Int J Clin Exp Pathol 2016;9(8):7995-8005
www.ijcep.com /ISSN:1936-2625/IJCEP0027571

Original Article

Btk and NFκB as prognostic biomarkers and potential therapeutic targets in B cell acute lymphoblastic leukemia

Shandong Tao*, Chunling Wang*, Yue Chen, Yuan Deng, Litao Zhou, Xin Zhang, Zhengmei He, Liang Yu

*Department of Hematology, Huai'an First People's Hospital, Nanjing Medical University, Huai'an, Jiangsu, P. R. China. *Equal contributors.*

Received March 6, 2016; Accepted June 29, 2016; Epub August 1, 2016; Published August 15, 2016

Abstract: Acute lymphoblastic leukemia (ALL) is a malignant disorder of lymphoid progenitor cells characterized by diverse cytogenetic and molecular abnormalities. Although chemotherapy can cure a major proportion of ALL patients, some may relapse and develop serious complications including death. Bruton tyrosine kinase (Btk), a cytoplasmic protein mainly expressed in hematopoietic cells, and nuclear factor kappa B (NFκB), a transcription factor that can bind to Btk promoters to induce its transcription. Both Btk and NFκB are involved in lymphoma, multiple myeloma, acute myeloid leukemia, breast cancer, non-small cell lung cancer, thyroid cancer and other tumors. Here we report that Btk and NFκB are widely expressed in bone marrow mononuclear cells of ALL patients and leukemia cell lines. We observed that Btk and NFκB expression were upregulated in newly diagnosed patients' samples, reduced to lower levels in complete remission patients' samples, and elevated again upon disease relapse. Furthermore, we found that patients with overexpression of Btk and NFκB have poor prognosis. Our findings show that Btk and NFκB are involved in the pathogenesis, development, progression and prognosis of ALL, and may shed light on targeting therapy for ALL treatment.

Keywords: Bruton tyrosine kinase (Btk), nuclear factor kappa B (NFκB), B cell acute lymphoblastic leukemia (B-ALL), Ibrutinib

Introduction

Acute lymphoblastic leukemia (ALL) is a malignant disorder of lymphoid progenitor cells characterized by diverse cytogenetic and molecular abnormalities. It affects both children and adults, with peak prevalence in children of 2 to 5 years old and adults older than 50 [1]. ALL cases originated from B lymphocyte progenitors represent 85% of childhood and 75% of adult cases, respectively. Risk-adapted chemotherapy can cure more than 80% of childhood cases, but still 20% to 30% of cases relapse, which develop serious complications including death [2, 3], Moreover, the outcomes of adults ALL patients are much poorer than that of children. Advances in the understanding of the pathobiology of ALL, fuelled by novel molecular technologies, suggest that drugs that specifically target the genetic defects of leukemia cells could revolutionize the management of this disease [1]. Deregulated gene expression of several key cellular pathways has been suggested as a useful tool to define prognosis and identify novel therapeutic targets for ALL [4].

Bruton tyrosine kinase (Btk), a member of the Tec family kinases, is a cytoplasmic protein, which expressed mainly in hematopoietic cells, except T cells [5, 6]. Btk can catalyse a variety of biological processes, such as protein phosphorylation of tyrosine residues, and play an important role in B lymphocyte development. Btk is involved in B-cell antigen receptor (BCR) signalling. Gene mutation or loss of function of Btk could block B lymphocyte differentiation and maturation, as well as inhibit the generation of mature B lymphocytes [6]. Overexpression of activated Btk enhances the potential transformation of normal cells to tumor cells [7]. Constitute activation of Btk signalling has been implicated in the pathogenesis of certain

Table 1. Patient characteristics

Patients' characteristics	Number (percentage)
Male	23 (45.10%)
Female	28 (54.90%)
Median age	32 years
White blood cell count	1.82-421.79×10^9/L
Mean ± SD	110.19 ± 98.23×10^9/L
Lymphadenectasis	
Present	15 (29.41%)
Absent	36 (70.59%)
Hepatosplenomegaly	
Present	22 (43.14%)
Absent	29 (56.86%)
Chromosome abnormality	
Present	24 (47.06%)
Absent	27 (52.94%)
BCR-ABL fusion gene	
Present	12 (23.53%)
Absent	39 (76.47%)
High risk	35 (68.63%)
Standard risk	16 (31.37%)
CR rate	38 (74.51%)
1 course CR rate	19 (37.25%)
Relapse	16 (31.37%)

CR: complete remission.

B cell malignancies. Previous studies showed that Btk is also expressed in lymphoma, multiple myeloma and other tumor cells [8, 9].

