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Effect of NPM1 type B mutation on the proliferation, invasion and chemosensitivity of THP-1 leukemia cells

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Acute myeloid leukemia (AML) is the most malignant myeloid disorder in adults. AML with mutated nucleophosmin (NPM1) is regarded as an independent leukemia subtype. According to previous studies, the role of NPM1 gene A mutation in AML has been well established; however, another major type, NPM1 gene B type mutation (NPM1 MutB) has been rarely reported. In the present study, we found that overexpression of NPM1 MutB enhanced the proliferation and invasion of THP-1 AML cells through the regulation of TIMP-2, MMP-2, Ang-1, c-myc and CCND1; led to no significant change of apoptosis rate with the absence of chemotherapy agents, while enhanced the chemosensitivity of THP-1 AML cells to chemotherapy agents DNR and Ara-C through the regulation of Bax, Bcl-2 and caspase-3. Further, we revealed that NPM1 MutB overexpression reduced the NF-κB activity of THP-1 cells upon drug treatment. Taken together, we demonstrated the detailed functions of NPM1MutB in THP-1 proliferation, invasion, apoptosis and chemo-sensitivity. We provided a novel understanding of prognosis of patients carrying the NPM1 B mutation.

1. Introduction

Acute myeloid leukemia (AML) accounts for the highest proportion of malignant myeloid disorder in adults, leading to the most annual deaths from leukemia (Schlenk et al. 2008; O'Donnell et al. 2013). Due to the distinctive genetic, pathological and/or clinical features, AML with nucleophosmin (NPM1) gene mutations, which occur in one third of the AMLs, has been regarded as a new leukemia entity (Vardiman et al. 2009).

NPM1 is a multifunctional nucleolar protein which is normally localized in nucleoli but also able to shuttle out of the nucleus to localize in the cytoplasm (Okuwaki 2008). As an essential regulator of cell cycle progression, NPM1 plays a key role in response to a variety of stress stimuli (Falini et al. 2008; Lindstrom 2011). The wild type NPM1 gene chromosomal translocations or mutations have been frequently reported and regarded to be related to the progression of leukemia or lymphoma (Colombo et al. 2011). Once the NPM1 mutation occurs, the balance between nuclear and cytoplasmic NPM1 is altered, resulting in an abnormal cytoplasmic localization of NPM1 (Bolli et al. 2007). The cytoplasmic nucleophosmin (NPMc+) in AML was firstly observed and reported in 2005 by Falini et al. (2005). They also revealed that the typeA NPM1 mutation was the most frequent in adults (75-80% of cases). NPM1 mutated protein plays a crucial role in leukemogenesis (Falini et al. 2009). Mutated NPM1 attenuates the p53-ARF tumor-suppressor pathway through causing cytoplasmic delocalization and degradation of ARF and Fbw7 (Colombo et al. 2006) and enhances the MYC oncogenic pathway (Bonetti et al. 2008). In addition, the cytoplasmic NPM1 mutant could directly interact with caspase-6 and -8, and suppressed caspase-6/-8-mediated myeloid differentiation (Leong et al. 2010).

So far, more than 50 kinds of NPM1 mutations have been found; most of them are due to the frame shift mutations caused by the insertion of different bases at the C-terminus of the NPM1 gene coding region. In the mutations of the NPM1 gene, six mutations are dominant, namely, mutation A to F (Pitiot et al. 2007; Luskin et al. 2015; Azari-Yam et al. 2016). However, most of the research on the NPM1 mutations is focused on NPM1 type A mutations, whereas NPM1 mutations such as NPM1 type B mutations (NPM1 MutB), accounting for about 10%, have rarely been reported. The NPMc + AML patients with different mutations had different NF-KB activity and Bax / bcl-2 ratio (Cilloni et al. 2008; Del Poeta et al. 2010). Clinical data also showed that although NPMc + AML patients overall showed a good prognosis, there are also differences in sensitivity to chemotherapy and disease-free survival in the NPMc + AML patients with different mutations (Verhaak et al. 2005).

In the present study, we focus on NPM1 MutB to investigate its detailed functions in the cell proliferation, invasion and apoptosis of human acute monocytic leukemia cell THP-1, the protein levels of proliferation-, invasion- and apoptosis-related factors, and the activity of NF-κB. Taken together, the aim of this study was to elucidate the effect of NPM1 B mutation on the phenotype of AML cells and its mechanism, to provide a novel theoretical basis for the treatment of AML.

