Efficacy of RNAi-induced down-regulation of wild-type FLT3 on NF-κB pathway in THP-1 cell line

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Received January 18, 2008

Abstract

Background. FMS-like tyrosine kinase 3 (FLT3) is a receptor tyrosine kinase that is constitutively activated in 80% – 90% acute myelocytic leukemia (AML) patients, and seems to have poor prognosis. *Objective.* To examine the potent relationship between FLT3 and NF-κB family in AML cell line THP-1. *Methods.* Short hairpin RNA targeting FLT3 (shRNA-FLT3) was designed and synthesized by *in vitro* transcription system. Flow cytometric analysis (FCM) was used to evaluate the suppressive effect of shRNA-FLT3. Cell counting kit-8 assay was used to evaluate the ability of cell proliferation. Semi-quantitative RT-PCR was used to detect the expression of P65, NF-κB, IκB mRNAs. Western blot was used to detect the expression of P65, NF-κB, IκB proteins. *Results.* 15 nM of shRNA-FLT3 suppressed the expression of FLT3 trans-membrane protein in THP-1 cells, and the inhibitory percentage was 79.67% 72 hours after transfection. The specific down-regulation of FLT3 resulted in markedly proliferation inhibition, and the percentage of proliferation inhibition was (36.66 ± 3.67)%. The down-regulation of FLT3 didn't affect the expressions of P65, NF-κB, IκB in both the mRNA and total cellular protein levels, but obviously reduced P65 protein in the nucleus. *Conclusion.* P65, NF-κB are key mediators in FLT3 signaling, and they may be involved in proliferative effects of FLT3-associated leukemogenesis. [Life Science Journal. 2008; 5(2): 15 – 20] (ISSN: 1097 – 8135).

Keywords: acute myelocytic leukemia; FMS-like tyrosine kinase 3; nuclear factor-κB; RNA interference

1 Introduction

Acute myelocytic leukemia (AML) is an aggressive disorder characterized by accumulation of immature malignant cells in the bone marrow[1]. Although therapy strategies for AML have been improved in the last few decades, the prognosis of AML patients is still poor. Obviously, a better understanding of the molecular mechanisms in leukemogenesis will be helpful. FMS-like tyrosine kinase 3 (FLT3) is a receptor tyrosine kinase (RTK) involved in the proliferation and differentiation of hematopoietic stem cells[2,3]. Aberrant activation of FLT3 has been reported in most of AML patients and predicts poor clinical outcome[4]. FLT3 pathway represents an attractive target for therapeutic inhibition[5–8]. However, the signaling mechanisms mediating FLT3 are incompletely elucidated. Nuclear factor-kappa B (NF-κB) family is an important signaling intermediate for FLT3-closely-related receptors, but the role of the NF-κB family in FLT3 signaling is not been determined.

Constitutive activation of NF-κB pathway has been identified in many AML patients, and NF-κB pathway has been identified to contribute to the uncontrolled proliferation and anti-apoptosis in AML cells[9,10]. It was reported that NF-κB can be activated by PI3kinase-Akt signaling[8,11], and PI3kinase-Akt was determined as mediator for FLT3 activation[12]. Therefore, we hypothesized that NF-κB family maybe play a role in FLT3 signaling. In the present study we designed and synthesized FLT3-targeting short hairpin RNA (shRNA-FLT3) by *in vitro* transcription system, identified that shRNA-FLT3 could effectively down-regulate the expression of wild-type

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FLT3 in human acute monocytic leukemia cell line THP-1. Thus, we investigated the effects of RNAi-induced down-regulation of wild-type FLT3 on cell proliferation, and on NF-κB family *in vitro*.

2 Materials and Methods

2.1 Cell line and culture

The human acute monocytic leukemia cell line THP-1 was purchased from Shanghai Institute of Cell Biology, and was cultuered in medium DMEM (Hyclone, Logan, USA) supplemented with 10% fetal bovine serum (FBS), 10 mM of HEPES, 1.0 mM of sodium pyruvate, 0.05 mM of 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 ºC with 5% CO_2.

