Regulatory effect of Act1 on the BAFF pathway in B-cell malignancy

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Abstract. The aim of the present study was to ascertain whether nuclear factor (NF)-κB Activator 1 (Act1) was involved in B cell-activating factor (BAFF) regulation in B-cell malignancy. The human B-cell malignancy cell lines Raji, Daudi and BALL-1 were cultured and the expression of BAFF receptor (BAFF-R) mRNA and protein was analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting, respectively. NF-κB signaling was also assessed using western blotting. Act1 silencing was performed using Act1 small interfering RNA. BAFF-R levels were assessed using flow cytometry. It was demonstrated that BAFF-R was upregulated in all three cell lines and RT-qPCR, and western blotting confirmed these results. Act1 overexpression was demonstrated to induce BAFF-R upregulation, whereas Act1 knockdown resulted in BAFF-R downregulation. Furthermore, the NF-κB pathway was activated by Act1 overexpression and inhibited following Act1 knockdown. The results of the present study demonstrated that Act1 can regulate BAFF via targeting NF-κB signaling, which suggests that Act1 may be a promising therapeutic target for the treatment of B-cell malignancy.

Introduction

B cell-activating factor (BAFF), also known as BLys, THANK, TALL-l, TNFsF13B or zTNF4, belongs to the tumor necrosis factor (TNF) family discovered in 1999. It serves a critical role in the differentiation and proliferation of B lymphocytes. By targeting peripheral B lymphocytes, BAFF promotes the differentiation, proliferation, antigen presentation, immunoglobulin (Ig) class switching and gene recombination of B lymphocytes, as well as enhancing the function of B cells, cluster of differentiation (CD)4$^+$ T lymphocytes and natural killer cells, therefore triggering the immune response (1-4). A total of three BAFF receptors (BAFF-Rs) have been identified, including B-cell maturation antigen (BCMA), transmembrane activator CAML interactor (TACI) and BAFF-R. These receptors are expressed in various cells: BCMA is present in T and B cells; TAC1 in T cells; and BAFF-R is highly expressed in the spleen and lymph nodes. However, BAFF-R exhibits a low expression in the thymus and peripheral blood. It has been reported that these highly specific BAFF-Rs, which are the main receptors mediating the regulatory effect of BAFF on B-cell differentiation, are expressed only in B cells and specifically bind to BAFF (5,6). Although the association between BAFF and hematological diseases has been extensively studied, it remains unknown whether BAFF-R is widely expressed in B-cell malignancy. In recent years, it has been reported that BAFF works mainly via two signaling pathways, namely the phosphoinositide 3 kinase (PI3K)/protein kinase B-mammalian target of rapamycin (Akt-mTOR) and nuclear factor (NF)-κB p65/p50 signaling pathways (7). The NF-κB p65/p50 signaling pathway contains a large number of signaling molecules and a number of positive/feedback signal regulation types are present. NF-κB Activator 1 (Act1), also known as connection to Iκκ-complex and stress-activated protein kinases, is a newly-discovered activator protein of the NF-κB signaling pathway and is involved in the formation of signaling complexes in the NF-κB and SAPK/Jun amino-terminal kinases signaling pathways (8). Act1 negatively regulates the NF-κB signaling pathway. Act1-deficient B cells are more sensitive to cell proliferation induced by CD40 and BAFF, as well as increased phosphorylation of IκB (9). Although the role of BAFF in the pathogenesis and development of hematological diseases has been extensively studied, whether Act1 regulates the expression of BAFF and the consequent activity of NF-κB in B-cell malignancy has yet to be reported. The aim of the present study was to detect the expression of BAFF in three cell lines derived from human B-cell malignancy. The effects of Act1 on the activity

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of BAFF and the NF-κB signaling pathway were investigated by overexpressing or silencing Act1. The results of the present study may provide a novel insight into potential treatments for B-cell malignancy.

Materials and methods

Cell culture. The normal lymphocytes as control was separated from XG's own peripheral blood. Inspection of certain indicators, including blood routine and lymphocyte typing, demonstrated that XG is healthy. All operations were carried out at the Medical Center in The Affiliated Hospital of Zunyi Medical College (Zunyi, China). Human B-cell cancer cell lines (Raji, Daudi and BALL-1) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were recovered from frozen liquid nitrogen at -196˚C and maintained in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). A. total of 100 U/ml of penicillin G sodium, and 100 µg/ml streptomycin sulfate (Gibco; Thermo Fisher Scientific, Inc.). Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2.

