SIRT2 mediates multidrug resistance in acute myelogenous leukemia cells via ERK1/2 signaling pathway

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Abstract. SIRT2, one of nicotinamide adenine dinucleotide (NAD+) -dependent class III histone deacetylase family proteins, has been found to be involved in the proliferation and survival of acute myeloid leukemia (AML) cells. However, its effect on drug resistance on chemoresistant AML cells is unclear. In the present study, we first found that SIRT2 was expressed at higher level in the relapsed AML patients than the newly diagnosed patients. Consistent with this observation, the expression level of SIRT2 was higher in HL60/A cells than that in HL60 cells. Depletion of SIRT2 by shRNAs in HL60/A cells resulted in decreased MRP1 level, enhanced drug accumulation and triggered more apoptosis. By contrast, overexpression of SIRT2 in HL60 cells led to increased MRP1 level, drug efflux and attenuated drug sensitivity. Moreover, the decreased expression of phosphorylated ERK1/2 was detected in SIRT2-depleted HL60/A cells and increased expression of phosphorylated ERK1/2 was observed in SIRT2 overexpressed HL60 cells. Furthermore, blockage of ERK1/2 signaling pathway with the chemical inhibitor PD98059, further induced apoptosis of HL60/A cells conferred by SIRT2 depletion. Importantly, ERK1/2 inhibition was able to reverse the drug resistance of HL60 conferred by SIRT2 overexpression. Thus, our findings collectively suggested that the expression level of SIRT2 has a positive relationship with DNR/Ara-C resistance and activity of ERK1/2 signaling pathway. SIRT2 might regulate DNR/Ara-C sensitivity in AML cells at least partially through the ERK1/2 pathway.

Introduction

Acute myeloid leukemia (AML) is a genetically heterogeneous disorder, characterized by uncontrolled clonal proliferation of immature myeloid cells in the bone marrow and blood with concurrent depletion of effective hematopoiesis (1,2). The standard treatment of AML with cytotoxic chemotherapy has remained mostly unchanged over the past few decades, with dismal clinical outcome (3). One of the key issues of this poor result is the development of resistance to chemotherapeutic agents, which can lead to clinical relapse (4). Hence, novel anti-leukemia agents, especially for relapsed leukemias resistant to existing chemotherapeutic drugs are needed.

Multidrug resistance (MDR) is a major problem in the treatment of various human hematological malignancies. Overexpression of the drug transporter P-glycoprotein (P-gp) and/or multidrug resistance-associated protein (MRP1) has generally been reported to correlate with prognosis in AML (5,6). MRP1, like P-gp belongs to the ATP binding cassette (ABC) superfamily of membrane transport protein (6). The difference between them is that the 190-kDa MRP1 can be localized on both the plasma and intracytoplasmic membranes, which can cause intracellular or cytoplasmic sequestration of the drug (5). MRP1 overexpression leads to the failure of numerous chemotherapy protocols when there is without P-gp overexpression, such as in HL60/A cells (7).

Sirtuins (SIRT1-7), the mammalian homologues of the silent information regulator 2 (Sir2) in yeast, are members of nicotinamide adenine dinucleotide (NAD+) -dependent class III histone deacetylase family proteins (8-10). Sirtuins have a critical role in genome stability, longevity, metabolism and aging (9,10). Among them, SIRT1 is the direct homologue of the yeast Sir2 and has a wide range of cellular functions (11). Previous studies have suggested that SIRT1 has oncogenic ability in hematological malignancies, such as CLL, AML and even can be seen as a therapeutic target for CML and AML treatment to overcome resistance (12-14). Little is known about SIRT2, which is the primary cytoplasmic Sirtuin but shuttles continuously between the cytoplasmic and nuclear compartments during interphase and thus, it has been reported to participate in cell cycle progression, cell differentiation, and oxidative stress by modifying other proteins and regulating gene expression (15-17,24,29). SIRT2 has been
suggested to participate in glioma, breast, lung cancer, hepatocellular carcinoma, though, there are few studies in leukemia (8,18-20). In the present study, we found that SIRT2 was overexpressed in the relapsed patients with AML compared to the newly diagnosed, and both groups were increased from the normal levels, and the MRP1-mediated multidrug resistance AML cell line HL60/A could be reversed by inhibition of SIRT2.