2.2 shRNA-FLT3 design, synthesis and transfection

The shRNA targeting FLT3 (shRNA-FLT3) was designed according to the guidelines given by Harborth J[13,14]. Nonspecific shRNA (shRNA-NC) was taken as negative control. shRNA-FLT3 as well as the shRNA-NC were synthesized using T7 RiboMAX™ Express RNAi System (Promega, USA). All of the steps were performed according to the recommendations of the manufacturer. The sequences of shRNA-FLT3 was 5'-GCUGUUCAUGUGAACCAUGucucuugaaCAUGGUUCACAUGAACAGCUU-3'; shRNA-NC was 5'-UUCUCCGAACGUGUCACGUucucuugaaACGUGACACGUUCGGAGAAUU-3'.

The concentration of shRNAs transfected into THP-1 cells was determined by dose-response studies as described in our previous article[15]. All the following tests were set 3 parallel groups which were treated with 15 nM shRNA-FLT3, 15 nM shRNA-NC and PBS, respectively. shRNAs were transfected into THP-1 cells using CodeBreaker transfection reagent (Promega, USA).

2.3 Flow cytometric analysis for FLT3 trans-membrane protein

After harvest, cells were incubated with FLT3 monoclonal antibody (Santa Cruz, USA) at 4 ºC for 1 hour. After washed with cold PBS, cells were incubated with goat anti-mouse IgG1-FITC for 30 minutes; then washed with cold PBS again. Finally cells were assayed by FacsCalibur flow cytometer (FACScan, Becton Dickinson, San Jose, CA). The percentages of FLT3 positive cells were quantified by Cell Quest Software.

2.4 Cell counting kit-8 (CCK-8) assay for cell proliferation

Cell proliferation was determined using CCK-8 solution (Beyotime, Beijing, China) according to the manufacture's instruction. Cells in 96-well plate were added with 10 μl CCK-8 solution, and incubated for another 4 hours at 37 ºC. Absorbances of each well were quantified at 450 nm using an automated ELISA reader (Bio-Tech Instruments, USA). Percentages of proliferation inhibition were calculated by (1 – A450 of transfected wells) / A450 of control wells) × 100%.

2.5 Semi-quantitative RT-PCR for P65 mRNA, and IκB mRNA

Total RNA was extracted from cells with Trizol reagent (Invitrogen, Carlsbad, USA) and reversely transcribed into cDNA using AMV first strand DNA synthesis kit (Sangon Corp, Shanghai, China) according to the manufacturer's instructions. The expressions of P65 mRNA and IκB mRNA were detected by semi-quantitative PCR amplification. Primers for P65 (195 bp): Forward: 5'-CCCCTTCCAAGTTCCTAT-3'; Reverse: 5'-TCACTTGGCTTTGAGACC-3'. Primers for IκB (316 bp): Forward: 5'-GAAGGAGCGGCTACTGGACG-3'; Reverse: 5'-AATTTCTGTGTGGCTGGTTGGTGA-3'. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was taken as an internal control. Primers for GAPDH (570 bp): Forward: 5'-GCACCGTCAAGGCTGAGAA-3'; Reverse 5'-AGGTCCACCACTGACACGTTG-3'. The total PCR mixtures were 25 μl, which consisted of 0.5 μl cDNA mixture, 0.5 U Taq DNA polymerase, 2.5 μl of 10 × PCR buffer, 2.5 mM dNTP mixture, and 50 pM sense and antisense primers each. The PCR were: 95 ºC for 3 minutes for initial denaturing, followed by 30 PCR cycles: 95 ºC for 30 seconds, 52 ºC for 30 seconds and 72 ºC for 1 minute; a final extension at 72 ºC for 5 minutes. The amplified products were subjected to electrophoresis on 1% agarose gels containing 0.2 μg/μl ethidium bromide and visualized under a UV light, and the relative levels of each mRNA were calculated by densitometry of the targets versus densitometry of GAPDH.