CCK-8 analysis for cell viability. Cell Counting Kit-8 (CCK-8) was used to calculate the proliferative ability of A549 cells. Cells ($2x10^3$/well) were seeded into 96-well plates with 100 µl complete RPMI-1640 in triplicate for each treatment and were then sustained in an incubator at 37˚C and 5% CO_2. A total of 10 µl CCK-8 solution was then added to each well and incubated for 4 h. Next, OD value was assessed by water-soluble tetrazolium salt analysis through microplate computer software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) following the instructions of CCK-8 analysis kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The absorbance at 450 nM (OD 450 nm) was read using a microplate reader, and the proliferation curves were plotted.

Analysis of BAFF-R expression using flow cytometry. For flow cytometry, $5x10^5$ cells were collected in the logarithmic growth phase, washed three times with cold PBS and resuspended in PBS. The cell suspension was incubated with the anti-BAFF-R antibody (fluorescein isothiocyanate anti-human CD268; BioLegend, Inc., San Diego, CA, USA) for 15 min in the dark following the manufacturer's protocols. Subsequently, cells were washed three times with cold PBS and analyzed using a flow cytometer BD FACSCalibur Cell Sorting System (version FACS101; BD Biosciences, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Unstained cells were used as a negative control.

RNA isolation and reverse transcription-quantittive polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells using the RNA PCR kit (AMV), version 3.0 (Takara Bio, Inc., Otsu, Japan) following the manufacturer's protocols. The cDNA synthesis reaction mixture (total volume of 30 µl) was prepared using 6.0 µl of 5X Prime Script™ Buffer, 1.5 µl of PrimeScript™ RT Enzyme Mix, 1.5 µl of random hexamers (100 µmol/l), 1.5 µl of oligo(dT) primers (50 µmol/l) and 19.5 µl of total RNA. Reverse transcription was performed at 37˚C for 15 min followed by heating at 85˚C for 5 sec to inactivate the enzyme. For qPCR (Sso Advanced SYBR-Green SuperMix; Bio-Rad Laboratories, Inc.), the 20 µl reaction mixture contained 1 µl of cDNA, 10 µl of SYBR® Premix Ex Taq™, 1 µl of the primer mixture and 8 µl of 1% DEPC water. Amplification was performed using the CFX96™ PCR system (Bio-Rad Laboratories, Inc.) and conditions were as follows: Initial denaturation at 95˚C for 30 sec followed by 40 cycles of 95˚C for 5 sec, 60˚C for 30 sec and 72˚C for 40 sec. The primers for BAFF-R and β-actin were: BAFF-R forward, [22 base pairs (bp)] 5'-AATCTCTGATGCCACAGCTCCT-3', BAFF-R and reverse, (19 bp) 5'-TATTGTTGCTCAGGGCCG G-3'; β-actin forward, (20 bp) 5'-CCACGAAACTACCTT CAACTCC-3' and β-actin reverse, (20 bp) 5'-GTGATCTCC TTCTGCATCCTGT-3'. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (10).

Construction of the pTT5-Act1 expression plasmid. Due to its ability to express foreign proteins, pTT5 (ATCC) was used to generate the overexpressed plasmid coding for the Act1 fragment coding sequence (CDS; 1,609 bp) and cloning was performed between the *Hind*III and *Not*I restriction sites. Total RNA was extracted from Raji cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol and reverse transcribed into cDNA, as aforementioned, with the Reverse Transcriptase Moloney Murine Leukemia Virus (Takara Bio, Inc.). RNA was assessed by electrophoresis on a 0.5% denaturing agarose gel. The specific Act1 primers were: Sense, 5'-CCCAAGCTTTTG ATCTTACTTGGCTTCGTAA-3' and antisense, 5'-AAC GCCGGCGTCACAAGGGAACCACCTGAACGG-3'. A total of 30 µl reaction mixture containing 1 µg of plasmid DNA (pTT5), 1.5 µl of *Hind*III/*Not*I, 3 µl of 10X FD buffer (Takara Bio, Inc.) and ddH_2O. The mixture was incubated at 37˚C for 2 h and heated at 80˚C for 10 min to inactivate the endonuclease enzyme.

The DNA ligase reaction mixture contained 50 ng of linearized vector, 40 ng of the target gene, 2.0 µl of 10X T4 ligase buffer, 0.2 µl of T4 DNA ligase and ddH_2O to give a total volume of 20 µl. The mixture was gently pipetted and incubated at 22˚C for 30 min, heated to 70˚C for 5 min to inactive the enzyme and chilled to 4˚C.