Materials and methods

Patients and cell culture. Following informed consent, we collected bone marrow samples of patients in the Hospital of Blood Diseases. The patients included 25 children with acute lymphoblastic leukemia (children-ALL), 30 acute lymphoblastic leukemia (ALL), 10 essential thrombocytopenia (ET), 11 chronic myeloid leukemia (CML), 8 polycythemia vera (PV), 11 myeloproliferative neoplasm (MPN), 25 acute myeloid leukemia (AML) and 6 healthy volunteers (Table I). All the patients were newly diagnosed, except the 10 relapsed AML patients. Inclusion criteria for the present study were based on the European Leukemia Net (ELN) criteria. All the patients were treated in accordance with the Helsinki Declaration.

HL60 cells were preserved by our laboratory and its MRP-mediated multidrug resistance HL60/A cells were provided by the Pharmacology Laboratory at Institute of Hematology, Chinese Academy of Medical Sciences. They were cultured in RPMI-1640 (Gibco-BRL Life Technologies, Inc., Burlington, ON, Canada) containing 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humid atmosphere with 5% CO₂. HL60/A cells were grown in the presence of 1 µg/ml doxorubicin (DOX) to maintain resistance activity, which was removed 1 week before each assay.

Quantitative real-time PCR and western blot analysis. Total RNA was extracted using TRIzol reagent (Invitrogen, Grand Island, NY, USA) and converted to cDNA by using the SuperScript II RT (Invitrogen). Premier software 5 was used to design the primers for real-time quantitative PCR. Human GAPDH primers used as an internal control were 5'-GAAGGTGAAGGTCGGAGTC-3' (forward) and 5'-GAA GATGGTGATGGGAGTT-3' (reverse). Human SIRT2 primers were 5'-ACGGTGTCCAGAGTCTCAT-3' (forward) and 5'-CGTCCAGGTGATCTAGTT-3' (reverse). Real-time quantitative PCR was performed with SYBR-Green PCR kit (Takara, Shiga, Japan) on the ABI PRISM 7500 Sequence detection system. Thermal cycling conditions were 95°C for 30 sec, followed by 40 cycles of 5 sec at 95°C, and 34 sec at 60°C. PCR reactions were performed in a total volume of 20 µl, containing 2 µl of sample cDNA, 0.2 µM of each primer and the SYBR-Green PCR kit following the manufacturer's instructions. The expression level of SIRT2 was analyzed by the RQ value calculated through 2^ΔΔCt method [ΔΔCt = (CtSIRT2 - CtGAPDH)_sample - (CtSIRT2 - CtGAPDH)_calibrator]. Data are the means ± SD of at least 3 independent experiments performed in triplicate.

Total protein was extracted using RIPA lysis buffer with 1 mM PMSF (Sigma, St. Louis, MO, USA) and other protease inhibitors. Protein concentration was determined by the BCA assay (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions. The total cell lysates were heat-denatured at 100°C for 10 min before being run on 8-12% gradient SDS-PAGE. After SDS-PAGE, the proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Blots were incubated with the indicated antibodies, incubated in secondary antibody and then developed by using enhanced chemiluminescence detection reagent (GE Healthcare, Buckinghamshire, UK) and determined by densitometric analysis with a Lynx video densitometer (Biological Vision Inc., San Mateo, CA, USA). Western blot analysis, we purchased antibodies against GAPDH (sc-665062), SIRT2 (sc-20966), multidrug-resistance protein 1 (MRP1) (sc-7773), anti-phosphospecific ERK1/2 (sc-101760) and non-phosphorylated ERK1/2 (sc-135900) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); p53 (9282), BCL-2 (2872) and Apoptosis Antibody Sampler kit (9915) from Cell Signaling Technology (Danvers, MA, USA).