2. Investigations and results

2.1. Establishment of THP-1 cells stably expressing NPM1 MutB

To establish the THP-1 cells that stably express NPM1 MutB, we firstly constructed lentiviruses expressing plasmid that contained NPM1 MutB. Compared with the wild-type NPM1 gene, the CDS region of the NPM1 MutB contains several different bases at the C terminus. By using the cDNA clone of the wild-type NPM1 gene as a template, and the NPM1 MutB specific primers, we obtained NPM1 MutB fragments through real-time PCR (Fig.S1); the NPM1 MutB fragments and the target vector were digested and connected. A skeleton diagram of pLVX-NPM1-MutB is shown in Fig. 1A.

Lentiviral expressing plasmid pLVX-NPM1-MutB or control plasmid pLVX-NC and lentivirus packaging plasmids pLP1, pLP2, pLP/VSVG were co-transfected into 293FT cells for lentivirus packaging; LV-NPM1-MutB over-expressing NPM1-MutB and control lentivirus LV-NC were obtained. THP-1 cells

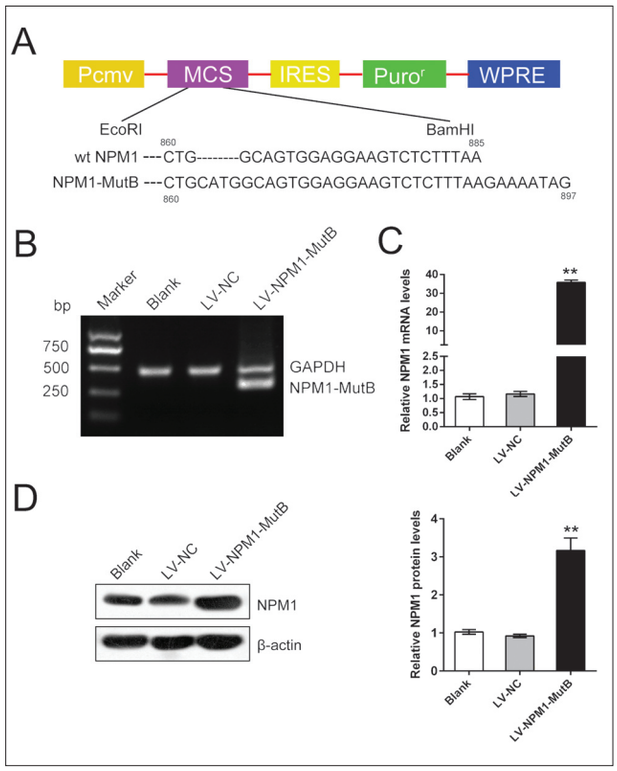

Fig. 1: Establishment of THP-1 cells stable expressing NPM1 MutB. (A) Skeleton diagram of pLVX-NPM1-MutB plasmid. (B) and (C) Verification of NPM1 MutB overexpression in THP-1 cells using real-time PCR assays analyzed by agarose electrophoresis and statistic analysis. (D) Verification of NPM1 MutB protein overexpression in THP-1 cells using Western blotting assays. The data are shown as mean± SD of three independent experiments. ** $P< 0.01$.

were infected with LV-NPM1-MutB or LV-NC; the expression of NPM1-MutB was then verified at both mRNA and protein levels using qRT-PCR and Western blot assays (Fig. 1B, C and D). The agarose electrophoresis of qRT-PCR products showed that NPM1 MutB was expressed in LV-NPM1-MutB group rather than the LV-NC group and Blank group (without any infection) (Fig. 1B). The statistic analysis of qRT-PCR and western blotting assays showed that NPM1 MutB significantly upregulated after infection of LV-NPM1-MutB, compared with the two control groups (Fig. 1C and D).

2.2. NPM1 MutB promoted THP-1 cells proliferation and invasion

After successfully establishing NPM1 MutB-expressing THP-1 cell line, we then evaluated the function of NPM1 MutB in THP-1 cell proliferation and invasion. 48 and 72 h after LV-NPM1-MutB infection, the proliferation of THP-1 cell was significantly promoted, compared to the LV-NC and blank group (Fig. 2A, P<0.01), as shown by the CCK-8 assays. Results from the Transwell assays showed that NPM1 MutB promoted the invasion capability of THP-1 cell, compared to the LV-NC group (Fig. 2B, $P<0.01$).