2.6 Western blot for P65 and IκB proteins

The total cellular protein and the nuclear protein were extracted according to instructions of nuclear and cytoplasmic extraction reagents kit (Beyotime, Beijing, China). The protein concentrations were determined by Bradford protein-binding assay. The total cellular extracts were for detecting P65 and IκB proteins using β-actin as internal control, and the nuclear extracts were for detecting P65 protein using Histone as internal control. 50 μg of protein was loaded per lane and separated by 10% sodium dodecyl sulfate-polyacrylamide gels electrophoresis (SDS-PAGE) and electrotransferred to supported nitro-

cellulose membranes (Amersham, Uppsala, Sweden) by a semi-dry transferor. The membranes were blocked in 5% skimmed milk in PBS-T containing 0.05% Tween 20 at 25 ºC for 2 hours, then incubated at 25 ºC for 2 hours with those specific antibodies such as P65, IκB, β-actin, and Histone, respectively (Santa Cruz, USA). After incubated with the appropriate HRP-linked secondary antibodies, the bands of specific protein on the membranes were developed with ECL solution (Amersham, Uppsala, Sweden). The relative levels of protein were obtained by densitometry using TotalLab 2.0 software.

2.7 Statistical analyses

All experiments results were obtained from three or four independent experiments and presented as mean ± SD ($\bar{X}$ ± SD). By statistical software SPSS13.0 (SPSS, Chicago, USA), comparison between groups were one-way ANOVA, and Tukey–Kramer multiple comparisons test. Two groups were compared with independent-samples *t* test. Differences were considered significant at $P \leq 0.05$.

3 Results

3.1 Effects of shRNA-FLT3 on the expression of FLT3 trans-membrane protein

FCM analysis showed that (53.55 ± 4.44)% THP-1 cells were FLT3 antigen positive. In order to identify the suppressive efficiency of shRNA-FLT3, 15 nM shRNA-FLT3 and 15 nM shRNA-NC were transfected to THP-1 cells, and cells were harvested at 48 hours and 72 hours, respectively. Results showed that shRNA-NC had no effect on FLT3 trans-membrane protein compared to those treated with PBS (Figure 1). Whereas, shRNA-FLT3 decreased FLT3 trans-membrane protein significantly ($P < 0.001$), and the inhibitory percentage were (79.67 ± 0.66)% at 72 hours.

3.2 Effects of FLT3 down-regulation on cell proliferation in THP-1 cells

Cells were aliquot into 96-well plates (4×10^4/ml) in triplicate, and treated with shRNA-FLT3, shRNA-NC, and PBS, respectively. Cell proliferations were assessed by CCK-8 assay at 24 hours, 48 hours and 72 hours, respectively. As shown in Figure 2, an obvious loss of cell proliferation was detected in cells treated with shRNA-FLT3 as well as shRNA-NC. Furthermore, cell proliferation in cells treated with shRNA-FLT3 decreased more obviously ($P < 0.001$), and the percentage of inhibition was up to (36.66 ± 3.67)% at 48 hours.

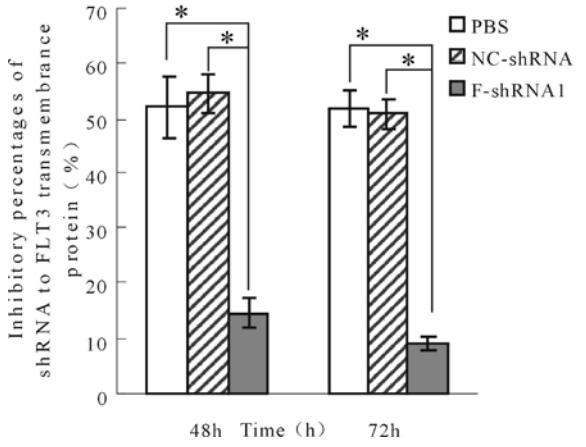

Figure 1. Effect of shRNA-FLT3 on FLT3 trans-membrane protein in THP-1 cells. $n = 3$. shRNA-FLT3 decreased the expression of FLT3 trans-membrane protein significantly. * $P < 0.001$.