Lentiviral shRNA vectors and transduction. Two different SIRT2-specific targeting sequences were designed with software from Ambion. The hairpins were synthesized and cloned into the lentiviral expression vector. The two shRNA vectors...
Lentiviral vector construction for SIRT2 overexpression and transfection. The complete coding region of SIRT2 gene was amplified by PCR and cloned into the lentiviral expression vector pCDH-CMV-MCS-EF1-GreenPuro-CD512B-1. The transfection protocol is described above.

Immunofluorescence assay. Cells with indicated treatments were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.5% Triton for 15 min. Cells were washed with ice-cold PBS, blocked with 0.5% BSA in PBS for 30 min and then incubated with a rabbit polyclonal antibody against SIRT2, followed by TRITC-conjugated goat anti-rabbit IgG antibody and stained nuclei with DAPI. The images were then visualized with Leica TCS SP2 confocal laser microscope (Perkin-Elmer). In another group, cells were incubated for 1.5 h in serum-free RPMI-1640 containing doxorubicin 10 µmol/l and analyzed by flow cytometry.

Cytotoxicity assay and analysis of apoptosis. Cells were seeded into 96-well culture plates at a density of 1x10^5 cells/ml and serial concentration of daunorubicin (DNR) and arabinocytidine (Ara-C) were, respectively, added in a final volume of 100 µl/well for 48 h. The cytotoxic effects of drugs were determined by VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 570 nm. The apoptosis of the cells with or without DNR and Ara-C were measured with Annexin V-APC/PI apoptosis analysis kit (Tianjin Sungene Biotech Co., Ltd., Tianjin, China) as recommended by the manufacturer and analyzed by flow cytometry.

Statistical analysis. Each experiment was repeated at least three times. The data were summarized and presented as mean ± SD. The statistical analyses were performed with Student's t-test using GraphPad Prism software (GraphPad Prism, San Diego, CA, USA). P<0.05 was considered as statistically significant.

Results

Expression of SIRT2 in leukemia. We first compared SIRT2 expression of patients with different leukemias by real-time PCR. The real-time PCR analysis revealed that the expressions of SIRT2 in leukemia patients was higher than that in healthy subjects, although the degree of discrepancy varied (Fig. 1A). Then, we detected the expressions of SIRT2 in 10 relapsed AML patients and 15 newly diagnosed AML patients...
and the result showed that the expressions of SIRT2 in the relapsed AML patients were higher than that in the newly diagnosed patients (Fig. 1B). In addition, we also detected the expression of SIRT2 in HL60 AML cells through real-time PCR and western blotting. Compared with SIRT2 expression in HL60 cells, SIRT2 level in HL60/A was higher (Fig. 1C and D). Both on the level of mRNA and protein the results showed that the expression of SIRT2 in HL60 cells and HL60/A cells were similar to our previous findings. These data not only indicate that the expression of SIRT2 in leukemia patients is higher than that in healthy volunteers, but also suggest that the overexpression of SIRT2 may associate with multidrug resistance in AML.

**SIRT2 knockdown decreased the MRPI level, enhanced drug accumulation and sensitivity to DNR and Ara-C.** Two different SIRT2 specific shRNA plasmids were constructed and transfected into HL60/A cells, which had a relatively higher expression level of SIRT2. As expected, stable transfection experiments showed both shRNA plasmids could significantly inhibit SIRT2 expression in HL60/A cells in mRNA and protein levels (Fig. 2A and B). As both cytoplasmic and nuclear SIRT2 exist, the expression of SIRT2 in HL60/A cells after transfection was detected by a confocal laser microscope. The results showed that the SIRT2 expression was significantly decreased, similarly to the results of western blotting (Fig. 2C).