To investigate the possible mechanism(s) by which NPM1 MutB affected the cell proliferation and invasion of THP-1 cell, we monitored the changes of the protein levels of proliferation- and invasion-related factors. Results from the Western blot assays showed that, the protein levels of c-myc and CCND1, which were both regarded as oncogenes related to cell proliferation (Rodrigo Tapia et al. 2001; Tian et al. 2005; Akinyeke et al. 2013; Chen et al. 2014), were upregulated by NPM1 MutB (Fig. 2C). The protein level of Ang-1, which highly expresses in cancer tissues, promotes tumor angiogenesis thus promotes cancer cell migration and invasion (Zheng et al. 2015), was upregulated by NPM1 MutB (Fig. 2C). In addition to the Ang-1/Tie2 pathway, the MMP2 pathway is also related to cancer cell migration and invasion (Shen

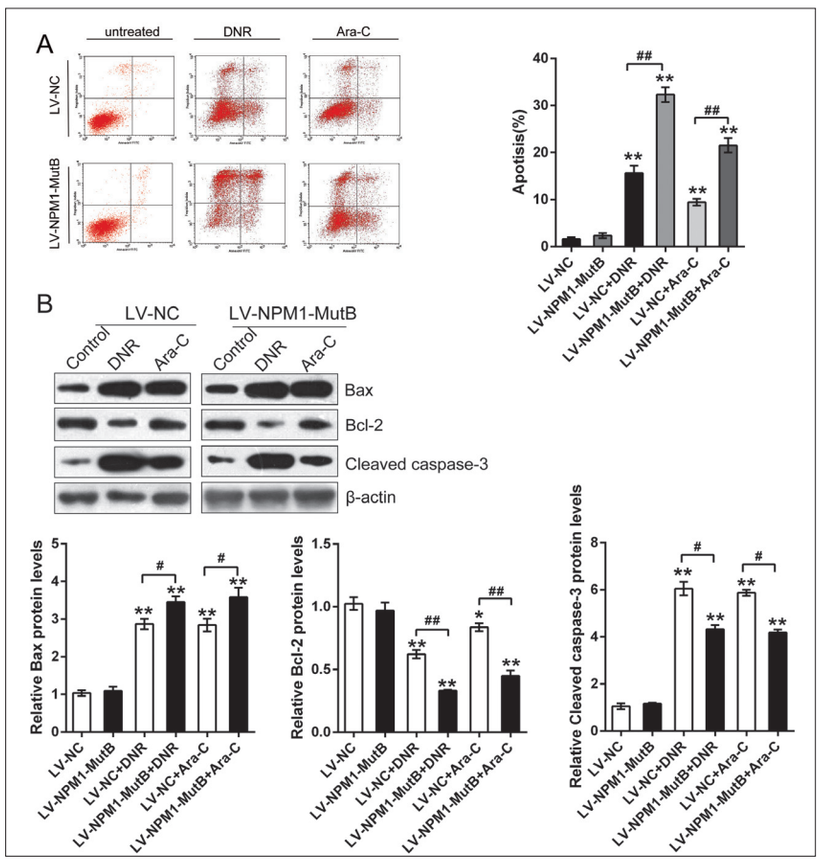

Fig. 3: NPM1 MutB enhanced the sensitivity of THP-1 cells to DNR and Ara-C. (A) The apotosis rate of LV-NPM-MutB-infected THP-1 cell, with the presence or absence of chemotherapy agents (DNR or Ara-C), compared to LV-NC group or LV-NC + DNR group or LV-NC + Ara-C group, as determined using Flow Cytometry assays. (B) The protein levels of Bax, Bcl-2 and Cleaved caspase-3 in LV-NPM-MutB-infected THP-1 cell, with the presence or absence of chemotherapy agents (DNR or Ara-C), compared to LV-NC group or LV-NC + DNR group or LV-NC + Ara-C group, as determined using Western blot assays. The data are shown as mean±SD of three independent experiments. ** $P< 0.01$ (compared to LV-NC group); # $P< 0.05$, ## $P< 0.01$ (compared to LV-NC + DNR group or LV-NC + Ara-C group).