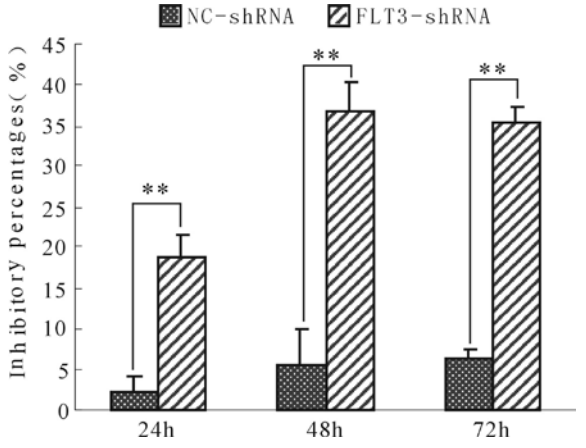

Figure 2. Effect of shRNA-FLT3 on cell proliferation. $n = 3$. Results showed that shRNA-FLT3 obviously inhibited cell proliferation compared to cells treated with shRNA-NC. * $P < 0.001$.

3.3 Effects of FLT3 down-regulation on the expression of P65 mRNA and protein

As shown in Figures 3A and 3B, results of RT-PCR showed that shRNA-FLT3 didn't affect the expression of P65 mRNA 48 hours after transfection. Results of Western blot showed that shRNA-FLT3 had no effect on the expression of P65 total protein 72 hours after transfection. However, shRNA-FLT3 decreased the expression of P65 nuclear protein about 2.3-fold compared to shRNA-NC control. The down-regulation of P65 nuclear protein might be caused by promoting the P65 shifting from the nucleus to the cytoplasm.