We detected the MRPI protein, which is known as a multidrug efflux pumps, and we found the MRPI expression decreased after inhibition of SIRT2 in HL60/A cells (Fig. 3A). After downregulation the expression of SIRT2, in HL60/A cells showed increased uptake of DOX compared with the control group HL60/A-NC cells (Fig. 3B). Flow cytometry was also used to measure the intracellular fluorescent of DOX, and similar result was obtained (Fig. 3C).
We evaluated the sensitivity of the HL60/A cells to DNR and Ara-C after the SIRT2 was downregulated. The cells were assessed for viability on exposure to DNR and Ara-C, respectively, by using MTT assay and a clear enhancement of sensitivity to DNR and Ara-C was observed in the HL60/A cells when the expression of SIRT2 was downregulated (Fig. 3G and H).

In order to detect and quantify apoptosis, we used Annexin V-FITC/PI double staining to determine the effect of SIRT2 knockdown on apoptosis in HL60/A cells with or
without DNR or Ara-C treatment. The results showed that SIRT2 downregulation increased slightly the percentage of apoptotic cells, but clearly only after incubation with 0.1 µM DNR or 0.5 µM Ara-C for 48 h compared with HL60/A-NC (Fig. 4A and B).

Caspase family proteins have been demonstrated as key mediators of apoptosis, and their activation is taken as a hallmark of apoptosis. We find that the expression of cleaved caspase-3, -7 and -9 and PARP were higher in SIRT2-sh1 and SIRT2-sh2 cells than that in HL60/A-NC after DNR or Ara-C treatment (Fig. 5A). These data indicated that knockdown of SIRT2 enhanced the sensitivity to DNR and Ara-C in HL60/A cells.

SIRT2 overexpression increases MRP1 level attenuated drug accumulation and sensitivity to DNR and Ara-C. In order to further study the effect of SIRT2 expression on multidrug resistance, we transfected HL60 cells with SIRT2-overexpression-plasmid and observed an obviously increased expression of SIRT2 in mRNA and protein levels (Fig. 2D and E). The expression of SIRT2 in the HL60 cells after transfection was also detected by a confocal laser microscope.
The MRP1 expression increased after overexpression of SIRT2 (Fig. 3E). Confocal laser microscope was used to detect the accumulation of the drug in the cells after SIRT2 overexpression. As expected, after upregulation the expression of SIRT2, in HL60 cells showed decreased uptake of DOX compared with the HL60-control cells (Fig. 3F). We also measured the intracellular fluorescence of DOX by using flow cytometry and obtained similar results (Fig. 3D). The cytotoxicity assay result showed that the sensitivity to DNR and Ara-C was attenuated in the HL60 cells when the expression of SIRT2 was upregulated (Fig. 3I and J). The overexpression of SIRT2, decreased the percentage of apoptotic cells with incubation of DNR and Ara-C (Fig. 4C and D), less cleaved caspase-3, -7 and -9 and PARP protein expression was observed in contrast to HL60-control (Fig. 5B). These results indicated that SIRT2

(Fig. 2F). The MRPI expression increased after overexpression of SIRT2 (Fig. 3E).

