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RNA interference-mediated knockdown of brain-derived neurotrophic factor (BDNF) promotes cell cycle arrest and apoptosis in B-cell lymphoma cells

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Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin superfamily that has been reported to be involved in a number of neurological and psychological situations. Recently, high expression level of BDNF is observed in diverse human malignancies, delineating a role of BDNF in tumorigenesis. Nevertheless, its effect on B-cell lymphoma remains unclear. In this study, RNA interference technology mediated by short hairpin RNA (shRNA) was performed to inhibit endogenous BDNF expression in B-cell lymphoma cells. Results showed that knockdown of BDNF reduced cell growth and proliferation of Raji and Ramos cells. Furthermore, down-regulation of BDNF induced a cell cycle arrest at G0/G1 phase in Raji cells, and consequently led to cell apoptosis *in vitro*. Meanwhile, down-regulation of Bcl-2 and up-regulation of Bax, activated caspase-3 and caspase-9 and cleaved poly (ADP-ribose) polymerase (PARP) were observed in Raji cells when endogenous BDNF was inhibited. Besides, we also found that suppression of BDNF in Raji cells increased their sensitivity to chemotherapeutic drug, 5-Fluorouracil (5-FU). Our research provides a promising therapeutic strategy for human B-cell lymphoma by targeting BDNF.

Key words: B-cell lymphoma, BDNF, RNA interference, cell proliferation, cell apoptosis

The lymphomas encompass an array of heterogeneous malignancies that originate in lymphocytes, and their incidence continues to rise [1-2]. Regarding about 95% of the lymphomas are of B-cell origin [3], we highlight the B-cell lymphoma in the present study. Earlier researches in understanding molecular pathogenesis of lymphoid malignancies have identified that lymphomas are associated with the deregulation of apoptosis and survival pathways, the alteration of the cell cycle, and the deregulation of the cell signaling or transcriptional regulation [4-5]. Though progresses have been made in studies of B-cell lymphoma using antitumor drugs [6], the effective treatments results however remain limited.

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin superfamily, was first identified as a survival factor for sympathetic and sensory neurons, and has since been shown to control a number of aspects of survival, development and function of neurons in nervous system [7-10]. BDNF has been shown to activate cellular biological effects mainly through a cell surface tyrosine kinase receptor, tropomyosin-related kinase B (TrkB) [11-12]. Recently, increasing evidence has proven that BDNF possesses also a range of functions outside the nervous system. The high expression level of BDNF is of great concern in cancer research. BDNF is found to be elevated in diverse tumors, including in hepatocellular carcimona [13], melanoma [14], and colorectal cancer [15], but not in nontumorous tissues or normal cell lines. Such previous researches suggest that BDNF may be involved in the development and progression of these malignancies. Moreover, as reported by Yang et al., BDNF aberrantly expresses in breast cancer, and inhibition of its expression by ribozymes induces a reduction of cell apoptosis [16]. Meanwhile, in xenografted rodents, anti-BDNF treatment results in myeloma growth inhibition, characterized by an increase in cell apoptosis [17]. These two prior studies indicate that BDNF may participate in tumorigenesis, probably through mediation of tumor cell proliferation and apoptosis. Nevertheless, its exact effects and

regulatory mechanisms in B-cell lymphomas remain to be fully elucidated.

The present study was performed to explore the role of BDNF in human B-cell lymphoma. Technology of short hairpin RNA (shRNA)-mediated RNA interference was conducted to inhibit the endogenous expression of BDNF in human B-cell lymphoma cells. Results showed that knockdown of BDNF by shRNA led to reduced cell proliferation and increased apoptosis in B-cell lymphoma *in vitro*. Our data suggest that BDNF may serve as a potential target for human B-cell lymphoma therapy.

Materials and methods

Cell lines and culture. Human B-cell lymphoma cell lines (Daudi, Raji, Ramos and Namalwa) were cultured in complete RPMI 1640 medium containing 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRC, Paisley, UK) at 37 ˚C and 5% CO_2. Cells in the logarithmic phase of growth were used for the experiments.