Cell transfection. Three types of B lymphoma cells in logarithmic growth were collected. In a 10-cm dish, $1x10^6$ cells were seeded and cultured for 6 h at 37˚C. The medium was then replaced with serum-free RPMI-1640 to starve the cells overnight. Cells were then maintained in fresh RPMI-1640 medium. Next, 2 µl of polyethylenimine (PEI; 1 mg/ml, pH=7.0, Sigma-Aldrich; Merck KGaA) and 2 µl of plasmid DNA were separately added to 100 µl of the cell suspension and mixed immediately by vortexing or pipetting. The mixture was incubated for 15 min at room temperature, added to the cells and maintained at 37˚C. The supernatant from the transfected cells was harvested 48 h post-transfection. The cell transfection efficiency was evaluated using fluorescence microscopy (Leica DMi8; Leica Microsystems GmbH, Wetzlar, Germany) to observe the green fluorescence protein intensity and flow cytometry to detecte fluorescent signal.

Western blotting. Three types of B lymphoma cells in the logarithmic growth phase were harvested, washed three times with PBS and suspended in radioimmunoprecipitation assay lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 mM deoxycholic acid and 1 mM EDTA) on ice for 30 min, followed by centrifugation at 26,475 x g for 30 min at 4˚C. Proteins in the supernatant were collected, quantified using a MiniDrop 2000 (Merck KGaA) and stored at -70˚C. Protein samples were mixed with loading buffer at a ratio of 1:4 and heated at 80˚C for 3-8 min. Next, 10-20 μl of the protein samples were separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). PVDF membranes were blocked with 5% reconstituted dry milk at 37˚C for 2 h and incubated with the primary antibody [AKT (cat. no. 4691,C67E7)/phosphorylated (P)-AKT (cat. no. 13083, Thr308)/ERK1/2 (cat. no. 4695, 137F5)/P-ERK1/2 (cat. no. 9101, Thr202/Thr204); Cell Signaling Technology, Inc., Danvers, MA, USA; 1:500 to 1:1,500] at 4˚C overnight, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (cat. no. 4410) and goat anti-rabbit IgG secondary antibodies (cat. no. 4414; Cell Signaling Technology, Inc.; 1:10,000) at room temperature for 1 h. Membranes were washed three times with TBS with 0.05% Tween-20 and the results were recorded by chimiDOC Touch Imaging (LI-COR; Bio-Rad Laboratories, Inc.), which is a bioimaging system (Bio-Rad Laboratories, Inc.). Protein expression levels were evaluated using β-actin (cat. no. 13E5) as a loading control (Cell Signaling Technology, Inc.; 1:500 to 1:1,500).

Statistical analysis. All experiments were repeated at least three times. Experimental data was presented as mean ± stand error of mean or in percentages. Comparison between different experimental groups was performed with the two-tailed Student's t-test and multiple group comparisons used one-way analysis of variance followed by Tukey's post hoc test. All analyses were performed using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

BAFF-R is upregulated in B-cell cancer cell lines. Flow cytometry was performed to assess the presence of BAFF-R in three cell lines of B-cell malignancy (Raji, Daudi and BALL-1; Fig. 1A). To confirm the expression of BAFF-R at the transcriptional level, normal B cells (obtained by the BD company's method of immunomagnetic beads separation from healthy people) were selected in the present study as a control (the study was approved by the Medical Ethics Committee of Affiliated Hospital of Zunyi Medical University) BAFF-R expression in the three cell lines of B-cell malignancy was significantly increased compared with the normal B cells ($P<0.05$; Fig. 1B). BAFF-R protein levels were considerably increased in Raji, Daudi and BALL-1 cells compared with the normal B cells (Fig. 1C). This suggests an association between BAFF-R expression and the occurrence of B-cell malignancy.

Table I. Results of the flowcytometry analysis of the transfection efficiency of polyethylenimine.

Cell line	Transfection efficiency (%)
Raji	78.21
Daudi	91.51
BALL-1	88.45

Construction of the pTT5-Act1 expression plasmid. As presented in Fig. 2A, RNA was assessed by electrophoresis on a denaturing agarose gel. DNA fragments from Act1 and pTT5 were purified by gel extraction following digestion by two restriction enzymes (Fig. 2B). Following the ligation reaction, a vector expressing Act1 CDS sequences was identified (Fig. 2C). At 48 h following transfection, the pTT5-Act1 expression plasmid was verified by restriction endonuclease digestion using agarose gel electrophoresis (Fig. 2D).