Confocal laser microscope was used to detect the accumulation of the drug in the cells after SIRT2 overexpression. As expected, after upregulation the expression of SIRT2, in HL60 cells showed decreased uptake of DOX compared with the HL60-control cells (Fig. 3F). We also measured the intracellular fluorescence of DOX by using flow cytometry and obtained similar results (Fig. 3D). The cytotoxicity assay result showed that the sensitivity to DNR and Ara-C was attenuated in the HL60 cells when the expression of SIRT2 was upregulated (Fig. 3I and J). The overexpression of SIRT2, decreased the percentage of apoptotic cells with incubation of DNR and Ara-C (Fig. 4C and D), less cleaved caspase-3, -7 and -9 and PARP protein expression was observed in contrast to HL60-control (Fig. 5B). These results indicated that SIRT2...
overexpression enhanced the resistance to DNR and Ara-C in HL60 cells, which had a relatively lower expression level of SIRT2, compared to the multidrug resistance HL60/A cells. The ERK1/2 signaling pathway is involved in SIRT2-mediated multidrug resistance in AML cell lines. We next analyzed the mechanism changing the multidrug resistance effects of the
SIRT2 expression in the HL60/A and HL60 cell lines. In our previous study we demonstrated that the MAPKs signaling pathways participate in mediation of the imatinib resistance in CML (21). In the present study, we detected the expression level of ERK1/2, an upstream regulator of p53 as well as BCL-2, an anti-apoptotic protein expressed in most cases of AML and may contribute to drug resistance in AML (22,23). It has been observed that downregulation of both SIRT1 and SIRT2 can enhance the sensitivity of chemotherapy in mammalian cells via p53-independent mechanism (24), thus, we also evaluated p53. We found that the expression of ERK1/2 and its phosphorylation as well as BCL-2 were downregulated, while p53 was upregulated in the SIRT2-depleted HL60/A cells (Fig. 6A and B). In contrast, overexpression of the SIRT2 in HL60 cells upregulated ERK1/2 and its phosphorylation and BCL-2, decreased the p53 level (Fig. 6C and D). Thus, these results implied the activation of ERK1/2 signaling might be important to this process.

To further measure the potential influence of manipulating ERK1/2 signaling pathway to this change of multidrug resistance induction exerted by the level of SIRT2, we took advantage of PD98059, a specific MEK1 pharmacological inhibitor, to block its activation and then measured the changes affected by SIRT2 depletion or overexpression. The inhibitor added to the HL60/A cells with SIRT2 depletion did not alone influence the viability of cells on exposure to DNR (P<0.05) and Ara-C (P<0.05). Pre-incubation with PD98059 additionally increased the sensitivity of HL60/A cells (Fig. 7A and B; P<0.05). The apoptosis results show that pre-incubation with PD98059 additionally increased the percentage of apoptotic cells under incubation with DNR and Ara-C (P<0.05). Pre-incubation with PD98059 additionally increased the percentage of apoptotic cells under incubation with DNR and Ara-C (Fig. 7E and F) as well as the caspase family proteins (Fig. 5A). Moreover, the inhibitor was added to the HL60 cells with SIRT2 overexpression, we found that pre-incubation with PD98059 abolished the resistance changes to DNR and Ara-C of HL60 cells (Fig. 7C and D; P<0.05), reversed the apoptosis changes under treatment with DNR and Ara-C (Fig. 7G and H) and the changes of caspase family proteins, which was caused by SIRT2 overexpressing HL60 cells after treatment with DNR or Ara-C (Fig. 5B). To further elucidate the role of ERK1/2 MAPK signaling in the regulation of MRPI expression in AML cell lines, we analyzed MRPI levels in the presence of PD98059 (50 µM). MRPI expression was reduced in HL60/A cells and HL60 cells, additionally decreased in HL60/A cells with SIRT2 depletion and reversed in HL60 cells with SIRT2 overexpression (Fig. 8). According to these results, we suggest that ERK1/2 signaling pathway is involved in these changes of multidrug resistance induction exerted by the level of SIRT2.

Discussion

Sirtuins, a complex family of proteins (SIRT1-7), are enzymes that require nicotinamide adenine dinucleotide (NAD+) to catalyze their reactions are present in organisms ranging from bacteria to plants to animals (8-10). Impressive recent progress has been shown with SIRT1, one of the most extensively studied sirtuins. It has been reported to deacetylate the histones H1, H3 and H4 and regulate transcription factors such as tumor suppressor p53, NF-κB and Foxo3a (12-14,24 -27).