Plasmid construction and transfection. The pRNA-H1.1 vector (GeneChem, Shanghai, China) was used to generate short hairpin RNA (shRNA) targeting BDNF (Genbank Accession: NM_170735.5). Two pairs of oligonucleotides encoding shRNAs for BDNF were designed and chemically synthesized as follows: BDNF-1 (forward, 5' GATCCCCggatagacacttcttgtgtTTCAAGAGAacacaagaagtgtctatccTTTTT 3'; reverse, 5' AGCTAAAAAggatagacacttcttgtgtTCTCTTGAAacacaagaagtgtctatccGGG 3'), BDNF-2 (forward, 5' GATCCCCggccaactgaagcaatactTTCAAGAGAagtattgcttcagttggccTTTTT 3'; reverse, 5' AGCTAAAAAggccaactgaagcaatactTCTCTTGAAagtattgcttcagttggccGGG 3'). The sequences in lowercase indicate two insert sequences targeting BDNF mRNA (1780-1798 bp and 1608-1626 bp). These oligonucleotides were then annealed and subcloned into the HindIII and BamHI sites of the pRNA-H1.1 vector (pRNA-H1.1-BDNF-1 and pRNA-H1.1-BDNF-2). Meanwhile, shRNA targeting scrambled sequence was designed and served as negative control (pRNA-H1.1-control). The accuracy of the recombinant vectors was confirmed by restriction enzyme analysis and sequencing.

For plasmid transfection, cells were plated in 6-well plates (3×10^5 per well) 30 min prior to transfection. Four point five microlitre attractene transfection reagent (QIAGEN, Shanghai, China) plus 2 μg of pRNA-H1.1-BDNF1/BDNF2 or non-silencing control plasmid was incubated in 100 μl RPMI-1640 (serum free) for 15 min, and then was used to transiently transfect cells. Additionally, cells without plasmid transfection served as the untreated control.

Cell proliferation assay. Cell viability was evaluated by colorimetric assay using Bromodeoxyuridine (BrdU) Cell Proliferation Enzyme-Linked Immunosorbent Assay (ELISA) Kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. Normal and shRNA treated cells were dispensed at a density of 2×10^5 cells/well in 96-well plates with RPMI1640 containing 10 % FBS. After various intervals of incubation, 200 μl BrdU was added into the medium and incubated for 4 h at 37 ˚C. Cells were fixed and denatured by incubation with 200 μl fixing solution for 30 min at room temperature (RT), and then the fixing solution was gently removed. Next, the detector anti-BrdU monoclonal antibody was pipetted into the wells and allowed to incubate for 60 min. Thereafter, horseradish peroxidase-conjugated goat anti-mouse antibody was used to incubate cells for 30 min at RT, and 100 μl chromogenic substrate tetramethylbenzidine (TMB) was subsequently added into the wells. Thirty minutes later, the absorbance was read at 450 nm. Data represented as percent reduction in metabolic activity, normalized to control shRNA.

Cell cycle and apoptosis analysis by flow cytometry. For cell cycle analysis, cells were collected at 48 h after transfection and fixed with ice-cold 70 % ethanol overnight. Then, the cells were washed twice with PBS and stained with 50 μg/ml propidium iodide (PI) in the presence of RNase A at 37 ˚C for 30 min. Intracellular DNA content was analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

For apoptosis analysis, apoptotic cells were quantified by flow cytometry using an Annexin V-FITC/PI Apoptosis Detection Kit (KeyGen Biotech, Nanjing, China) according to the manufacturer's instructions at 72 h after transfection. In brief, the normal or shRNA transfected Raji cells were collected and washed with PBS, and incubated with 500 μl Annexin V-binding buffer. Then, the samples were stained with 5 μl of fluorescein isothiocyanate (FITC)-labeled Annexin V and 5 μl of PI at room temperature for 15 min in the dark before analysis by flow cytometry. In addition, 5-Fluorouracil (5-FU) (Sigma-Aldrich) was added directly into the medium to a final concentration of 100 μg/ml 24 h before apoptosis assessment.

Quantitative real-time PCR . Total RNAs from cells were extracted using RNA simple total RNA kit (TianGen, Beijing, China), and cDNAs were synthesized using Super M-MLV reverse transcriptase kit (BioTeke, Beijing, China). SYBR Green (Solarbio, Beijing, China) was used for real-time PCR on ExicyclerTM 96 (Bioneer, Daejeon, Korea), and the mRNA expression levels of BDNF were quantified via $2^{-\triangle\triangle CT}$ [18]. Analytical data were normalized to the mRNA expression level of endogenous control β-actin (Genbank Accession: NM_001101.3). The primers used in quantitative real-time PCR were BDNF (forward 5' TAGAGCCCTGTATCAACCCA 3' and reverse 5' GGTAAATGCAATGCCAACTC 3'), and β-actin (forward 5' CTTAGTTGCGTTACACCCTTTCTTG 3'and reverse 5' CTGTCACCTTCACCGTTCCAGTTT 3').