Nuclear factor kappa B (NFκB) is present in various cells. In unstimulated cells, NFκB is blockaded in the cytoplasm. Following BCR stimulation, Btk, together with other components of the BCR signalosome, activates NFκB. The activated NFκB then translocates into the nucleus and binds to the target gene promoter to induce transcription [10, 11]. NFκB is involved in the regulation of cellular kinases, chemokines, growth factors and cell adhesion molecules, and plays an important role in the regulation of cell proliferation, differentiation, apoptosis and malignant transformation [12]. Dysfunction of NFκB is associated with a variety of malignancies, such as breast cancer, non-small cell lung cancer, thyroid cancer, lymphoma and leukemia [13]. We previously reported that Btk and NFκB expression levels are upregulated in leukemia cells of acute myeloid leukemia [14]. Although Btk and NFκB are associated with a variety of haematological malignancies, the expression levels of these proteins and their significance in ALL are unknown. In this study, we investigated Btk and NFκB expression (at both mRNA and protein levels) in bone marrow mononuclear cells (BMMCs) from B cell ALL (B-ALL) patients. We found that Btk and NFκB are overexpressed in BMMCs from both newly diagnosed (ND) and relapsed cases. The relationship between the expression level of Btk/NFκB and the disease progression was also investigated.

Materials and methods

Patients

Fifty-one adult B-ALL patients, with informed consent, were included in the present study,all of the experiments were performed in accordance with the guidelines of the Human Committee and with ethics approval from the Institutional of Huai'an First People's Hospital, Nanjing Medical University. The patients were diagnosed as ALL in Huai'an First People's Hospital (affiliated to Nanjing Medical University, China) during 2009 to 2014. The diagnosis of B-ALL was based on the morphological, biochemical, genetic and immunological features of the leukemia cells. According to National Comprehensive Cancer Network (NCCN) clinical practice guidelines on ALL, the 51 patients were divided into high risk group (35 patients) and standard risk group (16 patients). Among the 51 patients, 24 cases had chromosome abnormality, of which 12 were Philadelphia Chromosome and BCR-ABL fusion gene positive. Lymphadenectasis occurred in 15 cases, while hepatosplenomegaly occurred in 22 cases. The characteristics of the patients are summarized in **Table 1**.

The patients were treated with DVLP (Daunorubicin, vincristine, L-asparaginase and Prednisone) or DOCP (Daunorubicin, vincristine, cyclophosphamide and Prednisone) regimen [15]. BMMCs were collected from all patients when they were newly diagnosed. Moreover, BMMCs were also collected from 19 cases at ND and CR stage, 7 of which were collected at ND, CR and the relapsed stage as well. The clinical characteristics of these patients are described in **Table 2**. BMMCs obtained from healthy donors were used as control.

Table 2. Clinical characteristics of different disease stages in 19 (patients with ND and CR disease stage) out of 51 B-ALL patients for BM samples

Case	Sex	Age (years)	WBC in PB ($\times 10^9$/L)	Blast cells in BM (%)	Cytogenetic Abnormality	Fusion gene	Hepatospl-enomegaly or Lymphade-nectasis	Prognosis
1	M	36	24.8	94.5	+	BCR-ABL	+	CR-relapse
2	M	24	38.4	85	+	-	+	CR
3	M	18	27.6	90	-	-	-	CR
4	M	22	40.69	87	-	-	+	CR
5	F	16	42.07	76	-	-	-	CR
6	F	23	133.77	93	-	-	+	CR
7	M	41	57.17	74	+	BCR-ABL	-	CR
8	F	52	18.89	98	+	-	+	CR-relapse
9	F	16	1.82	90.5	-	-	+	CR
10	M	55	3.06	91	+	-	+	CR-relapse
11	M	25	19.71	78	-	-	+	CR
12	F	17	421.79	41	+	-	+	CR-relapse
13	M	30	4.62	40	-	-	-	CR-relapse
14	F	46	221	92	+	BCR-ABL	-	CR
15	M	49	38.55	76	+	BCR-ABL	-	CR
16	M	67	35.67	69	+	-	+	CR-relapse
17	F	24	96.4	88	-	BCR-ABL	-	CR-relapse
18	M	16	76.9	87	-	-	+	CR
19	F	62	2.39	79	+	-	-	CR

CR, complete remission; WBC, white blood cell; PB, peripheral blood; BM, bone marrow.

Preparation of BMMCs

Bone marrow (BM) samples were collected in ethylenediaminetetra- acetic acid (EDTA) tubes. BMMCs were isolated by Ficoll density gradient centrifugation then were preserved at -80°C. Mononuclear cells are mainly ALL primary cells at the ND and the relapsed stages.

RT-PCR

Total RNA from the BMMCs was extracted using Trizol reagent and RNA isolation kit (Invitrogen, Paisley, UK), according to the manufacturers' instructions. cDNA was synthesized from total RNA using random primers and superscript II reverse transcriptase. PCR primers for 331-Btk were 5'-TGGCAAGGATGTCTGTGAAG-3' (forward) and 5'-GCAATGTGTTCAGCAGTCTCA-3' (reverse); PCR primers for 489-p65 were 5'-CCTATGTGGAGATCATTGAGCA-3' (forward) and 5'-CAAAGATGGGATGAGAAAGGAC-3' (reverse). Primers were designed according to Btk cDNA and p65 cDNA sequences (NM000061 and NM001145138, respectively) that are available in GenBank. The reaction (25 μl) was composed of a template, primer and a Taq enzyme mixture with two-step amplification (94°C, 30 s, 66°C, 30 s, 72°C, 1 min, 35 cycles). PCR products were detected by agarose gel electrophoresis and analyzed using gel imaging analysis system (BIO-RAD ChemiDoc XRS, USA). The intensity for Btk, NFκB and β-actin on each PCR products band was used to indicate the relative mRNA expression level.