Transfection efficiency in cell lines. Suspended cells were transfected with the pCEP4-EGFP using 25 kDa PEI with the polymer/DNA ratio (N/P) ratio of 25. The transfection efficiency was characterized by green fluorescence protein intensity via fluorescence microscopy and flow cytometry measurement, respectively. As presented in Fig. 3 and Table I, successful transfection was observed in all three cell lines of B-cell malignancy.

Overexpression and silencing of Act1. To investigate the effect of Act1 in human B-cell malignancy, a eukaryotic expression plasmid was constructed and transfected into B-cell cancer cells to transiently overexpress Act1 and Act1 expression in B-cell malignancy cells was transiently silenced by small interfering (si)RNA transfection. The effects of Act1 expression on the NF-κB pathway were then studied. It was experimentally determined whether Act1 overexpression/silencing was successful. Under normal circumstances, self-designed siRNA sequences are synthesized by entrusted companies and the silencing efficiency is verified in pairs. However, the Act1 siRNA reagent used in the present study was an advanced product ordered from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) with a relatively high silencing efficiency (the sequences of these siRNAs were unknown) and the screening and optimization of the siRNA sequences were omitted.

As presented in Fig. 4, the expression of Act1 in B-cell malignancy cells was determined using western blotting. The results demonstrated that Act1 protein in Raji, Daudi and BALL-1 cell lines was effectively upregulated by transfection with Act1-expressing plasmids and effectively disrupted by Act1 siRNA interference.

Act1 silencing and overexpression in human B-cell lymphoma cell lines. Act1 was overexpressed or silenced in Raji, Daudi and BALL-1 cell lines to further investigate the regulatory role of Act1 in the growth of B-cell malignancies, and cell proliferation following the overexpression and silencing of Act1 was determined using the CCK-8 cell proliferation

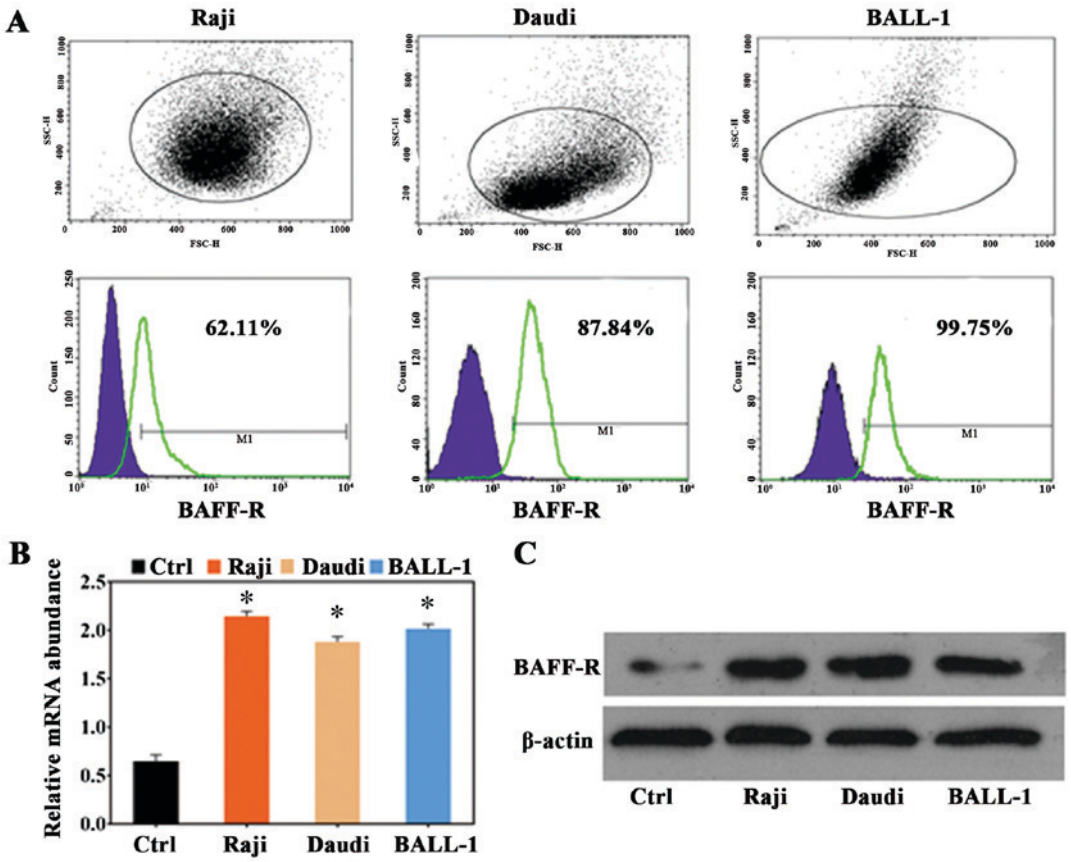

Figure 1. BAFF-R is upregulated in Raji, Daudi and BALL-1 cell lines. (A) BAFF-R upregulation was detected by flow cytometry. (B) Reverse transcription-quantitative polymerase chain reaction and (C) western blotting were performed to measure the expression of BAFF-R mRNA and protein, respectively. *P<0.05 compared with the control. The data was representative of at least three independent experiments. Normal B cells were used as a negative control. BAFF, B cell-activating factor; BAFF-R, BAFF receptor; Ctrl, control.