Western blot. Protein samples from cells were subjected to Western blot analysis using polyclonal antibodies for BDNF (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cyclin D1 (Santa Cruz Biotechnology), cyclin E (Santa Cruz Biotechnology), Bax (Boster, Wuhan, China), Bcl-2 (Boster), cleaved caspase-3 (Bioss, Beijing, China), cleaved caspase-9 (Santa Cruz Biotechnology), cleaved poly (ADP-ribose) polymerase (PARP; Santa Cruz Biotechnology), and proliferating cell

nuclear antigen (PCNA; Santa Cruz Biotechnology). Briefly, protein samples were extracted using RIPA lysis buffer (Beyotime, Shanghai, China) and then resolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis equally and transferred to polyvinylidene fluoride membranes. The membranes were incubated with appropriate antibodies, washed and incubated with horseradish peroxidase-coupled secondary antibodies. Protein blots were detected using the ECL-Plus Western blotting detection system.

Statistical analysis. All data were presented as the mean ± standard deviation (SD). Statistical analysis was carried out by one-way ANOVA analysis, and Bonferroni post test was used for multiple comparisons. A P value less than 0.05 was considered statistically significant.

Results

Knockdown of BDNF expression using plasmid-mediated shRNA. Human B-cell lymphoma cell lines (Daudi, Raji, Ramos and Namalwa) were examined for the presence of BDNF using real-time PCR and Western blot technologies (n = 3 per group). Our results showed relative high mRNA (Figure 1A) and protein expression levels (Figure 1B) of BDNF in all the above four cell lines. To further investigate the biological role of BDNF in B-cell lymphoma, RNA interference approach was performed to down-regulate BDNF expression in Raji and Ramos cells expressing higher level of endogenous BDNF.

Meanwhile, we evaluated the knockdown efficiencies of BDNF shRNAs in the two B-cell lymphoma cell lines by using real-time PCR and Western blot analysis (n = 3 per group). At 48 h post-transfection, mRNA expression of BDNF in cells transfected with pRNA-H1.1-BDNF-1 and pRNA-H1.1-BDNF-2 plasmids significantly decreased, as compared with the control vector transfected cells ($P < 0.001$, Figure 1C). Likewise, marked decreases in BDNF protein expression were found in cells transfected with BDNF-specific shRNAs ($P < 0.001$ versus control shRNA, Figure 1D). These findings indicated that the BDNF specific shRNAs could effectively suppress BDNF expression at both transcriptional and translational levels.

Knockdown of BDNF reduced cell growth and proliferation of B-cell lymphoma cells. Next, we evaluated the effects of BDNF knockdown on cell growth of the two B-cell lymphoma cell lines by detecting BrdU incorporation (n = 5 per group). The present data revealed that inhibition of BDNF resulted in a significant decrease in the proliferation rate of Raji and Ramos cells at 24, 48 and 72 h post-transfection (Figure 2A and 2B). Additionally, decreased protein level of PCNA was observed in these cells at 72 h after gene silencing of BDNF (n = 3 per group, Figure 2C). Collectively, the present results demonstrated that BDNF contributed to cell proliferation of B-cell lymphoma *in vitro*.

Suppression of BDNF induced cell cycle arrest in G0/G1 phase and repressed cyclin D1 and cyclin E expression in Raji cells. Knockdown of BDNF by shRNA reduced cell growth and proliferation of B-cell lymphoma cells, we therefore examined whether this anti-proliferative effect was mediated through cell cycle inhibition. Since further experiments were performed to determine the cell apoptosis by using Annexin V-FITC and PI double staining, gating parameters were set up to present the cell cycle distribution without showing an apoptotic sub-G1 peak. Results of flow cytometry analysis illustrated that genetic repression of BDNF expression caused a significant accumulation of cells in G1 phase (Figure 3A) as compared with non-sense shRNA infected cells (Figure 3A) at 48 h post-transfection. Furthermore, we found that suppression of BDNF induced down-regulation of cyclin D1 and cyclin E, which are the important regulators of G0/G1 phase progression and G1/S transition [19-20] (Figure 3B). These results suggested that down-regulation of BDNF altered cell cycle distribution and prevented mitosis in B-cell lymphoma Raji cells.