Unlike SIRT1, SIRT2 is mostly found in the cytoplasm and deacetylates a number of cytoplasmic proteins, such as α-tubulin (28). In addition to this, SIRT2 has also been observed in the nucleus and target histones, NF-κB, p53 and FOXO transcription factors (9,16,17). A recent study showed that SIRT2 expression is significantly upregulated in leukemic cells from AML patients and higher in the patients with high-risk AML, which demonstrates the involvement of SIRT2 in the resistance against chemotherapy (29).

In the present study, we not only demonstrated that SIRT2 expression was higher in the AML patients but also higher in other leukemia such as ALL, ET, CML, PV and MPN patients compared to healthy volunteers. As expected, we demon-
strated that SIRT2 was higher expressed in the relapsed patients suffering from AML than the newly diagnosed AML patients. The results on the expression of SIRT2 in the AML were consistent with the aforementioned report and further demonstrated that SIRT2 participates in the resistance against chemotherapy. To further understand this phenomenon, we used the AML cell line HL60 and its multidrug resistance cell line HL60/A as the cell model to develop the present study. First, we detected the expression of SIRT2 in HL60 and HL60/A cells and the result was consistent with in the patients. Based on this, we constructed two kinds of lentiviral vectors, one for knockdown of SIRT2 in the HL60/A cells and the other for overexpression of SIRT2 in the HL60 cells. We demonstrated that SIRT2-silencing HL60/A cells show more sensitivity to DNR and Ara-C. We observed that more DOX accumulated in SIRT2-silenced HL60/A cells than HL60/A-NC cells. The results we observed in the SIRT2 overexpressing HL60 cells were consistent with the SIRT2-silenced HL60/A cells, the SIRT2 overexpression enhanced the drug resistance ability in HL60 cells and more DOX was discharged. All of the data showed that SIRT2 enhanced to multidrug resistance in AML.

To gain more insights into how SIRT2 mediated AML cell multidrug resistance, we measured the activities of multiple signaling pathways after changing the SIRT2 level. We found that SIRT2 knockdown significantly downregulated the phosphorylation of ERK1/2. Overexpression of SIRT2 led to upregulation of the phosphorylation of ERK1/2. To further investigate whether the multidrug resistance of AML cells was mediated by SIRT2 though ERK1/2 pathway, the MEK1 specific inhibitor PD98059 was used. As shown in the results, inhibition of ERK1/2 by chemical inhibitor indeed assisted the SIRT2 effects. These results implied that SIRT2 mediated the multidrug resistance of AML cells though ERK1/2 signaling pathway.

Furthermore, the expression of p53 and BCL-2 proteins were evaluated. The classical function of p53 is regulating apoptosis of both the cytosol and mitochondria. These functions involve a direct interaction of p53 with members of the BCL2 family and allowing p53 to function as a so-called BCL2-homology domain-3 (BH3)-only protein (30,31). BCL2 family proteins have a central role in the intrinsic apoptotic pathway. BAX and BAK promote apoptosis by regulating mitochondrial membrane potential, BCL2 and BCL-XI negatively regulate them, whereas, the BCL2-homology domain-3 (BH3)-only proteins including p53 control these survival proteins (32). Based on the above, a number of p53 inhibitors have been considered as a patron to protect the normal tissues from many of the deleterious side effects of chemotherapies (33). Another report have shown that the balance of Mdm2/p53 ratio is involved in the sensitization in human breast-, endometrial- and hepatocellular-carcinoma cell lines via EGFR-Raf-MEK-ERK1/2-Mdm2 signaling pathway (34). We hypothesized that ERK1/2-Mdm2-p53-BCL2 may be a pathway related to the multidrug resistance in AML cell lines. However, this hypothesis needs further study.

In conclusion, our results demonstrated that lower expression of SIRT2 declines the multidrug resistance in chemoresistant AML cells. SIRT2 affects the multidrug resistance of AML cell lines via ERK1/2 signaling pathway. These findings shed light on the effect of SIRT2 on the multidrug resistance of AML cells and implied that targeting SIRT2 may yield therapeutic efficacy in the treatment of leukemia diseases.

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