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Correlation between p65 and TNF-α in patients with acute myelocytic leukemia

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Abstract. The correlation between the expression levels of p65 and TNF-α in patients with acute myelocytic leukemia (AML) and AML cell lines were investigated. The bone marrow samples of 30 AML patients and 10 non-leukemia controls were studied. The mRNA expression levels of p65 and TNF-α were detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and Pearson's Correlation test was used to demonstrate the correlation between TNF-α and p65 expression levels in AML specimens. Receiver operating characteristic (ROC) curves were plotted to determine whether TNF-α and p65 expression levels could be used to differentiate AML samples from non-leukemia samples. MG132 and anti-TNF-α antibody were used to inhibit the expression of p65 and TNF-α in the AML cell line, HL-60. The expression of p65 and TNF-α were detected by RT-qPCR and western blot analysis. The mRNA expression levels of p65 and TNF-α were significantly increased in AML patients compared with non-leukemia control bone marrow samples by RT-qPCR, and the two molecules expression pattern's exhibited sufficient predictive power to distinguish AML patients from non-leukemia control samples. Pearson's correlation analysis demonstrated that TNF-α expression was strongly correlated with p65 expression in AML bone marrow samples. In HL-60 cells, inhibition of TNF-α reduced the expression of p65; in addition, inhibition of p65 reduced the expression of TNF-α as assessed by RT-qPCR and western blot analysis. p65 and TNF-α were highly expressed in AML patients, and these 2 molecules were strongly correlated. The present study indicates that p65 and TNF-α have potential as molecular markers to distinguish AML patients from non-leukemia control samples, and that these 2 molecules may be useful prognostic factor for patients with AML.

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Introduction

Acute myelocytic leukemia (AML) is a hematopoietic disease, which results in excessive accumulation of myeloid precursor cells in the bone marrow. It is the most common form of adult leukemia and the survival rate is very low (1-3). Epidemiological studies from the United States National Cancer Institute reported that the overall incidence rate of AML in the USA between 2001 and 2005 was 3.6/100,000 individuals, and the mortality rate was 2.8/100,000 individuals (4). In China, the annual incidence rate of AML was 2.57/100,000 individuals in 2009 (5). In the past 30 years, the treatment of AML has made some progress. Although conventional chemotherapy has improved, the treatment of elderly patients with AML and AML patients with relapsed refractory remains a challenge (6-8). An improved understanding of the molecular events underlying the molecular mechanisms of AML may be helpful to identify therapeutic targets and improve the treatment efficacy to prolong the survival rates for patients, thus this area of research requires further investigation. Nuclear factor-κB (NF-κB) is a family of proteins comprising RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52), which form homo- and heterodimers (9). NF-κB family members are transcription factors that may mediate survival pathways in a number of types of tumor, including leukemia (10). These proteins induce the expression of genes involved in cell proliferation, angiogenesis, metastasis and serve important roles in carcinogenesis and chemoresistance (11). Tumor necrosis factor α (TNF-α) is a central regulator of inflammation. It has been demonstrated to upregulate molecules involved in cell growth, proliferation via NF-κB dependent and independent pathways in tumors (12). In the present study, the correlation between TNF-α and p65 expression levels and their association with AML were investigated.

Materials and methods

Patient samples. Bone marrow samples were obtained from 30 AML patients and 10 control patients, who were enrolled at the Hematology Department of the First Hospital of Lanzhou University (Lanzhou, China) for the present study. Pathological diagnosis was confirmed by two senior pathologists. The healthy individuals exhibited no signs of infection with hepatitis B virus, hepatitis C virus or human immunodeficiency virus. Abdominal ultrasonic, routine blood tests, and

biochemical examination findings were all normal. Written informed consent was obtained from all patients and the present study was approved by the Ethics Committee of the First Hospital of Lanzhou University. The serum samples were collected after obtaining informed consent and the clinical information of the samples are included in Table I (13).