Suppression of BDNF induced cell apoptosis. To explore potential effects of BDNF knockdown on apoptosis in Raji cells, we documented the proportion of apoptotic cells by flow cytometry at 72 h after transfection. Compared with control shRNA transfected cells, knockdown of BDNF via its specific shRNA induced significant cell apoptosis (Figure 4A). This result promoted us to explore the corresponding regulatory mechanisms of BDNF involved in the cell apoptosis. Protein expression levels of several apoptosis-associated proteins were determined by Western blot analysis in Raji cells (n = 3 per group). The obtained results indicated that inhibition of BDNF decreased anti-apoptotic Bcl-2 expression, whereas increased pro-apoptotic Bax (Figure 4B). Moreover, inhibition of BDNF induced the activation of caspase-3 and caspase-9, and subsequently caused PARP to cleave (Figure 4B). The above results suggested that inhibition of BDNF promoted cell apoptosis through mediation of these apoptosis related factors *in vitro*.

Inhibition of BDNF expression enhanced 5-FU cytotoxicity *in vitro*. A common chemotherapeutic agent, 5-FU is often used as an inducer of apoptosis in cancer cells [21]. In this study, 5-FU was used to induce apoptosis in Raji cells. To test whether inhibition of BDNF in Raji cells affects their sensitivity to 5-FU, BDNF shRNA transfected cells and control cells were treated with 5-FU for 24 h and then subjected to apoptosis analysis. The obtained data from flow cytometry analysis showed that the reduction of BDNF along with 5-FU remarkably increased cell death when compared with control cells. These findings illustrated that inhibition of BDNF expression enhanced the effect of 5-FU *in vitro* (Figure 5).

Discussion

Many previous studies have delineated a pro-proliferative role of BDNF in diverse cell types, including endothelial cells [22], neural stem cells [23] and smooth muscle cells [24]. Besides the pro-proliferative effects of BDNF observed in these normal cells, BDNF is currently believed to contribute to the cell growth and proliferation in tumor cells. Sun et al.