Cell lines

SUP-B15 (*BCR-ABL* positive B-ALL cell line) and RS4; 11 (*BCR-ABL* negative B-ALL cell line) were purchased from ATCC. Cells were cultured in IMDM medium (supplemented with 20% fetal calf serum) in a 5% CO_2 incubator. Cells were harvested and washed twice with PBS, incubated on ice for 30 min in 1× cell lysis buffer, and then sonicated. Following centrifugation at 4°C for 30 min, the supernatants were frozen at -80°C or used immediately.

Western blot

Western blot was performed as previously described [16]. Briefly, total proteins from BMMCs

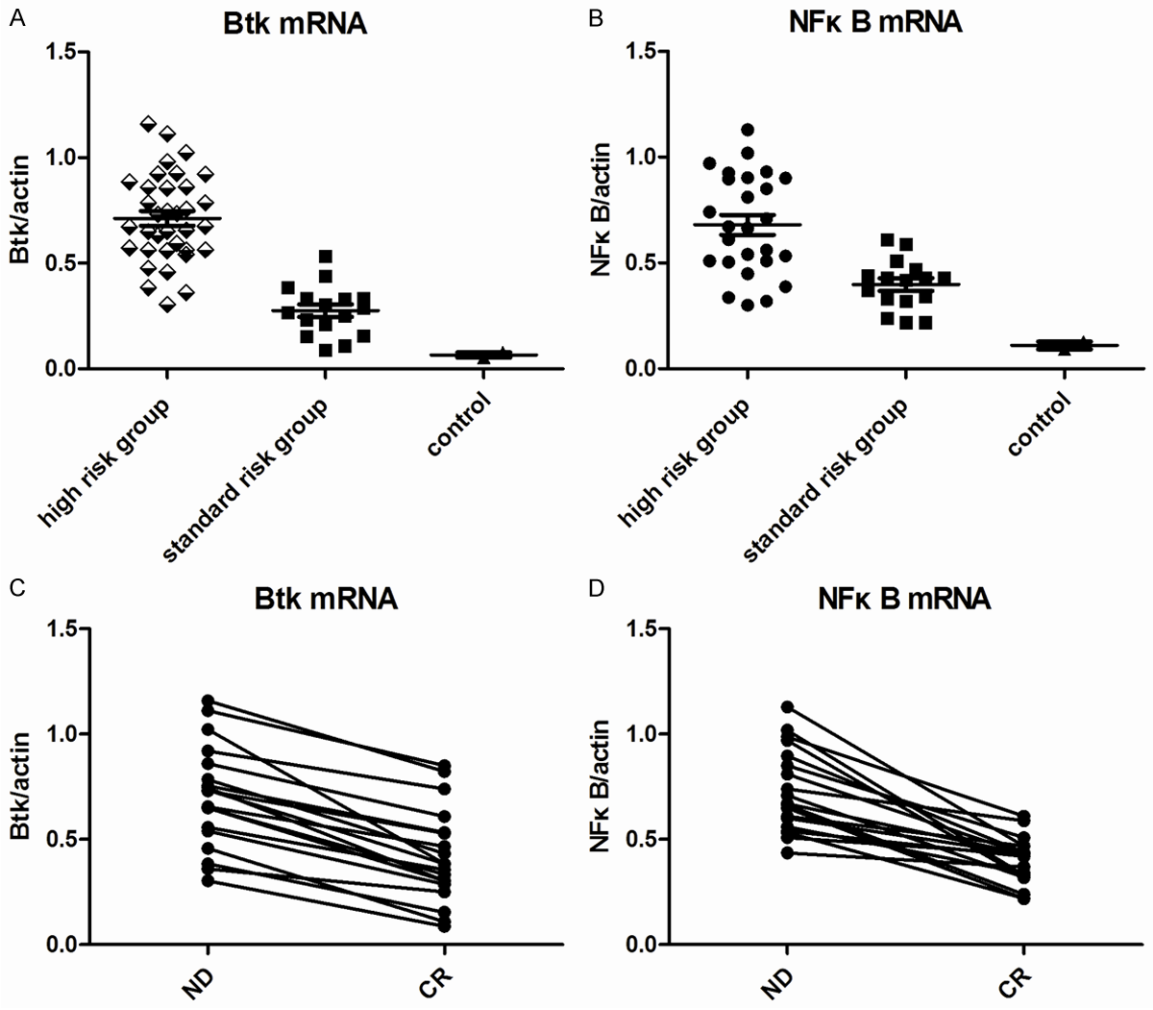

Figure 1. The mRNA expressions of Btk and NFκB in BMMCs from B-ALL patients. The mRNA levels in BMMCs from adult ALL patients (divided into high and standard risk groups) and healthy donors (control) were assessed by RT-PCR and electrophoresis, and normalized to actin. A. Btk mRNA level at ND stage. B. NFκB mRNA level at ND stage. C. Btk mRNA level of 19 paired B-ALL patients at ND and CR stages. D. NFκB mRNA level of 19 paired B-ALL patients at ND and CR stages.