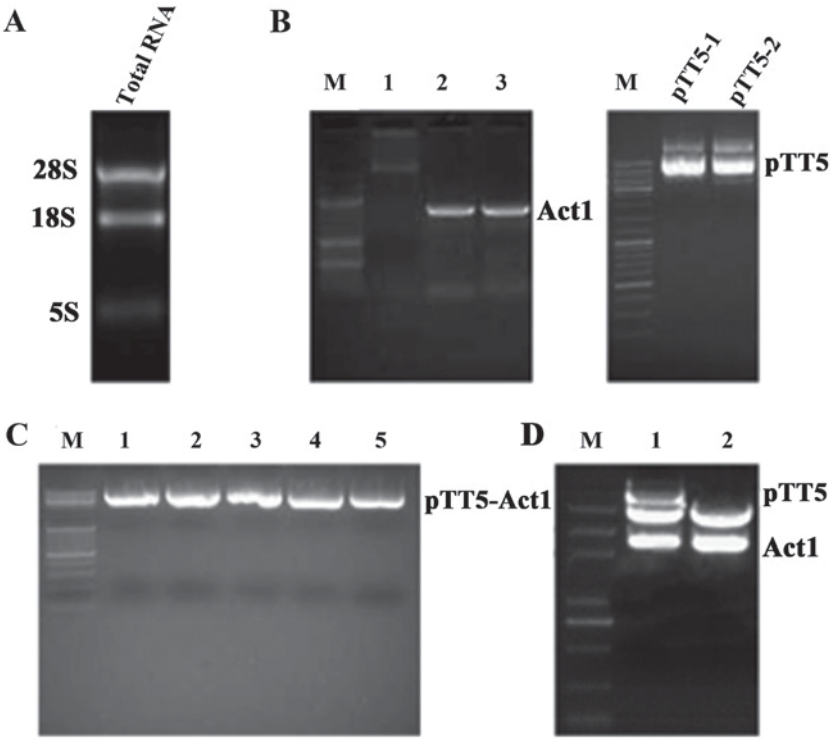

Figure 2. pTT5-Act1 expression vector construction. (A) Total RNA extraction from Raji cells. (B) A gel extraction kit was used to purify cDNA fragments of Act1 and pTT5 following dual restriction enzyme digestion (M, Marker; 1, 2 and 3, polymerase chain reaction products of Act1). (C) Construction of the pTT5-Act1 expression vector (M, Marker; 1, 2, 3 and 5, recombinant plasmid of pTT5-Act1). (D) A positive clone of pTT5-Act1 was verified using dual digestion (M, Marker; 1 and 2, sequences of pTT5 and Act1). Act1, nuclear factor-κB Activator 1.