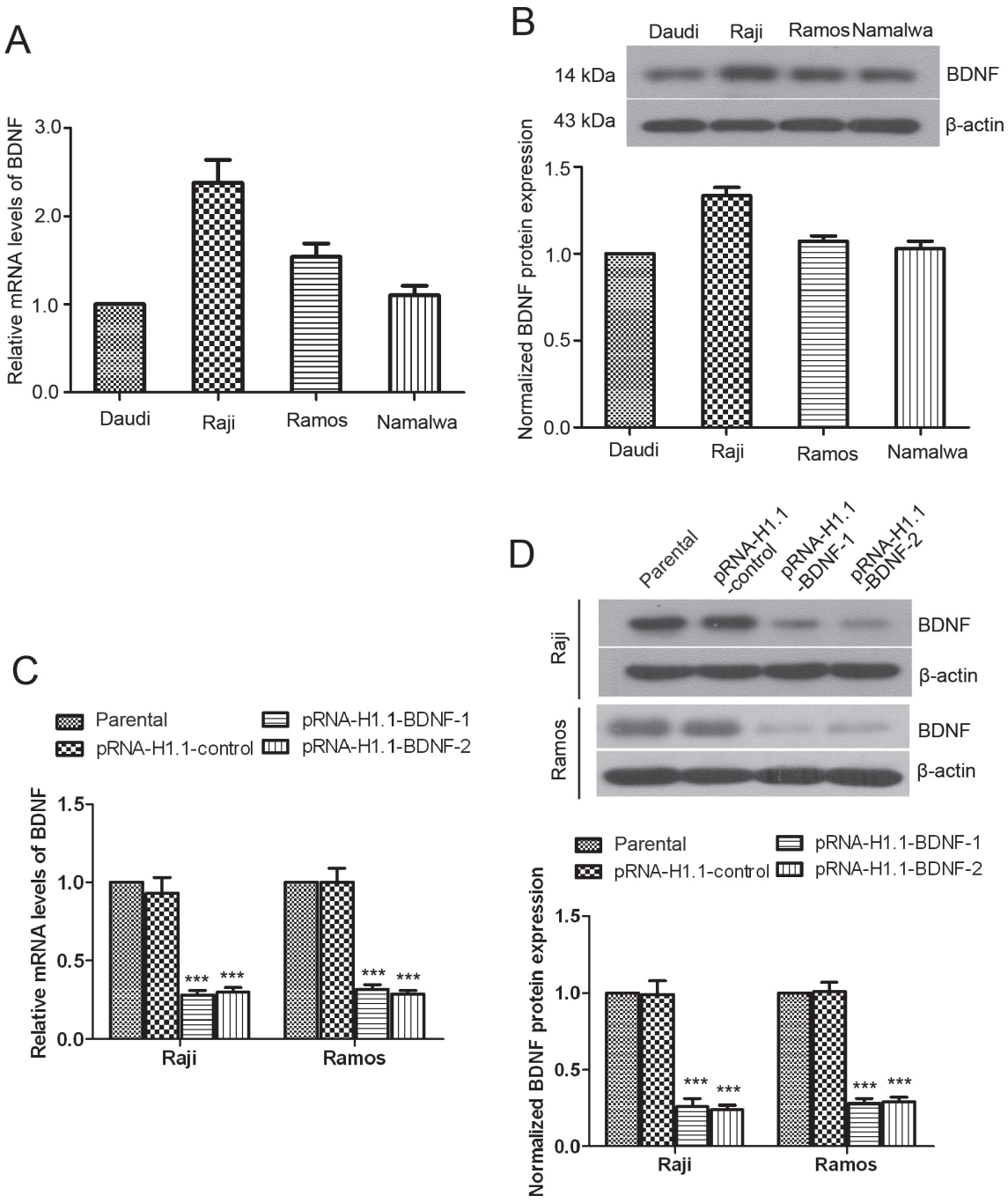

Figure 1. Knockdown of BDNF expression using specific shRNAs in B-cell lymphoma cells.
The mRNA (A) and protein (B) expression levels of BDNF in B-cell lymphoma Daudi, Raji, Ramos and Namalwa cells. BDNF expressed abundantly in these four cell lines. Two pairs of shRNAs reduced BDNF expression at both mRNA (C) and protein (D) level in Raji and Ramos cells. Data were presented as mean ± standard deviation (SD) (n = 3 per group). β-actin served as endogenous control. ***$P < 0.001$ versus control shRNA cells.

have demonstrated that a small dose of exogenous BDNF induces proliferation in human multiple myeloma cells, and they suggest a novel therapeutic strategy for multiple myeloma by targeting BDNF [25]. Nevertheless, the effect of BDNF in B-cell lymphoma remains to be elucidated.

In the present study, we demonstrated that BDNF was strongly expressed in four cell lines of B-cell lymphoma, indicating the involvement of BDNF in this disease. Thereby, we explored the role of BDNF in the cellular function in B-cell lymphoma cells via shRNA-mediated RNA interfering tech-

