt-P53 was detected by Western blot assay. C, PRAME siRNA/KG-1 cells was transfected with pGMp53, and PRAME, S100A4, wt-P53 was detected by Western blot assay.

Knockdown of PRAME Expression Promotes S100A4, Followed by p53 DownRegulation in K562 Cells

Next, we investigated whether PRAME inhibition downregulated p53 by S100A4 upregulation. We transfected K562 cells (high PRAME expression) with PRAME siRNA plasmid. We found that PRAME expression was significantly inhibited following PRAME siRNA transfection (Figure 2A). However, S100A4 expression was upregulated, and p53 was downregulated (Figure 2A). Similarly, S100A4 protein levels were suppressed in PRAME siRNA/K562 cells transfected with S100A4 siRNA and P53 was increased in the absence of PRAME expression (Figure 2B).

Overexpression of PRAME Induces S100A4/P53-dependent KG-1 Cell Death

The pCDNA3-PRAME transfected KG-1 cells exhibited a significant increase in the percentage of apoptosis compared with the pCDNA3-transfected population (Figure 3A). Furthermore, the survival rate of pCDNA3-PRAME transfected KG-1 cells was significantly decreased compared with the pCDNA3-transfected KG-1 cells (Figure 3B). However, when the pCDNA3-PRAME transfected KG-1 cells was transfected with pCDNA3-S100A4, the percentage of apoptosis was decreased (Figure 3A), and the survival rate was increased (Figure 3B). Similarly, when the pCDNA3-PRAME transfected KG-1 cells was

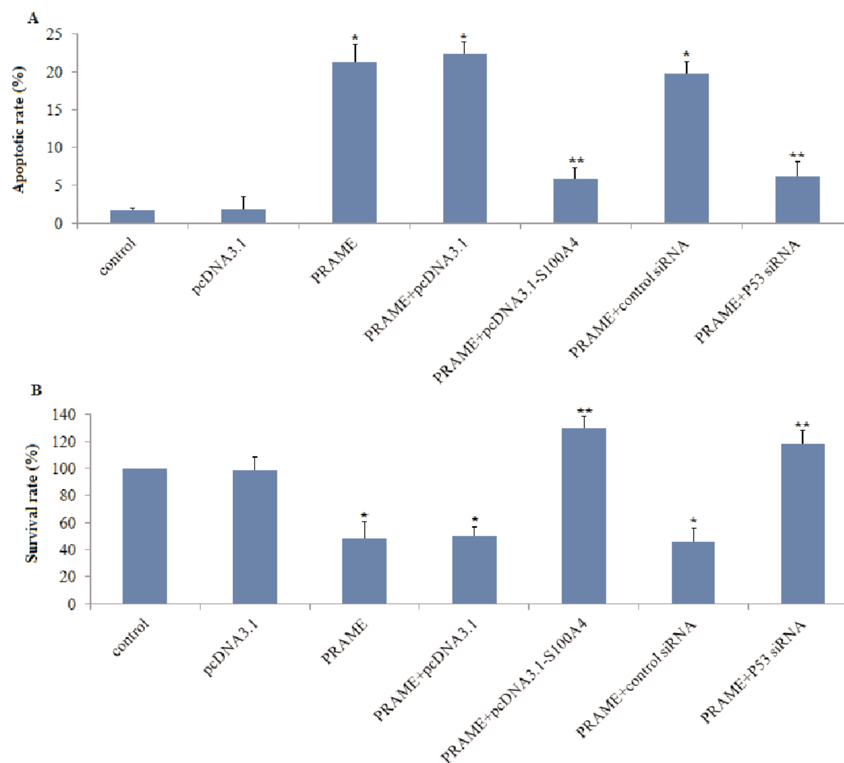

Figure 3. The effect of PRAME overexpression on KG-1 cells. ***A,*** The apoptosis assay was performed using annexin V/propidium iodide; ***B,*** Cell proliferation was performed using MTT assay. $^{*}p<0.05$.

transfected with wt-p53 siRNA to knockdown p53 (Figure 1C), the percentage of apoptosis was also decreased (Figure 3A), and the survival rate was increased (Figure 3B).

Knockdown of PRAME Induces S100A4/P53-Dependent K562 Cell Growth

The PRAME siRNA transfected K562 cells exhibited a significant increase in the cell survival rate compared with the control siRNA-transfected population (Figure 4). However, when the PRAME siRNA transfected K652 cells was transfected with S100A4 siRNA, the survival rate was decreased (Figure 4). Similarly, when the pCDNA3-PRAME siRNA transfected K562 cells was transfected with pGMp53-Lu to overexpress p53 (Figure 2C), the survival rate of K562 cells was significantly decreased (Figure 4).