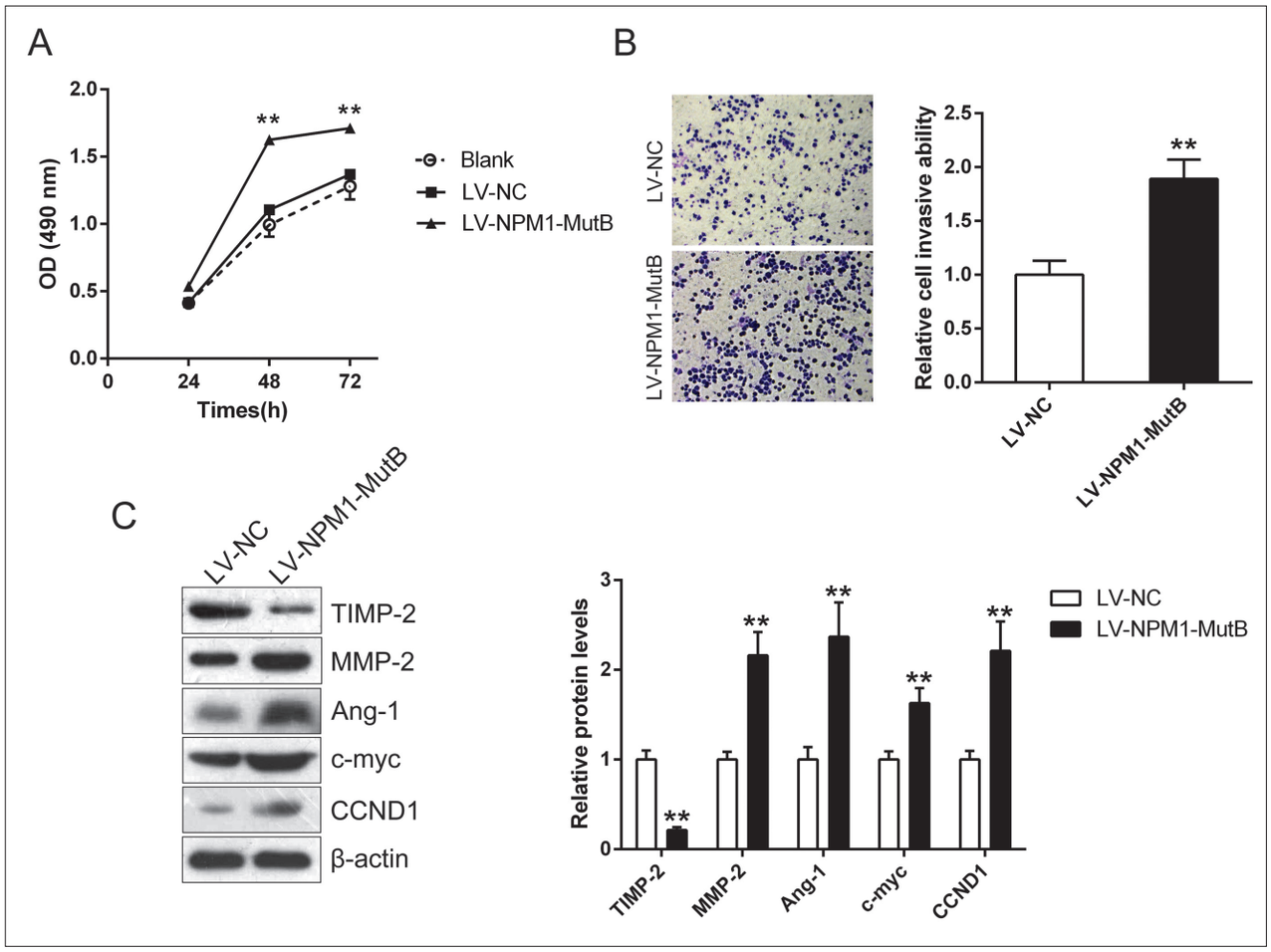

Fig. 2: NPM1 MutB promoted THP-1 cells proliferation and invasion. (A) The proliferation of LV-NPM-MutB-infected THP-1 cell, compared to Blank (without any infection) and LV-NC group, as determined using CCK-8 assays. (B) The invasion capability of LV-NPM-MutB-infected THP-1 cell, compared to LV-NC group, as determined using Transwell assays. (C) The protein levels of TIMP-2, MMP-2, Ang-1, c-myc and CCND1 in LV-NPM-MutB-infected THP-1 cell, compared to LV-NC group. The data are shown as mean± SD of three independent experiments. ** $P< 0.01$.

et al. 2015; Wang et al. 2016). Here we found that MMP2 protein was increased, while the protein level of an inhibitor of MMP2, TIMP2, was reduced by NPM1 MutB (Fig. 2C). These data suggested that NPM1 MutB could significantly promote THP-1 cell proliferation and invasion, through different pathways.