Reagents. RPMI-1640 and fetal bovine serum (FBS) were purchased from GE Healthcare Life Sciences (Logan, UT, USA); Rabbit anti-TNF-α antibody was obtained from Abcam (Cambridge, MA, USA); rabbit anti-p65 primary antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA); and the NF-κB inhibitor (MG-132) was purchased from Beyotime Institute of Biotechnnology (Jiangsu, China).

Cell culture and drug treatment. HL-60 human AML cells (Shanghai Institutes for Biological Science, Chinese Academy of Sciences, Shanghai, China) were grown in RPMI-1640 medium supplemented with 10% FBS, and maintained in humidified 5% CO_2 at 37˚C. For treatment with anti-TNF-α antibody and MG132, cells were seeded at $1.5x10^5$ cells/well in 2 ml RM1640 in a 6-well plate. After 24 h, the medium was changed and the anti-TNF-α antibody (10 ng/ml) and MG132 (3 μM) were added into the medium separately or together. The cells were incubated at 37˚C for 48 h and then used for further experiments.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted by TRIzol method (Takara, Dalian, China), according to the manufacturer's instructions, and reverse transcribed using a SuperScript III First-strand synthesis system (Invitrogen Life Technologies, Carlsbad, CA, USA) to generate cDNA by following the manufacturer's instructions. qPCR was performed using a LightCycler_ 480 SYBR Green I Master (Roche Diagnostics, Welwyn Garden City, UK) according to the manufacturer's instructions. The sequences of the primers used were as follows: β-actin, F 5'-TGGCACCCAGCACAATGAA-3' and R 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'; TNF-α, F 5'-CCATCTATCTGGGAGGGGTCT-3' and R 5'-CGTTTGGGAAGGTTGGATGT-3'; and p65, F 5'-TTCGTCCTCCTCCTCACACTCC-3' and R 5'-CCAGCCTGCTTCTCCAACAACA-3'. After an initial denaturation step of 5 min at 95˚C, 40 cycles of amplification for each primer pair were carried out. Each cycle included a denaturation step; 10 s at 95˚C, an annealing step; 20 s at 60˚C and an elongation step; 10 s at 72˚C. The final elongation temperature was 65˚C for 1 min. Relative levels of gene expression was measured using a LightCycler 480 (Roche Diagnostics) according to the manufacturer's instructions. The relative changes in the expression levels of TNF-α and p65 genes were normalized against the level of β-actin gene expression in each sample. Experiments were performed at least in duplicate for each data point.

Western blot analysis. Total cells were lysated with the buffer (1% SDS, 10 mm tris-Cl, pH 7.6, 20 g/ml aprotinin, 20 g/ml leupeptin and 1 mm AEBSF). The protein concentrations were determined using the Bradford method (14). Protein (20 μg) was separated on 12% of SDS-PAGE gels and transferred to PVDF membranes (Merck Millipore, Darmstadt, Germany). After blocking with 10% non-fat milk, the membranes were incubated with the first antibodies at 4˚C overnight. After washing 3 times with triethanolamine buffered saline solution (Sangon Biotech Co., Ltd., Shanghai, China), the membranes were incubated with goat anti-human IgG horseradish peroxidase-conjugated secondary antibodies (1:200 dilution in 5% non-fat milk) at room temperature for 1 h. The signals were developed with the ECL kit (Applygen Technologies, Inc., Beijing, China) and using anti-β-actin antibody as an internal control.

Statistical analysis. All statistical comparisons were performed using SPSS software, version 16.0 (SPSS, Inc., Chicago, IL, USA). Student's *t*-test was used to compare differences between the 2 groups or association. $P<0.05$ was considered to indicate a statistically significant difference. Pearson's Correlation test was used to show the correlation of TNF-α and p65 expression in AML specimens. Receiver operating characteristic (ROC) curves were plotted to determine the potential of TNF-α and p65 expression to differentiate AML samples from non-leukemic samples (15).