3.4 Effects of FLT3 down-regulation on the expression of IκB mRNA and protein

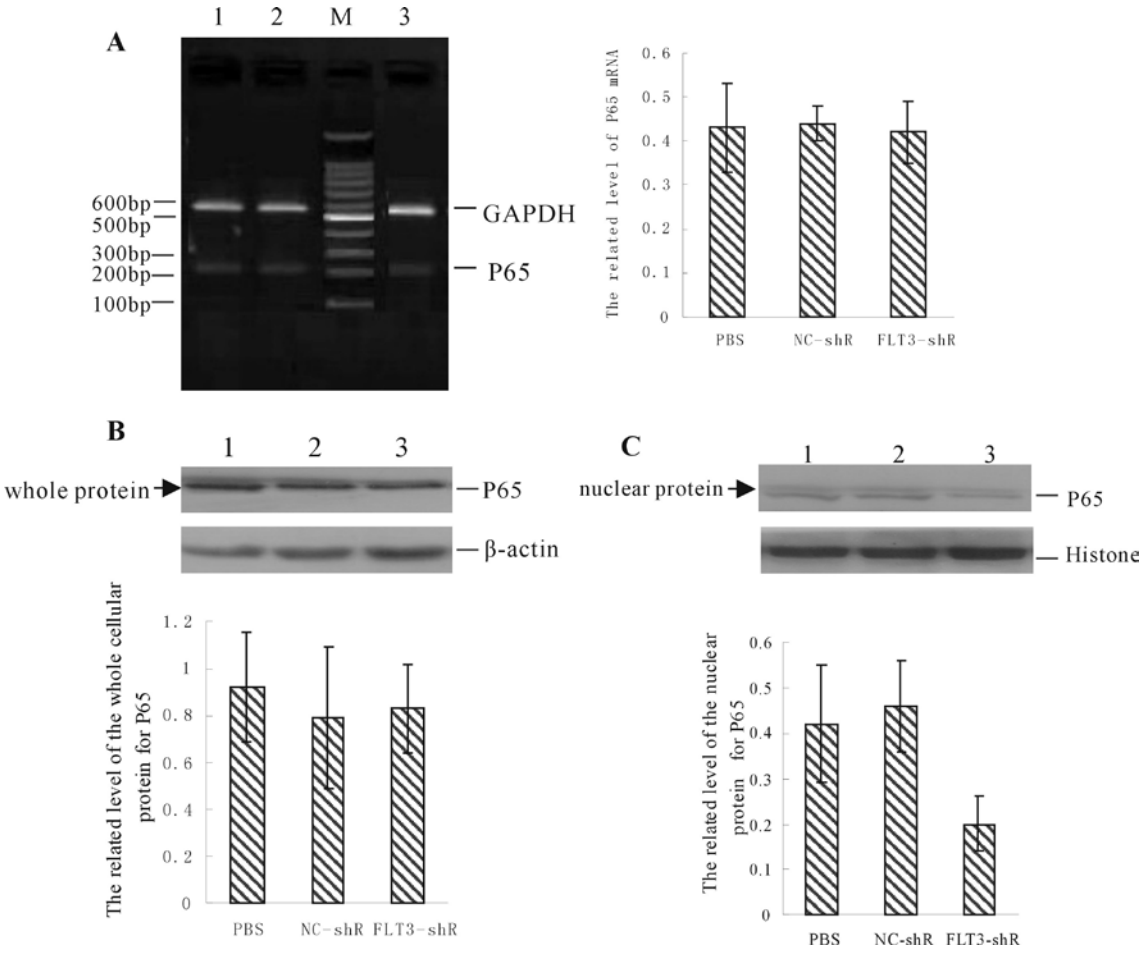

Figure 3. Effects of down-regulation of FLT3 on P65 expression in THP-1 cells. $n = 3$. A: shRNA-FLT3 had no effect on the P65 mRNA. $P > 0.05$. B and C: shRNA-FLT3 had no effect on P65 total protein ($P > 0.05$), but significantly decreased P65 nuclear protein. * $P < 0.01$.

As shown in Figures 4A and 4B, results of RT-PCR and Western blot showed that shRNA-FLT3 didn't have any effect on the IκB mRNA, and on IκB total protein, too.

4 Discussion

The NF-κB family includes P65 (RelA), P105/P50, P100/P52, RelB, c-Rel and the viral oncoprotein v-Rel. These members associate as homodimers or heterodimers. Classically, heterodimers of P65 with P50 (P50/P65) are predominant in various AML cells[10,15]. Because heterodimers of P50/P65 are tightly associated with their inhibitory protein IκB, they are sequestered in the cytoplasm and under nonstimulated conditions. When IκB is activated by a variety of stimuli such as cytokines, various stress signals, bacterial and viral products, heterodimers of P50/P65 are released and rapidly translocated to the nucleus, and touch off a series of transcriptional events. So transcriptional activity of NF-κB is specified by P65 proteins subcellular localization, as well as their association with their inhibitory protein IκB[10,11,16].

THP-1 is an AML cell line with over-expression of wild-type FLT3. We had reported in our previous paper that constitutive activation of NF-κB signaling existed in THP-1 cells. For one, P65, P50 and IκB proteins were detected in the THP-1 cells using immuno-cytochemistry and Western blot analyses. P65 proteins mainly located in the cytoplasm and few in the nucleus, and IκB proteins located just in cytoplasm. For another, parthenolide, known as a NF-κB inhibitor, could obviously inhibited cell proliferation in a concentration-dependant manner in THP-1 cells (data not shown)[17]. Thus it's reasonable to use THP-1 to study the potent relationship between FLT3 and NF-κB family.

RNA interference (RNAi) is a powerful tool for investigating gene function. We chose RNAi in our study