2.3. NPM1 MutB enhanced the THP-1 cell chemo-sensitivity to DNR and Ara-C

To evaluate the function of NPM1 MutB in inducing chemo-sensitivity of THP-1 cells, THP-1 cells expressing NPM1 MutB were incubated under DNR (0.25 μM) or Ara-C (8.5 μM) treatment for 24 h. Then the cell apoptosis was evaluated using Flow cytometric assays. As shown in Fig. 3A, without durg treatment, the apoptosis rate of THP-1 cells expressing NPM1 MutB showed no significant difference compared to the LV-NC group, indicating that NPM1 MutB made almost no impact on THP-1 cell apoptosis. Under either DNR or Ara-C treatment, the apoptosis rate of LV-NC-infected THP-1 cells was significantly promoted compared to non-treated LV-NC group (15.54% in LV-NC + DNR group, 9.45% in LV-NC + Ara-C group); in addition, after either DNR or Ara-C treatment, the apoptotic percentage of NPM1 MutB-expression THP-1 cells was significantly higher as compared to LV-NC + DNR or LV-NC + Ara-C group (32.32% in LV-NPM1 MutB + DNR group, 21.52% in LV-NPM1 MutB + Ara-C group) (Fig. 3A). These data indicated that NPM1 MutB made almost no impact on THP-1 cell apoptosis; however, NPM1 MutB amplified the promotive effect of DNR and Ara-C on THP-1 cell apoptosis.

To investigate the possible mechanism(s) by which NPM1 MutB amplified the promotive effect of DNR and Ara-C on THP-1 cell apoptosis, we monitored the changes of protein levels of apoptosis-related factors using Western blot assays. Consistent with the results of apoptosis assays, NPM1 MutB led to no significant changes of apoptosis-related proteins (Fig. 3B). Either DNR or Ara-C treatment increased the protein levels of pro-apoptosis factors, Bax and Cleaved caspase-3, reduced the protein level of anti-apoptosis factor Bcl-2 (Fig. 3B). Consistent with the cell apoptosis, NPM1 MutB amplified the effect of DNR and Ara-C on the indicated apoptosis-related proteins (Fig. 3B). These data suggested that NPM1 MutB amplified the promotive effect of DNR and Ara-C on THP-1 cell apoptosis most possibly through Bax/Bcl-2 and caspase-3.

2.4. NPM1 MutB reduced the NF-κB activity in THP-1 cells after drug treatment

Recently, NF-κB activation has been regarded as a key response of leukemia cell to chemotherapy (Turco et al. 2004; Jacamo et al. 2014). To investigate whether NF-κB activity is involved in the NPM1 MutB-mediated chemosensitivity of THP-1 cells, luciferase reporter assays were employed to evaluate the NF-κB activity of THP-1 cells with the presence or absence of chemotherapy agents. The changes of NF-κB activity were not statistically significant ($P>0.05$) in untreated cells. The activity of NF-κB in both LV-NPM1-MutB and LV-NC cells was higher after DNR or Arc-C treatment, compared to untreated cells ($P<0.01$); however, there was a certain

degree of increase of the NF-κB activity in the chemotherapeutic agents-treated LV-NPM1-MutB cells, both the magnitude of increase and the NF-κB activity in the LV-NPM1-MutB cells were significantly lower than those of the LV-NC cells (Fig. 4). These results indicate that the treatment of chemotherapeutic agents can stimulate or induce NF-κB activation in THP-1 cells, whereas overexpression of NPM1-MutB significantly attenuates or inhibits NF-κB activation induced by the chemotherapeutic agent.

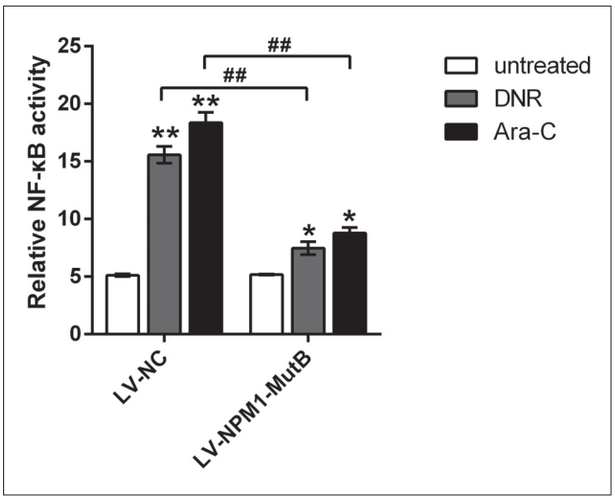

Fig. 4: NPM1 MutB reduced the NF-κB activity in THP-1 cells after drug treatment. The activity of NF-κB in THP-1 cells, with the presence or absence of chemotherapy agents (DNR or Ara-C), compared to LV-NC group or LV-NC + DNR group or LV-NC + Ara-C group, as determined using Luciferase reporter assays.The data are shown as mean± SD of three independent experiments. * $P< 0.05$, ** $P< 0.01$ (compared to LV-NC group); ## $P< 0.01$ (compared to LV-NC + DNR group or LV-NC + Ara-C group).