Results

The mRNA expression levels of TNF-α and p65 were increased in AML patient samples. A total of 40 samples were analyzed in the study: The mRNA expression levels of TNF-α and p65 in bone marrow samples from non-leukemia controls (n=10) and patients with AML (n=30) were assessed by RT-qPCR. The results demonstrated that the expression levels of p65 were significantly increased in AML patients compared with non-leukemia control bone marrow samples ($P=0.001$) (Fig. 1A). The upregulation of TNF-α ($P<0.01$) in AML patients was also confirmed (Fig. 1B). The mRNA expression levels of p65 and TNF-α in each AML case are presented in Fig. 1C and D, and demonstrate the variation in mRNA expression levels between the patient samples.

To further evaluate whether the expression levels of TNF-α and p65 could distinguish AML patients from non-leukemia control samples, receiver operating curves (ROC) were plotted. The results demonstrated that the area under the curve (AUC) values of TNF-α and p65 were 0.970 and 0.901 respectively, which demonstrated that the 2 molecules exhibited sufficient power to distinguish AML patients from non-leukemia control samples (Fig. 2).

The expression of TNF-α was correlated with p65 in AML patients. To assess the correlation between TNF-α and p65 expression in AML specimens, Pearson's correlation analysis was used. The results demonstrated that TNF-α expression was strongly correlated with p65 expression in AML bone marrow samples (Fig. 3, $R=0.901$).

Inhibition of TNF-α reduced the expression of p65 in HL-60 cells. HL-60 cells were treated with anti-TNF-α antibody (TNF-α inhibitor), and the mRNA and protein expression levels of TNF-α and p65 were detected by RT-qPCR and western blot analysis, respectively. The results demonstrated that anti-TNF-α antibody reduced the expression of TNF-α; the reduction in TNF-α expression resulted in reduced expression levels of p65 mRNA and protein levels (Figs. 4 and 5).

Table I. Clinical characteristics of bone marrow sample patients.

Characteristic	Patients (n=30)		Control (n=10)	
	n	%	n	%
Age				
<50	13	43.3	4	40
≥50	17	56.7	6	60
Gender				
Male	15	50	5	50
Female	15	50	5	50

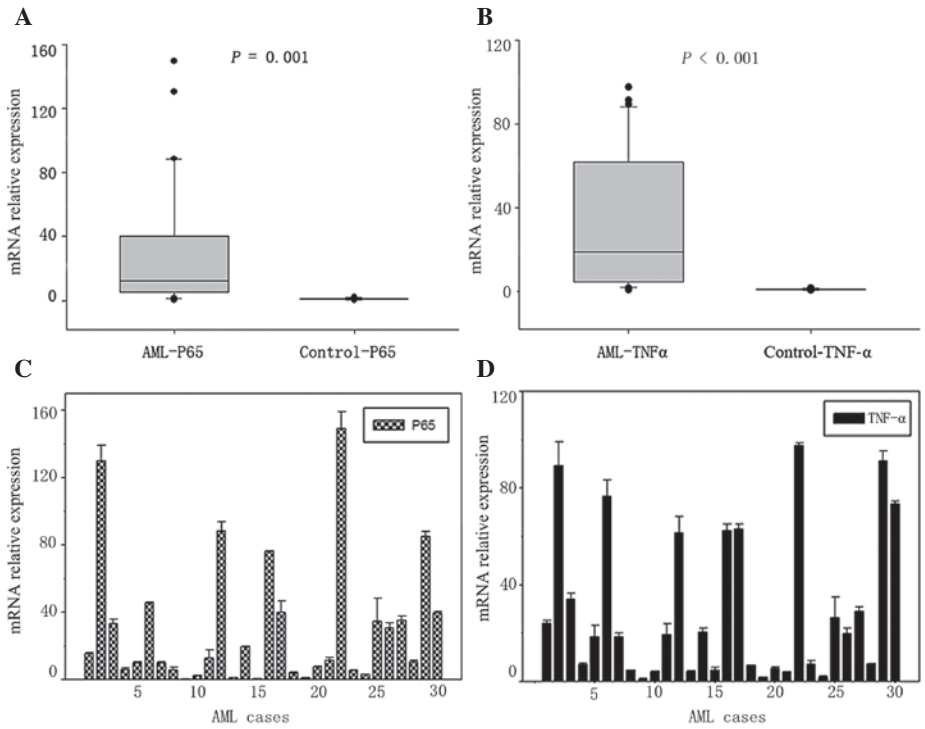

Figure 1. TNF-α and p65 were highly expressed in AML patients. The bone marrow levels of (A) p65 and (B) TNF-α in non-leukemia controls and patients with AML were assessed by reverse transcription-quantitative polymerase chain reaction. The expression levels of (C) p65 and (D) TNF-α in all AML cases.