and cell lines were extracted using cell lysis buffer (Life technologies, Foster, CA). Samples containing 20 μg of total proteins were separated in 8%~10% SDS-PAGE gel, and proteins were transferred onto polyvinylidene difluoride (PVDF) membranes in transfer buffer (25 mM Tris, 40 mM glycine, and 20% methanol) using a Mini Trans-Blot Cell (BIO-RAD) at 250 mA for 45 min. Btk was detected with an anti-Btk monoclonal antibody (Cat# SC-81159, Santa Cruz Biotechnology, Santa Cruz, CA) and NFκB-p65 was detected with an anti-p65 polyclonal antibody (Cat# 1546-1, Epitomics, California, USA), while actin was probed with a mouse monoclonal antibody (Cat# AP0060, Bioworld Technology, Inc., Louis Park, USA). The target protein bands were visualized by using the ECL chemiluminescence detection system and quantified by densitometry using Gel-Pro Analyzer 4.5 (Media Cybernetics).

Statistics

Statistical analysis was performed with the SPSS 16.0 software (Applied Biosystems, USA) and Graphpad prism 5 software (GraphPad Software Inc,USA), A P value <0.05 was considered statistically significant. The results of semi-quantitative evaluation were compared between ND and CR stage groups with pared student's t-test and among ND, CR and relapse stage groups were compared with Student-Newman-Keuls Test.

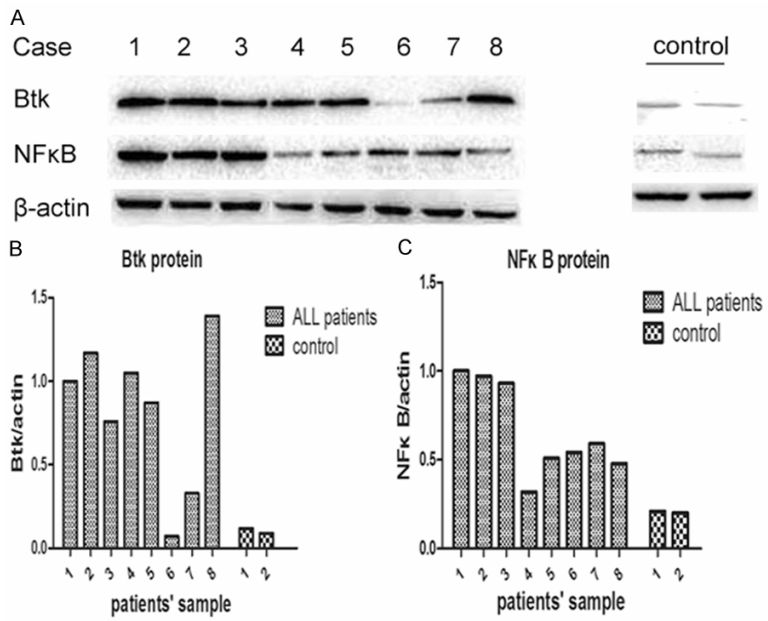

Figure 2. Btk and NFκB protein expression of B-ALL patients.The lysates of BMMCs from adult B-ALL patients and healthy donors were separated by SDS polyacrylamide gel electrophoresis and transferred to a PVDF membrane for Western blot. 8 patients (the representive results are shown here). A. Protein bands probed with anti-Btk, anti-p65 and anti-actin antibodies. β-actin was used as a loading control. B and C. The expression levels of Btk and NFκB were semi-quantified by analyzing with Gel-Pro Analyzer software and normalized to β-actin.

Results

Patient characteristics

A total of 51 B-ALL patients, 23 male and 28 female, were recruited in the current investigation. The age of the patients ranged from 26 to 73 years, with a median age of 32 years. The patients' white blood cell count ranged from 1.82×10^9/L to 421.79×10^9/L ($110.19 \pm 98.23\times10^9$/L) upon diagnosis. The detailed characteristics of the patients are presented in **Table 1**.

Elevated Btk and NFκB-p65 mRNA levels in B-ALL patients

Although Btk and NFκB are involved in a variety of haematological malignancies, their expression levels and function in ALL are not clear. In the present study, the Btk and NFκB mRNA were analysed in 51 newly diagnosed B-ALL patients. As shown in **Figure 1A** and **1B**, Btk and NFκB mRNA levels in BMMCs from the 51 newly diagnosed B-ALL patients were both higher than those in the heathy donors. Notably, Btk and NFκB mRNA levels were significantly higher ($P<0.05$) in the high-risk patients' group (35 cases) than in the standard risk patients' group (16 cases). Among the 51 patients, 19 experienced the ND and the complete remission (CR) stages. The mRNA levels of Btk and NFκB were upregulated at the ND stage but were decreased to lower levels at the CR stage (**Figure 1C** and **1D**). These data indicate that Btk and NFκB mRNAs were ubiquitously expressed in adult B-ALL patients, which is in agreement with previous findings [17]. Our result also implied that Btk and NFκB mRNA levels are associated with different disease stages and correlate with the disease severity.

