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Decreased expression of nucleophosmin/B23 increases drug sensitivity of adriamycin-resistant Molt-4 leukemia cells through mdr-1 regulation and Akt/mTOR signaling

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ABSTRACT

Nucleophosmin/B23 (NPM) is a nuclear protein with prosurvival and ribosomal RNA processing functions. However, the potential role of NPM involved in drug-resistance in leukemia has not been investigated clearly. In this study, we generated an adriamycin (ADM)-resistant lymphoblastic cell line Molt-4/ADR (MAR) by stepwise induction. Cell proliferation, sensitivity to chemotherapy agents and expressions of drug resistance related molecules were assessed. The IC50 of Molt-4 cells were 0.58 ± 0.11 μmol/L and MAR cells were 22.56 ± 1.94 μmol/L, meaning MAR cells were 38.63 fold resistant to Molt-4 cells. Furthermore, MAR cells gained an expression of mdr-1 (P-gp) and a higher expression of NPM compared to Molt-4 cells. Knockdown of NPM by RNA interference (RNAi) suppressed the viability of both Molt-4 and MAR cells. After NPM RNAi, the IC50 of MAR and Molt-4 cells were 3.83 ± 0.38 μmol/L and 0.19 ± 0.02 μmol/L respectively. Both of them revealed an increase of drug sensitivity with down-regulation of mdr-1 and Akt/mTOR signaling. Knockdown of mdr-1 could also reverse the drug resistance, with no change in NPM expression. It could be concluded that knockdown of NPM reversed the drug resistance by down-regulating P-gp and Akt/mTOR signal pathway, indicating that NPM may serve as a potential modulator in drug resistance.

Introduction

Adult acute lymphoblastic leukemia (ALL) is a heterogeneous hematological malignancy. There are two subtypes of ALL, B-lineage ALL (B-ALL) accounts for 85% of all cases and the remaining 15% are T-lineage ALL (T-ALL). Compared to children, who can approach a cure rate of 90%, a majority of adult patients failed to attain disease eradication, especially the prognosis in patients with relapsed or refractory disease is uniformly poor (Pui et al. 2008).

Many factors have been proved to be associated with ALL prognosis, such as immunologic subtype, and clinical, genetic, and molecular features (Lazarus and Advani 2012), several of which have clear implications for risk stratification and targeted therapeutic intervention (Mullighan 2012). T-ALL is known to have less favorable prognosis and relapse is more common probably due to emergence of chemo-resistance. The principal source of ALL relapse is the survival and expansion of leukemic cells resistant to chemotherapy. Researches over past three decades have identified a number of ways in which cancer cells can elude chemotherapy (Gottesman et al. 2002); including ATP-binding cassette (ABC) transporters mediated drug efflux and other mechanism.

Nucleophosmin (NPM, also known as B23, numatrin, or N038) is regularly identified as multifunctional nuclear protein (Maggi et al. 2008; Yu et al. 2006; Okuwaki 2008; Mukudai et al. 2008), not only an important player in ribosome biogenesis but also a potential regulator for cell proliferation. Aberrant expression of NPM, such as mutation, deletion, over-expression or rearrangement, could lead to malignant transformation in tumor cells (Grisendi et al. 2006). It is reported that over-expression of NPM in solid tumor had been detected as a poor prognostic factor and related to drug-resistance development (Yang et al. 2007). While in adult de novo

Abbreviations: NPM/B23, nucleophosmin/B23; ALL, acute lymphoblastic leukemia; MDR, multidrug resistance; P-gp, P-glycoprotein; ADM, adriamycin; DNR, daunorubicin; CTX, cyclophosphamide; VCR, vincristine; VP16, etoposide; IC50, half inhibitory concentration; RNAi, RNA interference; shRNA, short hairpin RNA; qRT-PCR, quantitative real-time reverse transcription chain reaction.

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acute myeloid leukemia (AML), NPM1 mutations occurs in about 30% of patients, and approximately 85% of AML patients with NPM1 mutation show a normal karyotype subgroup (AML-NK) (Verhaak et al. 2005). Researchers found that mutated NPM without FLT3-ITD showed a good response to chemotherapy in AML, while patients carrying both NPM1/FLT3-ITD mutations predicted poor responses (Schnittger et al. 2005).

Although a great deal of knowledge of NPM has been accumulated, little information on the role of NPM in drug resistance in leukemia is available. According to our previous investigation, NPM levels are up regulated in drug-resistant cell lines and primary leukemic cells. It is revealed that an expression of NPM together with nucleolin/C23 (NCL), another important nuclear protein, has relationship to drug resistance. High expressions of NPM and NCL could be observed in refractory or relapsed acute leukemia patients, which significantly correlated with poor prognosis (Hu et al. 2011). Moreover, the expressions of both proteins were confirmed in 9 hematologic cell line, especially in drug resistant cell line HL60/ADR, K562/ADR and KG/01. It is confirmed that in resistant myeloid leukemia HL-60/ADR cells, knockdown of NPM could reverse multidrug resistance (Lin et al. 2013).