3. Discussion

The efficiencies of AML treatment in patients are different due to the genotypic leukemia-specific differences (Lowenberg 2008). In the pathogenesis of leukemia, NPM1 is a major object of research. As we have mentioned, NPM1 mutations frequently occur in AML and play crucial roles in leukemogenesis (Falini et al. 2009). Previously, NPM1 MutA has been frequently reported to a play key role during AML. In the present study, we focused on another important NPM1 mutation, NPM1 MutB, to evaluate its functional roles in THP-1 cell line and the underlying mechanism.

In the present study, we used human monocytic leukemia THP-1 cells without NPM1 mutation as a cellular model. In the cell lines stably expressing NPM1 MutB, we observed that NPM1 MutB significantly promoted the cell proliferation and invasion capability of THP-1 cells. To investigate the underlying mechanism, we monitored the changes of protein levels of a series of proliferation- and invasion-related factors.

The disorder of c-myc, a member of the myc gene family with multiple functions, has been frequently reported in some tumors. C-myc is an essential regulator of many physiological processes including cell cycle control, apoptosis, protein synthesis, and cell adhesion as a vital transcription regulator (Dang 2012). In addition to c-myc, elevated cyclin D1 protein due to overexpression of CCND1 mRNA in cancers is also frequently observed (Tian et al. 2005). In the present study, NPM1 MutB increased the protein levels of c-myc and CCND1 in THP-1 cells, indicating that NPM1 MutB might promote THP-1 cell proliferation through increasing c-myc and CCND1 protein expression.

Ang-1, a member of the Ang family, binds to the tyrosine kinase receptor Tie2 and participates in a variety of physiological or pathological angiogenesis, including tumor angiogenesis (Shantha Kumara et al. 2010). Recent studies have revealed that Ang-1 is highly expressed in a variety of tumor tissues, and thus promote tumor angiogenesis (Enholm et al. 1997; Shantha Kumara et al. 2010). Since tumor growth and metastasis is largely dependent on tumor angiogenesis, Ang-1 can thus make contribution to tumor growth and metastasis. In the present study, Ang-1 protein level and the invasive potential of THP-1 cells were both significantly promoted by NPM1 MutB overexpression, indicating that NPM1 MutB might contribute to the invasion of THP-1 cell through Ang-1.

In addition to Ang-1, MMP2 has also been regarded as an essential regulator of cancer invasion (Kalhori and Tornquist 2015; Xuan et al. 2015). Increased MMP2 expression leads to increased degradation of type IV collagen, which in turn affects the integrity of the basement membrane. The destruction of the basement membrane leads to less restrictions of invasion and metastasis of the tumor cells, leading to increased tumor invasion and metastasis. In the present study, we monitored the protein levels of MMP2 and its inhibitor, TIMP2 in NPM1 MutB-overexpressing THP-1 cells. After NPM1 MutB overexpression, MMP2 protein was increased, whereas TIMP2 protein was reduced, indicating that NPM1 MutB might decrease the expression of TIMP-2, decrease the inhibition of MMP-2, and promote the expression of MMP2, which can enhance the degradation of THP-1 cells to extracellular matrix, leading to THP-1 cell invasion and extrame dullary in filtration.