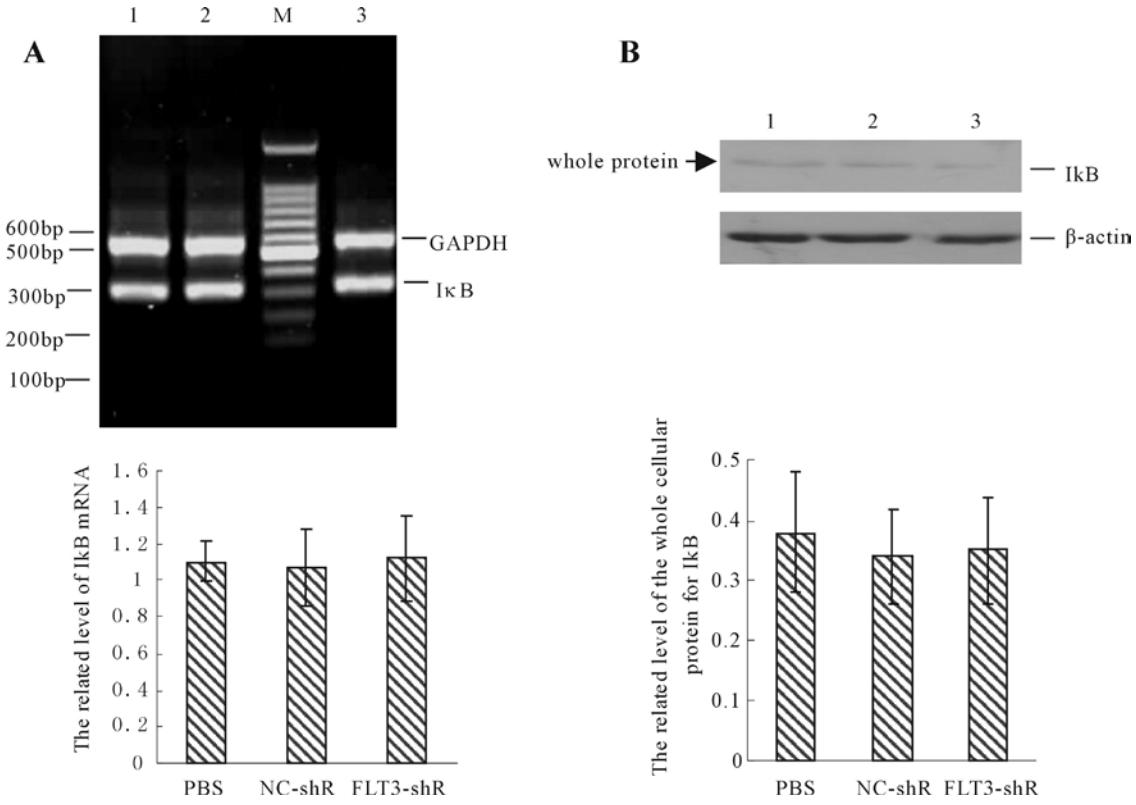

Figure 4. Effects of FLT3 down-regulation on IκB expression in THP-1 cells. $n = 3$. A: shRNA-FLT3 had no effect on IκB mRNA; B: IκB protein, $P > 0.05$.

mainly based on its specific characteristic that it just silences FLT3 gene while others weren't affected[18,19]. Our data showed that shRNA-FLT3 could effectively suppress FLT3 expression. Results of FCM showed that the percentage of FLT3 positive cells was (53.55 ± 4.44)%. After transfected with 15 nM of shRNA-FLT3 for 72 hours the percentage of FLT3 positive cells was down to (9.0 ± 1.42)%, and the inhibitory percentage was (79.67 ± 0.66)%. More importantly, we determined that the down-regulation of FLT3 receptor could availably inhibit the cell proliferation, and the inhibitory percentage was (36.66 ± 3.67)%. So, we concluded that FLT3 signaling is activated in THP-1 cells.

After down-regulating FLT3 expression using FLT3 specific RNAi in THP-1 cells, we analyzed the potent changes in NF-κB family. Our data showed that FLT3 suppression didn't affect the expressions of P65 and IκB in both the mRNA level and the total cellular protein level. Whereas, FLT3 suppression resulted in 2.3-fold decrease of P65 nuclear protein compared to those treated with shRNA-NC, suggesting that it induced P65 protein shifting from the nucleus to the cytoplasm. Previous reports have mentioned that the subcellular localization of P65 protein is key for NF-κB transcriptional activity[10,16], thus, we speculated that the down-regulation of P65 nuclear protein might suppress NF-κB activation. Of course, further studies are needed to confirm it.

5 Conclusion

We reported here that RNAi-induced down-regulation of wild-type FLT3 could effectively inhibited THP-1 cell proliferation, and decrease the expression of P65 nuclear protein. Because the subcellular localization of P65 protein is important for NF-κB activation, thus, we speculated that NF-κB pathway might play a role in the mediation of FLT3 signaling and be involved in the proliferation of FLT3-associated leukemogenesis. Nowadays, AML treatments are mostly unsuccessful, and FLT3 is an attractive molecular target. Based on NF-κB roles in FLT3 signaling pathway, we suggested that FLT3 inhibition in combination with NF-κB inhibition might represent a potent therapeutic option for AML patients.

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