Btk and NFκB are differentially expressed in different stages of B-ALL at protein level

As both Btk and NFκB were ubiquitously expressed in adult B-ALL patients at the mRNA level, next we explored the effects of Btk and NFκB protein levels on progression and prognosis in B-ALL patients. We quantified Btk and NFκB proteins in BMMCs from all of the 51 newly diagnosed B-ALL cases. Compared with the control group, 38 of the 51 cases had increased expression levels of Btk, while those in the remaining 13 were lower or undetectable (Btk/actin <0.5; **Figure 2**).

We previously found that Btk, together with other components of the BCR signalsome, activates the transcription factor NFκB following BCR stimulation in B cells. NFκB translocates into the nucleus, binds to the Btk promoter and induces Btk transcription, thus forms a positive feedback loop [11]. Additionally, Btk and phospholipase Cγ2 (PLC-γ2) are essential for the activation of NFκB in response to BCR engagement [18-20]. In the present study, 34 out of the 51 cases showed increased expression level of NFκB in BMMCs compared with the control. The NFκB expression levels in the remaining 17 patients were lower or undetect-

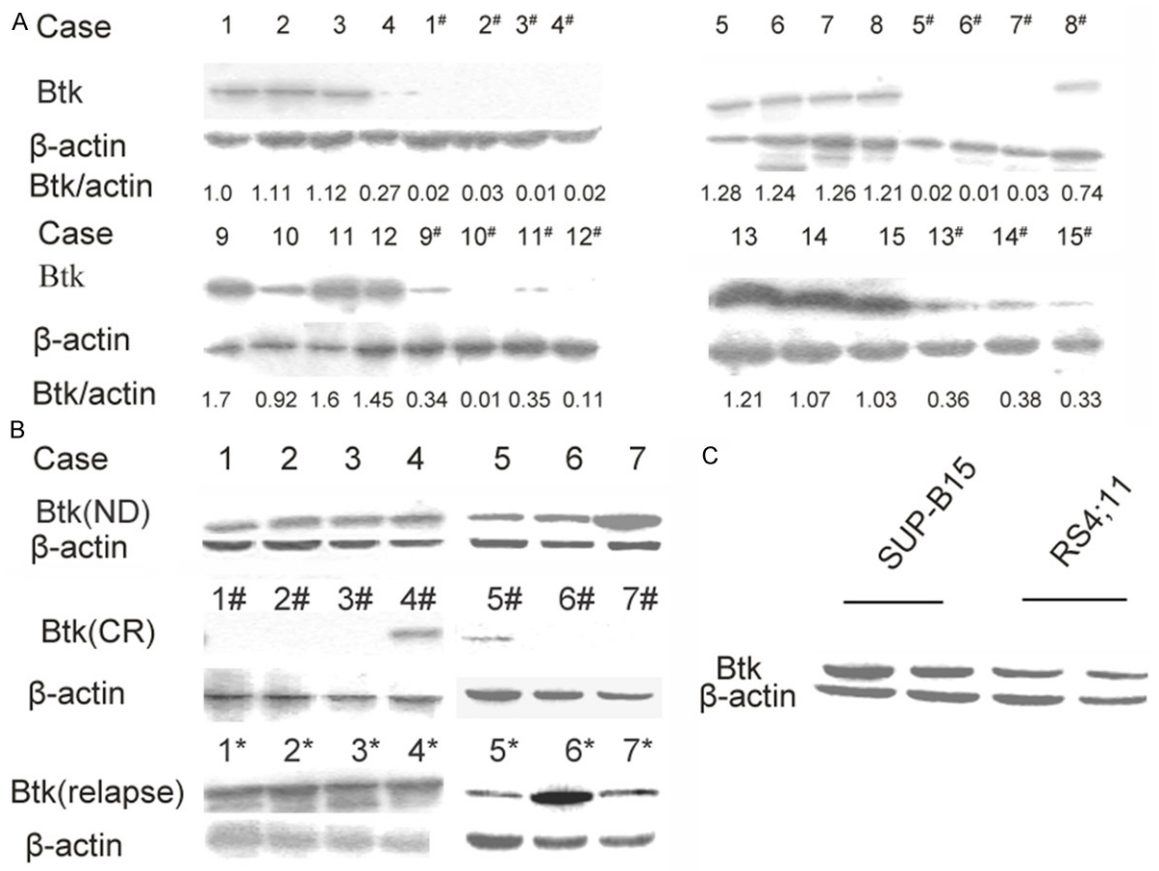

Figure 3. Btk expression in B-ALL at different disease stages. A. Western blot analysis of paired samples from 15 B-ALL patients (n=30) were shown. The Btk index (Btk/actin expression ratio) was normalized to that of sample No. 1 and is shown below each blot. B. Btk expression was detected in 7 relapsed patients. C. Btk expression in SUP-B15, a *BCR-ABL* positive cell line, and RS4; 11, a *BCR-ABL* negative cell line. β-actin was measured as loading control.

able (NFκB/actin <0.5) (**Figure 2**), and most of the samples (32/51) with higher Btk expression also have elevated level of NFκB.