Apoptosis involves a series of gene activation, expression and regulation, in which Bcl-2 family is one of the most important regulator of apoptosis playing a decisive role in the process of apoptosis (Siddiqui et al. 2015). Bcl-2 family can be divided into anti-apoptotic factors and pro-apoptotic factors according to their roles, of which the most important anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax (Zhang and Saghatelian 2013, Volkmann et al. 2014). The ratio of Bax/Bcl-2 can reflect the apoptosis of cells; the ratio of Bax/Bcl-2increases, the apoptosis rate increases, the ratio of Bax/Bcl-2 decreases, the apoptosis rate decreases (Sakinah et al. 2007, Skala et al. 2016). In addition, either death receptor pathway-mediated apoptosis or mitochondrial pathway-mediated apoptosis induces cell apoptosis by activating caspase-3 ultimately, therefore, the expression of cleaved caspase-3 in cells can directly reflect the level of apoptosis (Mukai et al. 2005).In the present study, we found that in the absence of external stimuli, NPM1-MutB had little effect on apoptosis of THP-1 cells. Correspondingly, when NPM1-MutB gene was overexpressed in THP-1 cells, Bax and Bcl-2 protein expression did not change significantly, nor did the expression of cleaved caspase-3 protein. These data indicated that NPM1-MutB did not directly affect THP-1 cell apoptosis or apoptosis-related factors. However, after treatment with chemotherapy agents DNR or Ara-C, different results were observed. Either DNR or Ara-C treatment significantly increased the apoptosis rate; more strongly increased the apoptosis rate of NPM1-MutB-overexpressing THP-1 cell. The changes of the apoptosis-related factors were consistent: either DNR or Ara-C treatment increased the protein levels of Bax and cleaved caspase-3, reduced the protein level of Bcl-2; the amplitude of changes was stronger in NPM1-MutB-overexpressing THP-1 cells. All these data suggested that NPM1-MutB might improve the sensitivity of THP-1 cells to DNR or Ara-C through Bax/Bcl-2 and caspase-3.

After treatment with chemotherapy agents, NF-κB abnormal activation and continuous rising in leukemia cells frequently appears, leading to decreased Bax/Bcl-2 ratio and inhibition of apoptosis, which is also the main reason of leukemia cell chemo-resistance (Vaskivuo et al. 2002; Zhang,et al. 2016). In the present study, we monitored the activity of NF-κB in THP-1 cells in the presence or absence of chemotherapy agents. In response to chemotherapy agents' treatment, NF-κB activity in both LV-NC-infected or NPM1-MutB-overexpressing THP-1 cell was significantly upregulated; however, in NPM1-MutB-overexpressing THP-1 cells, the extent of variation of NF-κB activity was lighter than that of the LV-NC-infected THP-1 cells. As we have mentioned, the mutant NPM1 protein is abnormally localized in the cytoplasm (Al-Husseinawi 2013); it might bind to the cytoplasmic NF-κB protein through N-terminal domain, thereby prevent the NF-κB protein

from entering the nucleus, or result in degradation of NF-κB protein (Cilloni et al. 2008), thus reduce the NF-κB activity, inhibit its regulation of downstream genes such as Bax and Bcl-2, weaken its inhibition of apoptosis, finally enhance the sensitivity of THP-1 cells to chemotherapeutic drugs.

Taken together, we demonstrated the detailed functions of NPM1 MutB in THP-1 proliferation, invasion, apoptosis and chemo-sensitivity. We provided a novel understanding of prognosis of patients carrying the NPM1 B mutation. Further studies are needed to investigate the role of NPM1-MutB in leukemia chemo-sensitization in a proper animal model and clinical study.

4. Experimental

4.1. Cell line, cell culture and agents

THP-1 cells were obtained from Shanghai Institutes for Biological Sciences (Shanghai, China), maintained in RPMI-1640 medium (Gibco, USA)with a supplement of 10%fetal bovine serum (Gibco, USA) and 100 U of penicillin and streptomycin (Sangon Biotech, Shanghai, China) in a 5% CO_2-humidified incubator at37 °C. Lentivirus packaging cells 293FT were purchased from Invitrogen, USA. Chemotherapy agents Daunorubicin (DNR) andcytarabine (Ara-C) were purchased from Sigma.

4.2. NPM1-MutB slow virus expression plasmid construction and cell infection

pLVX-NPM1-WT and pLVX-NC (negative control) were purchased from YRBio (Changsha, China). Using the cDNA clone of wild-type NPM1 as a template, the NPM1-MutB--f and NPM1-MutB--ras primers to amplify theNPM1-MutB CDS region (NPM1-MutB-f: 5'-GGAATTCGCCACCATGGAAGATTCGATGGACATGGA-3'; NPM1-MutB-r: 5'-CGGGATCCCTATTTTCTTAAAGAGACTTCCTCCACTGC-CATGCAGAGATCTTGAATAGC-3'; 918 bp). Lentivirus packaging plasmids pLP1, pLP2, pLP/VSVG were purchased from Invitrogen, USA. The lentiviral expressing plasmid pLVX-NPM1-MutB or control plasmid pLVX-NC and the lentivirus packaging plasmids pLP1, pLP2, pLP/VSVG were co-transfected into 293FT cells, and lentivirus packaging was performed to obtain NPM1-MutB over-expressing LV-NPM1-MutB and control lentivirus LV-NC by using Lipofectamine2000 (Invitrogen, USA) following the manufacturer's instructions. The lentivirus in supernatants wash arvested, concentrated and the viral titers were determined.