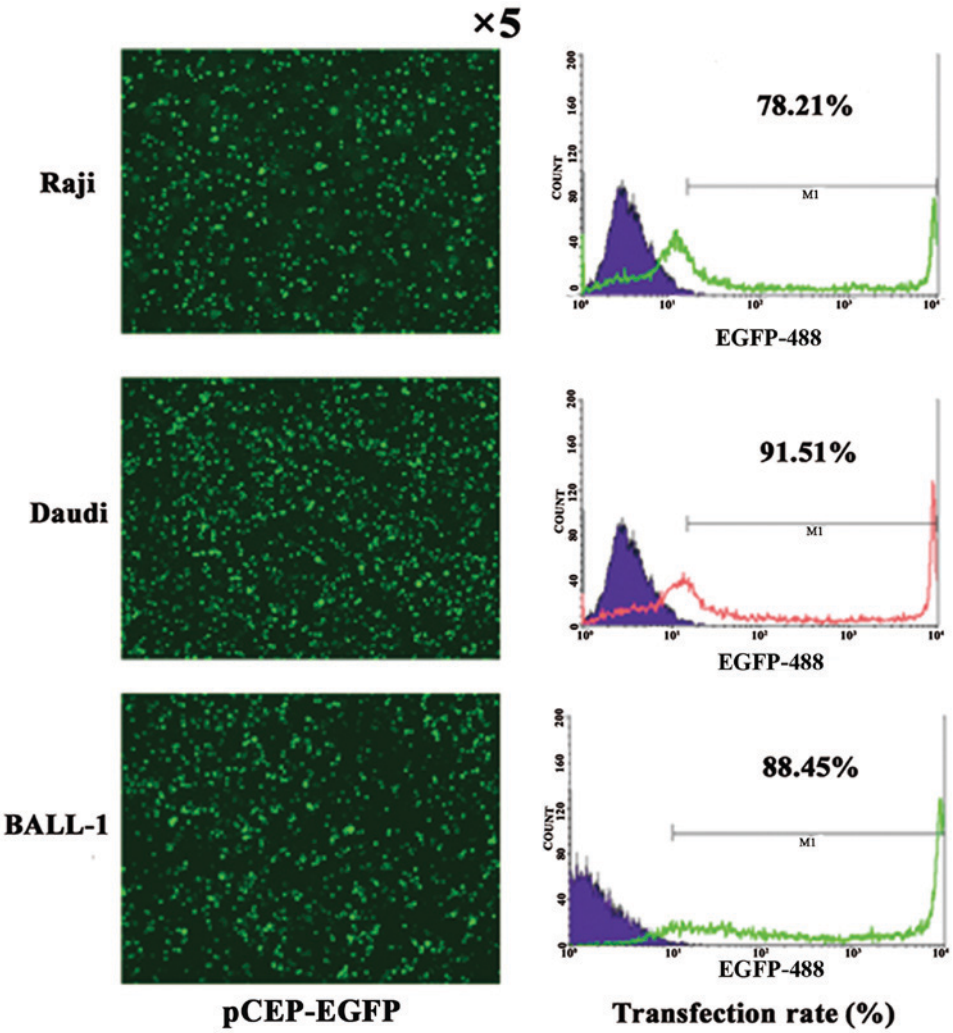

Figure 3. Transfection methodology used for B-cell cancer cell lines. Fluorescence microscopy and flow cytometry were used to measure the transfection efficiency.

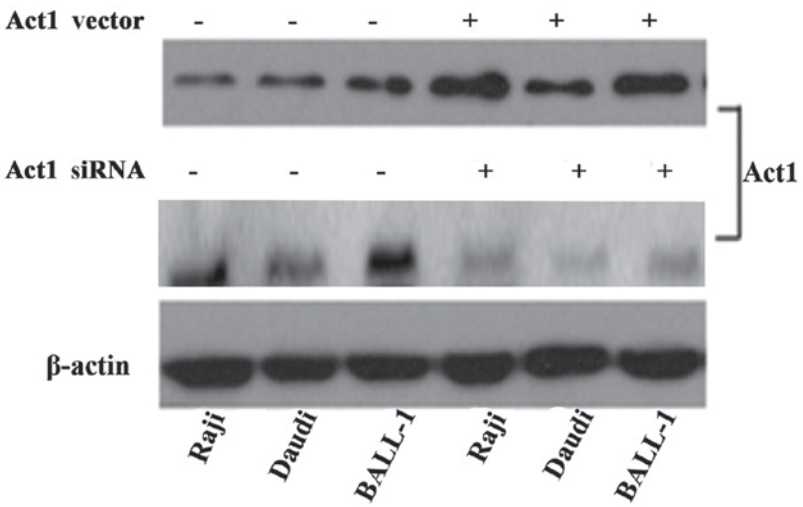

Figure 4. Alterations in Act1 expression following transfection with Act1 expression vector or siRNA in Raji, Daudi and BALL-1 cells were identified using western blotting. Act1, nuclear factor-κB Activator 1; siRNA, small interfering RNA.

assay. The results demonstrated that the proliferation of B-cell malignancy cells was increased following Act1 silencing and inhibited following Act1 overexpression (Fig. 5A). The expression of BAFF-R protein and relevant signaling proteins in the NEMO-independent NF-κB signaling pathway were detected using western blotting. The results revealed that