Our data demonstrated that Btk and NFκB were differentially expressed in newly diagnosed B-ALL patients. To investigate their impact on the disease prognosis, 26 paired samples from both ND and CR stages of B-ALL patients were analysed by Western blot, the results showed that Btk and NFκB proteins were present at high levels in ND samples but decreased to very low levels in CR samples (**Figures 3A** and **4A**, **Figures 3B** and **4B**). Clinical features of these 26 paired patients are described in **Table 2**, among these patients, 7 patients eventually relapse, We compared the Btk and NFκB protein level of these 7 patients at ND-, CR- and relapse stages. Btk and NFκB were expressed ubiquitously at high levels in ND samples and were reduced to lower levels upon CR, interestingly, Btk and NFκB expression levels increased again after disease relapse (**Figures 3B** and **4B**). These results suggest that Btk and NFκB might serve as important prognosis biomarkers for B-ALL.

Btk and NFκB expression levels affect the survival and prognosis of B-ALL patients

Given that Btk and NFκB were expressed in the majority of B-ALL patients, and in particular, at various levels among different risk groups, they may have different roles in the development and progression of B-ALL. We observed the CR and relapse rates as well as PFS of 51 B-ALL patients. Among the 38 newly diagnosed B-ALL cases who had higher expression of Btk, 27 experienced CR (71%), and 12 of which achieved CR after one course chemotherapy (one course CR) (31%). Moreover, 16 out of the 27 CR patients relapsed after a short period

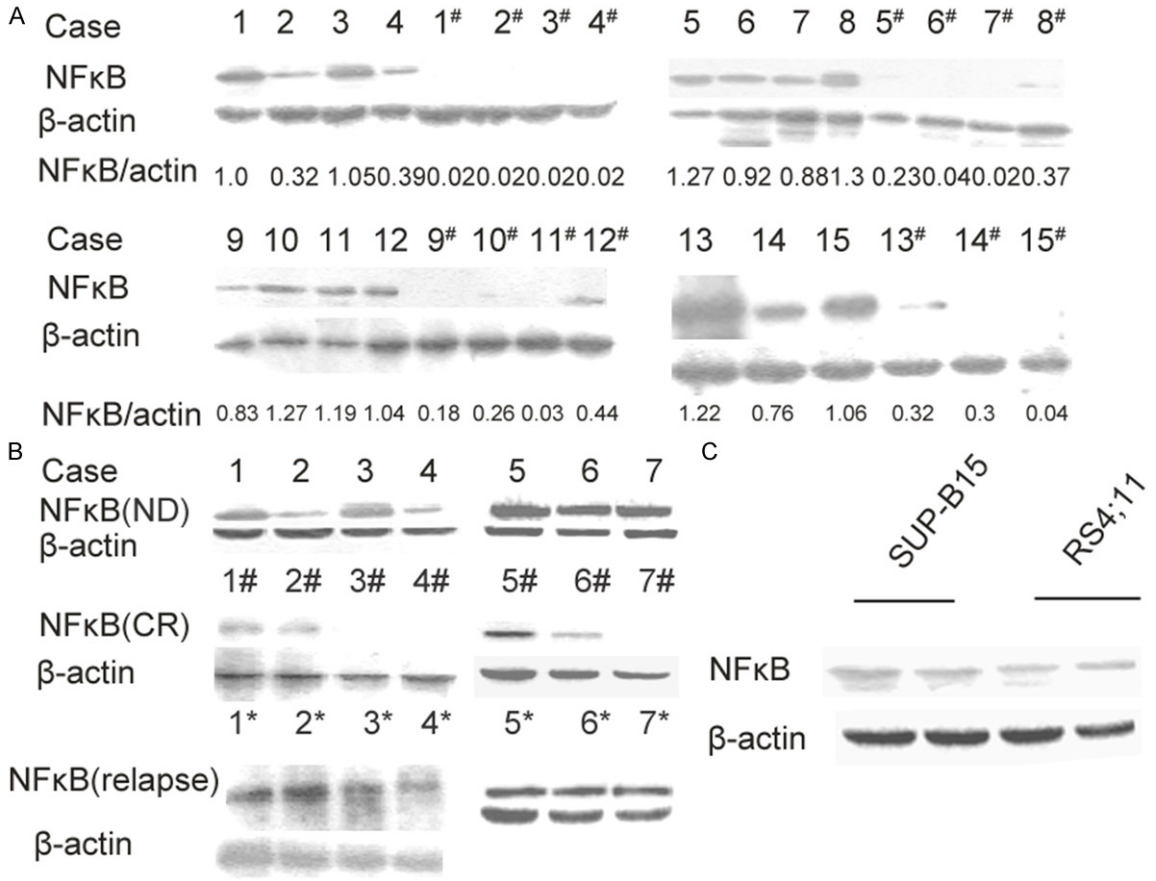

Figure 4. NFκB expression in B-ALL at different stages. A. Western blot analysis of paired samples from 15 adult B-ALL patients (n=30) were shown. NFκB index (NFκB/actin expression ratio) was normalized to that of sample No.1 and is shown below each blot. B. NFκB expression was detected in 7 relapsed patients. C. NFκB expression in SUP-B15, a *BCR-ABL* positive cell line, and RS4; 11, a *BCR-ABL* negative cell line. β-actin was measured as loading control.