In this study, we wanted to evaluate whether NPM is involved in drug resistance in lymphocytic leukemia cells. Since the lymphocytic cell lines with multidrug resistance are not common in laboratory, we had to induce the drug resistant cell line by induction with increasing concentration of Adriamycin. We also investigated the potential involvement of NPM in cell drug resistance and explored the possibility of modulating drug sensitivity by knocking down NPM.

Materials and methods

Cell culture and drug-resistance induction

Molt-4 cell line kept in our laboratory (Fujian Institute of Hematology, Fuzhou, China) was cultured in RPMI-1640 medium (Hyclone, UT, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, UT, USA) at 37 °C in a 5% CO_2 incubator (Thermo Fisher Scientific, Rockford, IL, USA). Adriamycin (ADM, Sigma–Aldrich, St. Louis, MO, USA) was dissolved in normal saline (NS) with a concentration of 2 mg/ml. To establish an adriamycin-resistant leukemia cell line, Molt-4 cells were exposed to stepwise increasing concentrations of adriamycin. The medium were changed every other day, and drug sensitivity of resistant cells and parental cells were compared using MTT assay (described as followed). Single-cell clones were developed by a standard limiting dilution method, and the ADM resistant Molt-4 cell clone was named as Molt-4/ADR (short as MAR).

50% inhibiting concentration (IC50) detection

MTT assay was employed to detect cells' sensitivity toward Adriamycin. Cells at logarithmic phase cells were collected, and the cell suspension was adjusted to 2×10^4 cells per well in different concentrations of drug. 20 μl of the MTT solution was added to each well (5 mg/ml, Sigma–Aldrich, MO, USA). After incubation for 4 h, 100 μl dimethyl sulfoxide (DMSO) was added to each well to dissolve crystal completely. Plates were read on Elx808 Absorbance Microplate Reader (BioTek, UT, USA) at 490 nm with a reference wave-length at 630 nm. IC50 values were calculated by SPSS 19.0 software. The experiment was repeated for three times.

Intracellular ADM imaging by confocal microscopy

Intracellular drug accumulation images in Molt-4 and MAR cells were acquired by confocal microscopy (Leica Microsystems GmbH, Wetzlar, Germany), and quantitative analysis was done by LAS AF Lite (Leica). Molt-4 cells, MAR cells and MAR cells with 20 μM Verapamil Hydrochloride were cultured with 2 μg/ml (8.62 μmol/L) ADM for 24 h, then cells were collected for confocal microscopy analysis. Gray values were evaluated to quantify the accumulation of ADM.

Intracellular ADM detection by flow cytometry

Intracellular ADM accumulation was also evaluated by flow cytometry using a BD FACS Calibur (Becton Dickinson, CA, USA). Molt-4 cells, MAR cells and MAR cells with 20 μM Verapamil Hydrochloride (Sigma–Aldrich, St. Louis, MO, USA) were cultured with 0, 0.5, 1, 2 μg/ml ADM respectively for 24 h, then cells were collected. The ADM fluorescence intensity and ADM-accumulated cell populations were determined using the Cell Quest Pro Software (Becton Dickinson, CA, USA) and analyzed by FlowJo Software, Version 7.6.1.

Construction of vectors for RNA interference (RNAi)

Plasmids encoding short hairpin RNAs (shRNA) against NPM and mdr-1 were designed respectively. Oligo DNA sequences were inserted between U6 promoter and CMV promoter with green fluorescent protein (GFP) in a GV113-1 basic vector (Genechem, Shanghai, China). Vectors containing shRNA against non-sense sequence were used as control (Hu et al. 2011). The sequences for target gene were as follows:

Sequences targeted to NPM (Lin et al. 2013):

- Sense: 5′-CCGGTACGAAGGCAGTCCAATTAAATTCAAGAGATTTAATTGGACTGCCTTCGTATTTTTG-3′.
- Antisense: 5′-AATTCAAAAATACGAAGGCAGTCCAATTAAATCTCTTGAATTTAATTGGACTGCCTTCGTA-3′.

Sequences targeted to mdr-1 (Zhong et al. 2007):

- Sense: 5′-T GATGTGTGCTTTCCTCAAATTCAAGAGATTTGAGGAAAGCACACATCTTTTTTC-3′.
- Antisense: 5′-TCGAGAAAAAAGATGTGTGCTTTCCTCAAATCTCTTGAATTTGAGGAAAGCACACATCA-3′.

Lentivirus-based RNA interference

Infectious viral supernatants (DMEM media with 10% FBS) were derived by transient co-transduction of plasmids into HEK-293 T (4×10^6 in 100 mm^3 petri-plates) cells using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's recommendations. The viral vector titers were 2.0×10^9 transducing units (TU)/ml assayed by infection of 293 T cells at different dilutions in a 96-well plate.

To produce the RNAi cells, Molt-4 and MAR cells were seeded at 1.0×10^5 cells per well in 24-well plates. The cells were transfected with 100MOI each lentivirus stock (MOI refers to multiplicity of infection, a parameter for viral infectivity in a population of target cells), and then incubated for an additional 48–72 h prior to identifying the GFP positive cells by fluorescence microscopy.

For RNAi experiment, 3 groups of cells were defined: BC (blank control) meant cells without transfection; NC (negative control) meant cells transfected with vector contains non-sense sequence; KD (knockdown) referred to cells transfected with shRNA against target RNA.