The target cells were plated and incubated overnight (16 h) for infection. On the day of infection, 1.5 ml/well viral supernatant was used to replace the culture medium. After incubated at 37 °C for 10 h, the viral supernatant was replaced with fresh media. 48 h after infection, the target cells were selected using puromycin (2 mg/ml) and maintained in G418 (Invitrogen, USA).

4.3. Real-time PCR assays

Total RNA was extracted with TRIzol reagent (TaKaRa, Japan). Total RNA was reverse-transcribed for cDNA synthesis. Quantitative real-time PCR (qRT-PCR) analysis was performed on a MJ Mini™ Gradient Thermal Cycler Real-Time PCR machine (Bio-Rad, USA) with the SYBR Green reaction (TaKaRa, Japan) kit.

4.4. Cell counting Kit-8 (CCK-8) assay

Cells were seeded into 96-well plates (Corning, USA) at a density of 1×10^3 cells per well with RPMI-1640 containing 10% FBS. The cell number was quantified 0, 24, 48, 72 h with the Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay; at 1 h before the endpoint of incubation we added 10 µl CCK-8 reagents to each well. A microplate reader was used to determine OD_{490nm} value in each well. Each experiment was performed in triplicate.

4.5. Transwell assay

THP-1cells with NPM1-MutB were detected for their invasive ability. THP-1cells were suspended in medium without serum and medium supplemented with serum was used as a chemo-attractant in the bottom chamber. The cells were incubated at 37 °C for 48 h. The non-invasive cells in the top chambers were removed with cotton swabs. The invaded cells on the lower membrane surface were fixed in 100 % methanol for 10 min, air-dried, then stained with 0.1% crystal violet solution (Beyotime Institute of Biotechnology, Haimen, China), and counted under a microscope. The experiments were performed in triplicate.

4.6. Cell apoptosis assay

For apoptosis analysis, quantification of apoptotic cells was performed with Annexin V-FITC apoptosis detection kit (Keygen, China). Briefly, the cell samples were harvested with 0.25% trypsin without EDTA 48 h after infection and then washed twice with ice-cold PBS and re-suspended in 500 µl binding buffer. Then cells were incubated with 5 µl Annexin V-FITC specific antibodies and 5 µl propidium iodide (PI) then incubated for 15-20 min in the dark and detected by BD Accuri C6 flow cytometer (BD, USA) with the excitation wavelength of Ex = 488 nm and emission wavelength of Em = 530 nm. Each experiment was repeated three times in triplicate.

4.7. Western blotting

Treated THP-1 cells were washed with cold PBS and lysed in radio immune precipitation assay (RIPA) buffer. Protein concentration was determined by the bicinchoninic acid (BCA) protein assay, and denatured proteins were separated in 15% SDS polyacrylamide gel electrophoresis and transferred onto PVDF membranes. Nonspecific binding was blocked with 5% milk in TBST buffer for 2 h, followed by incubation with primary antibodies (MMP-2, TIMP-2, Ang-1, c-myc, CCND1, Bax, Bcl-2 and cleaved caspase-3 monoclonal antibodies from Inc. Santa Cruz, USA; NPM1MutB monoclonal antibodies from Abcam, UK; β-actin monoclonal antibodies from Inc. Santa Cruz, USA) at 4 °C overnight and secondary antibodies at room temperature for 2 h. Blots were visualized using ECL detection reagents. Protein expression quantification was normalized against the β-actin protein expression using image software.

4.8. Luciferase reporter assays

Nuclear factor-κB basal activity was determined by the ELISA method. 10^6 cells in each group was placed on a 6-well plate, transfected with a firefly luciferase-reporter construct (pNF-κB-TA-luc, Beyotime, Shanghai, China), or a control vector containing Renilla luciferase (pRL-TK Promega, Madison, USA). 24 h after the transfection, cells were incubated with 0.25 µM of DNR or 8.5µM Ara-C. Another 24 h after chemotherapy agents' treatment, luciferase activities were determined using the Dual-Luciferase® Reporter (DLR™) Assay System (Promega, Madison, USA). The ratio was set as the relative activities of NF-κB. The final numerical value is expressed as mean absorbance values±SD.

4.9. Statistical analysis

Results were presented as the means±SD for three independent experiments. Data assessing and statistical analysis was generated using SPSS17.0 software. A Student's t-test was employed to assess to compare averaged values. A P value of <0.05 were considered statistically significant.

Conflicts of interest: None declared.

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