(less than 6 months) (59%). On the contrary, among the 13 patients with low Btk expression, 11 achieved one course CR (84.6%) and 1 relapsed (8 months after CR) (7.6%). Higher one course CR rate and lower early relapse rate were observed in patients with lower Btk expression level than the patients with high Btk expression (**Figure 5B**). A similar pattern of NFκB expression was observed. As indicated in **Figure 5**, the group with lower level of Btk and NFκB, their PFS is longer than that of the group with higher level. Taken together, these data demonstrate that Btk and NFκB expression levels could affect the survival and prognosis of B-ALL patients.

Discussion

ALL treatment strategies that adopt risk-adapted chemotherapy cure more than 80% of childhood cases. However, approximately 20% to 30% of patients relapse and develop serious complications including death [2, 3]. In adults, ALL accounts for about 20% of all forms of acute leukemia. In recent years, substantial progress has been made in the treatment of adult ALL. It was reported that about 80% of adult ALL patients under 55-65 years could achieve CR [20]. However, the problems of shorter PFS and high relapse rate remain to be addressed, and traditional chemotherapy is prone to various serious complications. Advances in the understanding of the pathobiology of ALL, fuelled by emerging molecular technologies, suggest that drugs that specifically target the genetic defects of leukemia cells could revolutionize the management of this disease [1]. Deregulated gene expression of several key cellular pathways has been suggested as a useful tool to define prognosis and

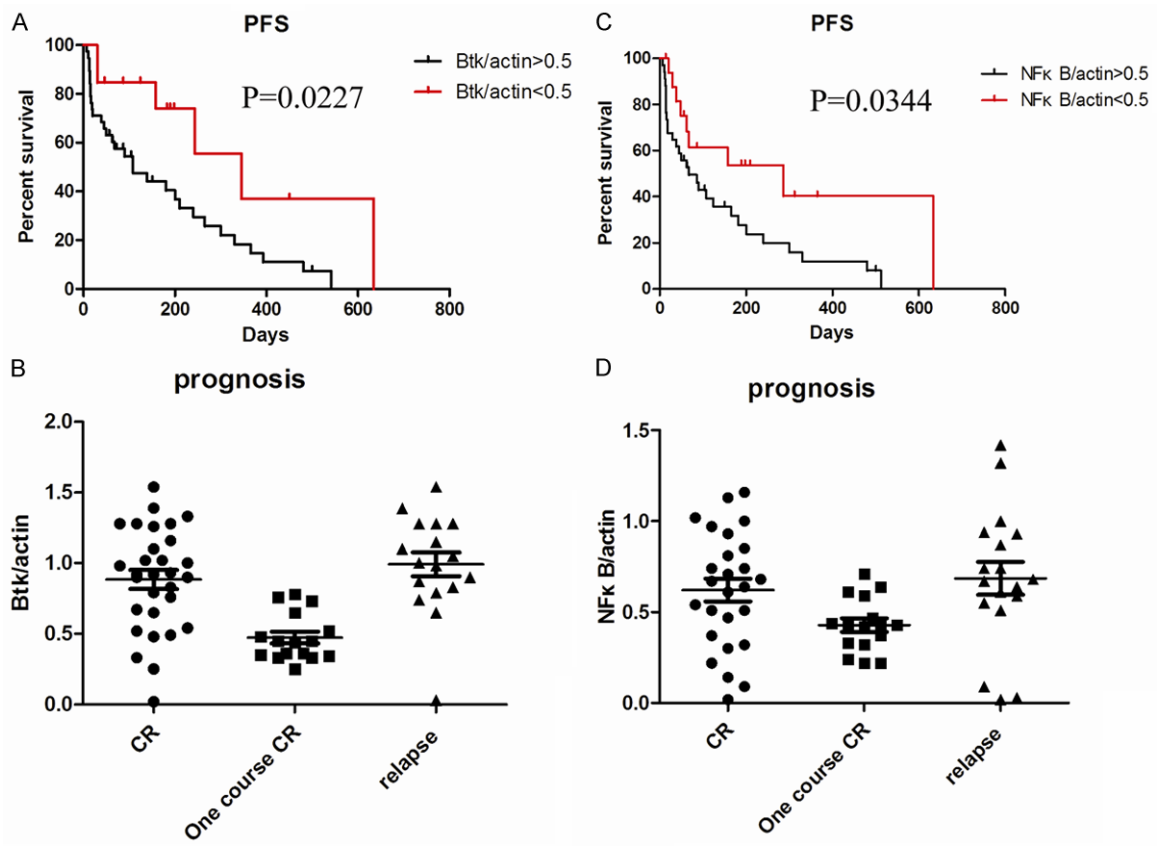

Figure 5. Correlation between Btk/NFκB expression levels and the survival of B-ALL patients. The correlation between the expression levels of Btk/NFκB and prognosis of B-ALL was analysed with the 51 ALL cases. A and C. The PFS of ALL patients with higher or lower expression level of Btk (P=0.0227) and NFκB (P=0.0334), Btk or NFκB / actin >0.5 show higher expression, and Btk or NFκB/actin <0.5 show lower expression. B and D. Btk and NFκB expression levels in B-ALL samples at ND stage was correlated with the prognosis of patients, the relapse rate was elevated in patients with highly level of Btk and NFκB, and the one course CR rate increased in patients with lower level of Btk and NFκB. The overall CR rate of the patients with higher level of Btk and NFκB is similar to that of those with lower level.

identify novel therapeutic targets for ALL [4]. Btk and NFκB are both important molecules in the BCR signalling pathway. Previous studies showed that the abnormal expression levels of Btk and NFκB are associated with many cancers, including a variety of solid tumors and haematological malignancies [17, 18, 21]. Sustained activation and overexpression of Btk and NFκB in these malignancies were also observed [13, 19]. To investigate the biological function and prognosis of Btk and NFκB in ALL, we analyzed their expression levels in bone marrow samples from ALL patients at different disease stages.