Discussion

PRAME expression has been characterized for a wide variety of solid tumors[11,18-24] and hematological malignancies[24-28]. In solid tumors, overexpression of PRAME was associated with poor prognosis[19-22,24]. However, overexpression of PRAME seems to be associated with significantly higher rates of overall and disease-free survival and lower relapse rate, compared with patients with no or low PRAME expression[25-26]. However, Tanaka et al[17] has reported that PRAME expression in acute leukemia does not seem to be of prognostic significance, whereas it might represent a candidate marker for the monitoring of minimal residual disease. In Hodgkin's lymphoma, PRAME expression is considered as a poor prognostic parameter[12]. PRAME inhibits retinoic acid signaling in HL cells and that the knock-down of PRAME increases retinoic acid signaling and cytotoxic drug sensitivity of Hodgkin lymphoma cells[27]. Preclinical studies aimed at decreasing PRAME expression in cancer cell lines by delivery of PRAME-specific siRNA resulted in a cell cycle arrest and apoptosis[25]. A recent study has proven that PRAME protein has antigenicity *in vitro*, and stimulates the proliferation and activation of specific CD8+CTL[29]. However, Tajeddineet al[13] has reported that PRAME exhibited a decreased proliferation rate. This raises the possibility that PRAME may have different roles in oncogenesis or tumour suppression dependent on the tumour type. The function of PRAME and its effect on gene expression in leukemic cells remains controversial due to conflicting observations in the literature.

In the present study, we show that overexpression of the PRAME induced apoptosis and inhibited proliferation in KG-1 cells *in vitro*. We also show that suppression of PRAME gene by siRNA promotes proliferative potential of human K562 cells. Although PRAME gene is a regulator of proliferation and apoptosis in the study, little is known about the mechanisms involved.

The S100A4 protein is mainly overexpressed in human metastatic tumor cells, and high level of S100A4 is correlated with a poor prognosis in several types of cancer[30]. However, the mechanisms that associate S100A4 expression with tumor progression and development of metastasis

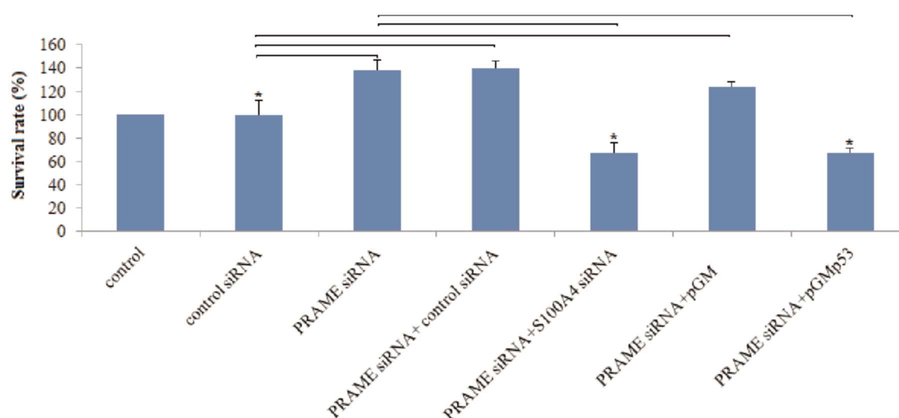

Figure 4. The effect of PRAME inhibition on K562 cells proliferation performed using MTT assay. *$p<0.05$.

remain incompletely understood. In a series of 28 acute myeloid leukemia pediatric patients, Tajeddine et al[13] observed that PRAME expression was associated with an increased leukemia-free survival. The favorable prognosis of PRAME could be mediated, at least in part, by the modified expression of S100A4. It has been reported that S100A4 sequesters and disables the tumor suppressor p53[30-32]. The down-regulation of S100A4 could, therefore, abrogate its inhibiting effect on p53, thus favoring apoptosis. Based on these biological evidences, the favorable prognostic effect of PRAME in AML and ALL could be explained by a down-regulation of S100A4 and a consecutive induction of activation of P53.

In our study, we first observed that overexpression of PRAME in KG-1 cells (low PRAME expression) inhibited the induction of S100A4 expression and upregulated the p53 expression. When S100A4 was restored by S100A4 cDNA transfection, P53 expression was significantly reduced. The results showed that PRAME upregulated p53 expression by down-regulation of S100A4. We also found although overexpression of the PRAME induced apoptosis and inhibited proliferation in KG-1 cells, when S100A4 was restored or p53 was knockdown by wt-p53 siRNA transfection, the percentage of apoptosis was decreased, and the survival rate was increased. The results showed that PRAME promoted apoptosis and inhibited proliferation in KG-1 cells by down-regulation of S1004 followed by up-regulation of P53. Similarly, knockdown of PRAME showed increased S100A4 expression, resulting in decreased endogenous P53 protein. Suppression of PRAME promotes the proliferative potential of human K562 cells. When S100A4 was inhibited or p53 was overexpressed by wt-p53 transfection, the survival rate was significantly decreased. The results showed that knockdown of PRAME promoted proliferation in K562 cells by up-regulation of S1004, followed by down-regulation of P53.

Conclusions

This study shows that overexpression of PRAME in cultured cells induces S100A4/P53-dependent cell apoptosis and proliferation inhibition, inhibition of PRAME in cultured cells promotes S100A4/P53-dependent cell proliferation. We suggest that these observations could explain the good prognosis of PRAME-expressing childhood acute leukemias.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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