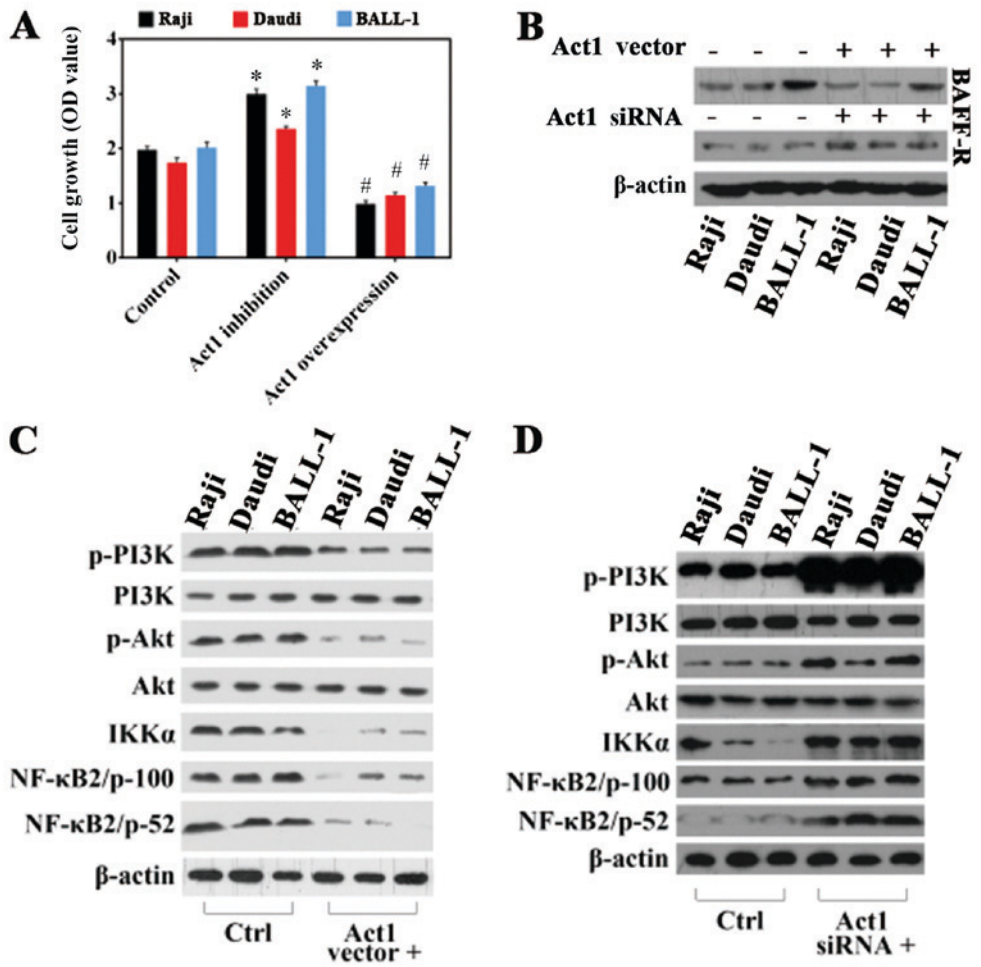

Figure 5. Alterations in NF-κB expression were detected by western blotting following overexpression or knockdown of Act1 in the three B-cell cancer cell lines. Act1 was demonstrated to regulate the (A) proliferation and (B) BAFF-R levels of B-cell malignancy cell lines. NF-κB expression was (C) activated following Act1 knockdown and (D) inhibited following Act1 overexpression. The data was representative of at least three independent experiments. β-actin was used as the internal control. *P<0.05 compared with the control and #P<0.05 compared with the control. NF, nuclear factor; Act1, NF-κB Activator 1; BAFF-R, B cell-activating factor receptor; p, phosphorylated; PI3K, phosphoionsitol 3 kinase; Akt, protein kinase B; IKKα, inhibitor of NF-κB; si, small interfering.

Act1 was able to regulate the expression of BAFF-R in all three cell lines (Fig. 5B). In addition, the expression of NEMO-independent NF-κB pathway-associated proteins, including PI3K, Akt, IKKα, NF-κB2/p-100 and NF-κB2/p-52, was downregulated following Act1 overexpression (Fig. 5C) and upregulated following Act1 silencing (Fig. 5D). These results suggest that Act1 regulates the proliferation of Raji, Daudi and BALL-1 cell lines by targeting the BAFF and the NF-κB signaling pathways in these cells.

Discussion

NF-κB Activator 1 (Act1) is a recently identified activator protein in the NF-κB signaling pathway that comprises multiple domains with 574 amino acids. Act1 is widely expressed in various tissues and organs, including the thymus, heart, lungs, liver, kidney, colon and placenta, and serves an important role in regulating the immune response of T and B cells (10). Previous studies have demonstrated that Act1 is an important factor in the immune-associated interleukin (IL)-17 signaling pathway, interacting with TNF receptor associated factor 4 (TRAF) molecules (11,12). Act1 also serves a negative regulatory role in the CD-40L/BAFF-R-mediated activation of B lymphocytes (13). As such, Act1 regulates the signaling pathways and cell proliferation of cells in two distinct ways.