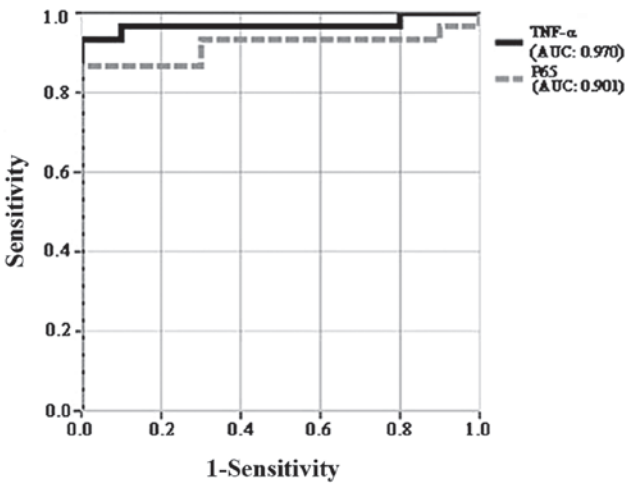

Figure 2. TNF-α and p65 expression levels could distinguish AML patients from non-leukemic control samples. The area under the curve values of TNF-α and p65 were 0.970 and 0.901 respectively.

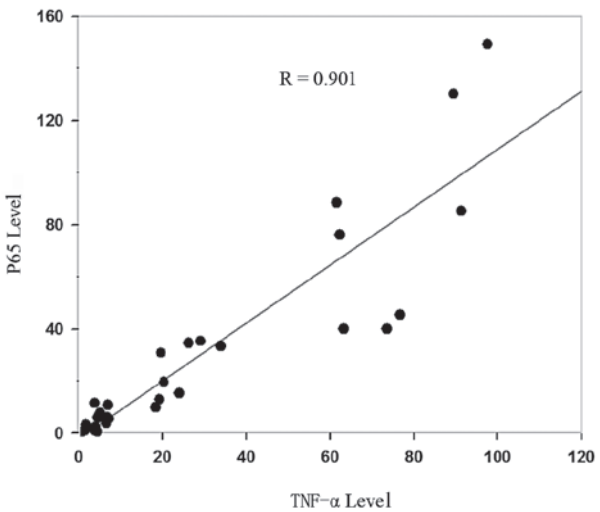

Figure 3. TNF-α was correlated with p65 in AML patients. Pearson's correlation analysis was utilized. R=0.901.

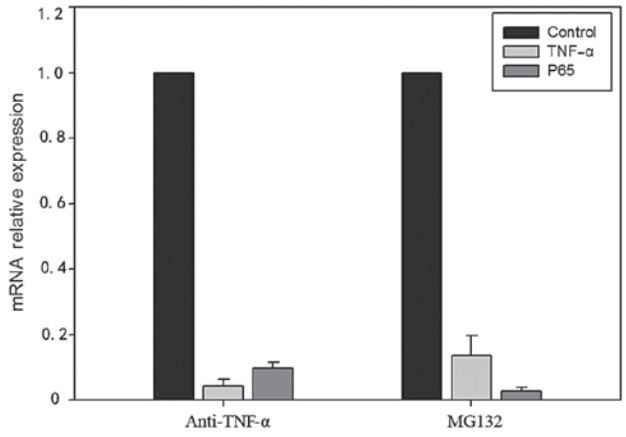

Figure 4. Reverse transcription-quantitative polymerase chain reaction was used to detect the mRNA expression levels of TNF-α and p65 following MG132 and anti-TNF-α antibody treatment.