In this study, the expression levels of Btk and NFκB in *de novo* adult B-ALL were analysed. Both Btk and NFκB mRNA expression levels were higher in the high-risk group than in the standard risk group ($P<0.05$). Similarly, at the protein level, Btk and NFκB expression was higher in the high-risk group than in the standard risk. These results indicated that Btk and NFκB mRNA were ubiquitously expressed in adult B-ALL, and both proteins might be involved in the development of B-ALL. Btk expression level was higher in patients with chromosome abnormality or BCR-ABL positive (data not shown). Thus, it is plausible to speculate that Btk and BCR-ABL may interact with each other in the BCR signalling pathway. However, the mechanism of Btk and BCR-ABL interaction in leukemia cells of ALL needs further investigation.

In recent years, intensive chemotherapy combined with potent supportive care has improved

the survival of ALL patients, but the overall cure rate has not been significantly increased. To explore the correlation of Btk/NFκB with disease progress, we detected the Btk and NFκB expression in adult B-ALL BM samples at different disease stages. The results show that at both mRNA and protein level, Btk and NFκB were upregulated at the ND stage, and then decreased to lower level or even undetectable at the CR stage. Interestingly, Btk and NFκB expression levels increased again after disease relapse. Thus, Btk and NFκB may serve as promising indicators of B-ALL progression and predict the treatment response.

The dynamic changes of Btk and NFκB expression in different disease stages and risk groups of ALL patients may indicate that Btk and NFκB play significant roles in the development and progression of B-ALL. A follow-up investigation was conducted to determine the relationship between the expression level of Btk/NFκB and CR rate, relapse rate and PFS of ALL. Higher one course CR rate and low relapse rate were observed in patients with lower Btk or NFκB expression group than in the patients with higher expression group, and PFS was prolonged in patients with lower Btk and NFκB expression. Thus, we conclude that Btk and NFκB expression may affect the prognosis and survival of B-ALL patients.

Recent studies have shown that the inhibitory activity of Btk in leukemia cells of B cell origin can enhance the sensitivity to vincristine-induced apoptosis *in vitro* [21]. Given the important role of Btk in B-cell tumors, in recent clinical trials, the Btk and NFκB inhibitors have been used for the treatment of non-Hodgkin's lymphoma, chronic lymphocytic leukemia, multiple myeloma, acute myeloid leukemia and other B-cell proliferative tumors, and achieved promising results [22-24]. Ibrutinib is a selective and irreversible small-molecule inhibitor of Btk with strong-targeted inhibition of B-cell malignancies [25]. Additional research has shown that ibrutinib is cytotoxic to myeloma and potently enhances bortezomib and lenalidomide activities through NFκB [23]. In chronic lymphocytic leukemia, ibrutinib can significantly inhibit DNA synthesis, immune response, tissue chemokine migration and survival of leukemia cells [26]. In the present study, we showed that both in mRNA and protein level, Btk and NFκB were upregulated in ND samples, reduced to normal levels in CR samples following chemotherapy, and increased again upon disease relapse, and also, when B-ALL patients with high expression of Btk and NFκB, they have high relapse rate and poor one course CR rate and PFS. It is well-known that high one course CR rate and low early relapse rate are the most important prognosis factors that affect PFS and OS. So Btk and NFκB may be involved in the pathogenesis, development, progression and prognosis of ALL, and they may serve as newly potential therapeutic target for ALL patients.

In conclusion, we found that Btk and NFκB are widely expressed in BM cells from ALL patients. Btk and NFκB were upregulated in ND samples, reduced to normal levels in CR samples following chemotherapy, and increased again upon disease relapse. Moreover, ALL patients with overexpression of Btk and NFκB had a very poor prognosis and PFS. Therefore, Btk and NFκB may be the potential targets for ALL treatment. The mechanisms of Btk and NFκB inhibitors, either alone or combined, should be explored in clinical trials.

Acknowledgements

This work was funded by Natural Science Foundation of China (grant #81170489), Natural Science Foundation of Jiangsu Province (grant #BK20141254), "333 Projects" Foundation of Jiangsu Province (grant #BRA2015152), "Six Talent Peaks Project" Foundation of Jiangsu Province (grant #2013-WSN-017 and 2015-WSN-099) and Key Laboratory of Hematology Foundation of Huai'an City (grant #HAP201-423).

Disclosure of conflict of interest

None.

Address correspondence to: Liang Yu, Department of Hematology, Huai'an First People's Hospital, Nanjing Medical University, 6 Beijing Road West, Huai'an 223300, Jiangsu, P. R. China. Tel: (86) 517-8087 Ext. 2603; Fax: (86) 517-8087 Ext. 2603; E-mail: yuliangha@163.com

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