The proliferation and metastasis of B-cell cancer cells are regulated by the BAFF signaling pathway. BAFF exists in membranous and soluble forms *in vivo*. Membranous BAFF is expressed in myeloid-derived cells, including dendritic cells, T cells and macrophages and is stimulated and regulated by interferon-γ and IL-10. The peptide bond between amino acids 133-134 in the extracellular domain of membranous BAFF can be hydrolyzed by proteases to give soluble BAFF. The extracellular domain of BAFF is a conserved domain containing ~150 amino acids, which forms a β-sheet trimer and binds to BAFF-Rs on the surface of cells (14). A total of three BAFF-Rs have been identified, namely the BCMA, the TACI and the recently identified BAFF-R. These receptors are expressed in various cells, i.e., BCMA is present in T cells and B cells; TAC1 is in T cells; and BAFF-R is highly expressed in spleen and lymph nodes but with a low expression in the thymus and peripheral blood. Studies (15-17) have demonstated that these highly specific BAFF-Rs specifically bind to BAFF, but not to other members of the TNF family and are the principal receptors mediating the regulatory effect of BAFF on B cell differentiation (18). Abundant evidence has demonstrated

that BAFF serves an important role in regulating the growth and differentiation of B cells. Schneider *et al* (19) found that BAFF stimulated the growth of B cells. Schiemann *et al* (20) also demonstrated that BAFF serves a key role in the growth of B cells via a BCMA-independent signaling pathway. Claudio *et al* (21) demonstrated that BAFF regulates B cell maturation via the NEMO-independent NF-κB2 pathway. In the present study, associated studies (22-24) demonstrated that the expression of BAFF-R in the B malignancy cells can be detected and BAFF can promote cell proliferation, however how Act1 affects the expression of BAFF and regulates of the growth of B-cell malignancy has not yet been reported. Therefore, three B malignancy cell lines were selected for the functional study, including Raji, Daudi (derived from Burkitt's lymphoma) and BALL-1 (derived from acute lymphoblastic leukemia), they are standard cell lines for B-cell malignancy research and have been widely used in previous studies (25-30). The purpose of the present study was to investigate the association between Act1 and B-cell malignancy by overexpressing and silencing Act1. The results revealed that the proliferation of these cell lines was increased by Act1 silencing and inhibited by the overexpression of Act1, suggesting that Act1 may serve a negative role in regulating the proliferation of B-cell cancer. Furthermore, the expression of BAFF-R and associated signaling proteins in the NEMO-independent NF-κB signaling pathway in these cell lines was detected using western blotting, and the results revealed that Act1 negatively regulated BAFF-R expression. Western blotting also demonstrated that NEMO-independent NF-κB signaling pathway associated proteins, including PI3K, Akt, IKKα, NF-κb2/p-100 and NF-κb2/p-52, were downregulated following Act1 overexpression and were upregulated following Act1 silencing. These results suggest that Act1 controlled the proliferation of the Raji, Daudi and BALL-1 cell lines by regulating the BAFF and NF-κB signaling pathways. In conclusion, the results of the present study confirmed the high expression of BAFF-R in three human cell lines of B-cell cancer, Raji, Daudi and BALL-1, suggesting that the BAFF pathway is associated with the proliferation of B-cell malignancy cells. Act1 overexpression led to BAFF-R downregulation, while Act1 silencing resulted in BAFF-R upregulation. Furthermore, Act1 negatively regulated the activity of the NF-κB signaling pathway in B-cell malignancy cell lines. The results of the present study suggest that Act1 serves a negative regulatory role in B-cell malignancy cells, suggesting that Act1 may serve as a possible therapeutic target with potential value for future development.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

XJG and YLW contributed to the study design and major laboratory work. YPW and ZXF contributed to the data analysis and data interpretation. LL, MYL and JYJ contributed to the total RNA extraction, protein extraction, cell culture and RNA interference. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of Affiliated Hospital of Zunyi Medical University (the reference number is 56). The normal lymphocytes used came from XG's own peripheral blood. Inspection of certain indicators, including blood routine and lymphocyte typing, demonstrated that XG is healthy. XG volunteered for the research and signed a consent form. All operations were carried out at the medical center in the affiliated hospital of Zunyi Medical College (Zunyi, China).

Patient consent for publication

Written informed consent for publication was obtained.

Competing interests

The authors declare that they have no competing interests.

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