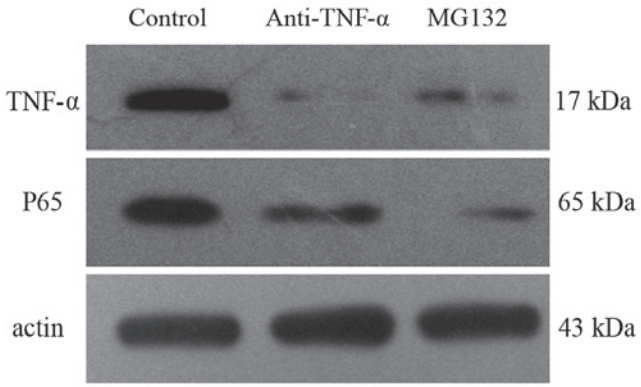

Figure 5. Western blot analysis was used to detect the protein expression of p65 and TNF-α following anti-TNF-α antibody and MG132 treatment.

Inhibition of p65 reduced the expression of TNF-α in HL-60 cells. HL-60 cells were treated with MG132 (an NF-κB inhibitor) (16), and the mRNA and protein expression levels of TNF-α and p65 were detected by RT-qPCR and western blot analysis, respectively. The results demonstrated that treatment with MG132 reduced the expression levels of p65; the reduction in p65 expression resulted in concurrent reduced expression of TNF-α at both the mRNA and protein level (Figs. 4 and 5).

Discussion

Nuclear factor-κB (NF-κB) are a group of transcription factors that induces the expression of genes involved in cell proliferation, angiogenesis and metastasis. It is a family comprising RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52), which form homo- and heterodimers (10). NF-κB mediates survival pathways in a number of types of tumor and serves important roles in carcinogenesis and chemotherapy (11). Abdullah *et al* (17) detected the expression of p65 in colorectal cancer cases and tumor-adjacent normal tissues from the same subjects by immunohistochemical analysis, and demonstrated that p65 was expressed at increased levels in colorectal cancer case. A previous study demonstrated that the activity of NF-κB could be detected in almost all leukemic cells, and its expression was significantly increased in leukemic cells compared with normal bone marrow cells (18).

Tumor necrosis factor α (TNF-α) is a central regulator of inflammation (19). It is also important for the development and progression of a number of types of cancer. A previous study demonstrated that TNF-α activated stromal COX-2 signalling and promoted the proliferative and invasive potential of colon cancer epithelial cells (20). Another previous study demonstrated that TNF-α acting on TNFR1 promotes breast cancer growth via p42/P44 MAPK, JNK, Akt and NF-κB-dependent pathways (21).

The present study detected the expression levels of p65 and TNF-α in bone marrow samples of AML patients and non-leukemic controls by RT-qPCR. The mRNA expression levels of p65 and TNF-α were significantly increased in AML patients compared with that of non-leukemic control bone marrow samples. NF-κB and TNF-α may therefore active survival signaling pathways and serve roles in AML development and progression. ROC curve analysis revealed that these 2 molecules have potential as molecular markers to distinguish AML patients from non-leukemic control samples and thus act as a potential biomarker for AML.

TNF-α has been demonstrated to upregulate molecules involved in cell growth and proliferation via NF-κB dependent or independent pathways in tumors. TNF-α upregulates PTEN expression via NF-κB signaling pathways in human leukemic cells (22). Positive feedback between NF-κB and TNF-α promotes leukemia-initiating cell capacity (23). The present study also analyzed the correlation between TNF-α and p65 expression in AML specimens. Pearson's correlation analysis results demonstrated that TNF-α expression was strongly correlated with p65 expression in AML bone marrow samples. This correlation was also observed in an AML cell line. Inhibition of TNF-α reduced the expression of p65 in HL-60 cells, and inhibition of p65 reduced the expression of TNF-α in HL-60 cells.

In conclusion, the present study demonstrated that p65 and TNF-α were expressed at high levels in AML patients, and these 2 molecules were strongly correlated. p65 and TNF-α have potential as molecular markers to distinguish AML patients from non-leukemic control samples, and these 2 molecules may be useful to predict prognostic factor for patients with AML.

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