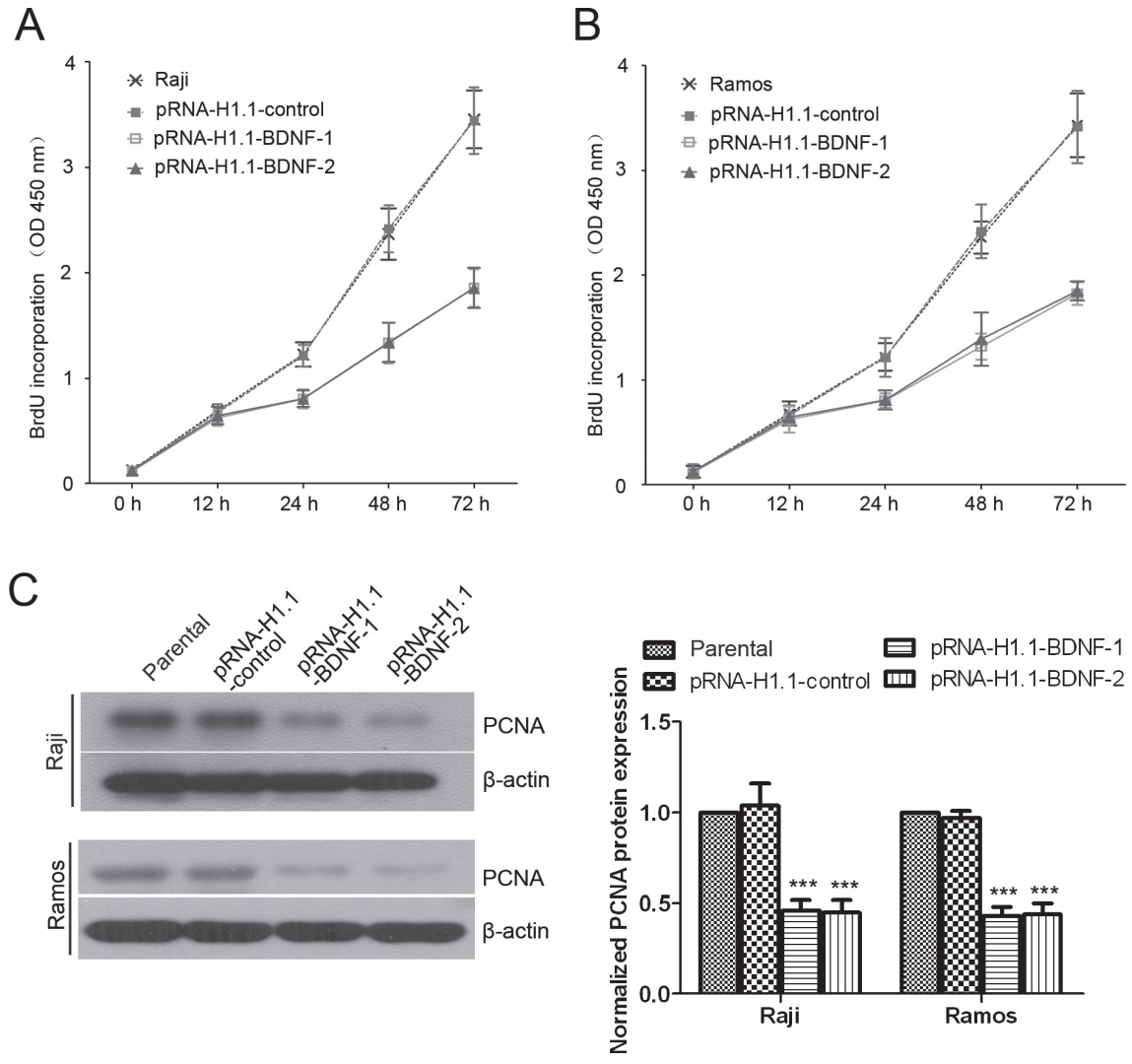

Figure 2. Knockdown of BDNF reduced cell growth and proliferation of Raji and Ramos cells.
(A, B) Cell proliferation determined by using BrdU Cell Proliferation ELISA Kit and presented as the absorbance values at 450 nm. BDNF silencing suppressed Raji and Ramos cells growth (n = 5 per group). (C) Protein expression of PCNA determined by Western blot analysis (n = 3 per group). PCNA expression markedly decreased when BDNF was inhibited in the two B-cell lymphoma cell lines. Data were presented as mean ± standard deviation (SD) (n = 3 per group). β-actin served as endogenous control. ***$P < 0.001$ versus control shRNA cells.

nology *in vitro*. We first confirmed that the designed specific shRNAs were able to efficiently reduce BDNF expression at both mRNA and protein levels in two B-cell lymphoma cell lines, Raji and Ramos cell lines. As compared with control groups, decreased growth rate was observed in these cells when the endogenous BDNF was down-regulated. Additionally, decreased PCNA expression was also found in BDNF knockdown cells. These results implied that the down-regulation of BDNF reduced cellular proliferation of B-lymphoma cells, corresponding to a prior research from Yang et al [16].

Moreover, because the deregulated cell cycle progression is one of the primary characteristics of cancer cells [26], we examined the cycle distribution of Raji cells after inhibition of BDNF. Our data showed that knockdown of BDNF caused a G0/G1 phase arrest of the cell cycle. We continued to investigate whether BDNF regulated G1/S transition by mediating the expression of cyclin D1 and cyclin E in Raji cells. These two cyclins were found to be decreased in cells when endogenous BDNF was down-regulated by shRNA. Since inhibition of cyclin D1 and/or cyclin E has been reported to prevent S-phase entry [19-20], we concluded that suppression of BDNF may reduce B-cell lymphoma proliferation by inhibiting G1/S transition of cell cycle.

We further explored the effect of BDNF on Raji cell apoptosis with emphases on Bcl-2 and caspase singling pathways. When compared with normal controls, the apoptotic cell

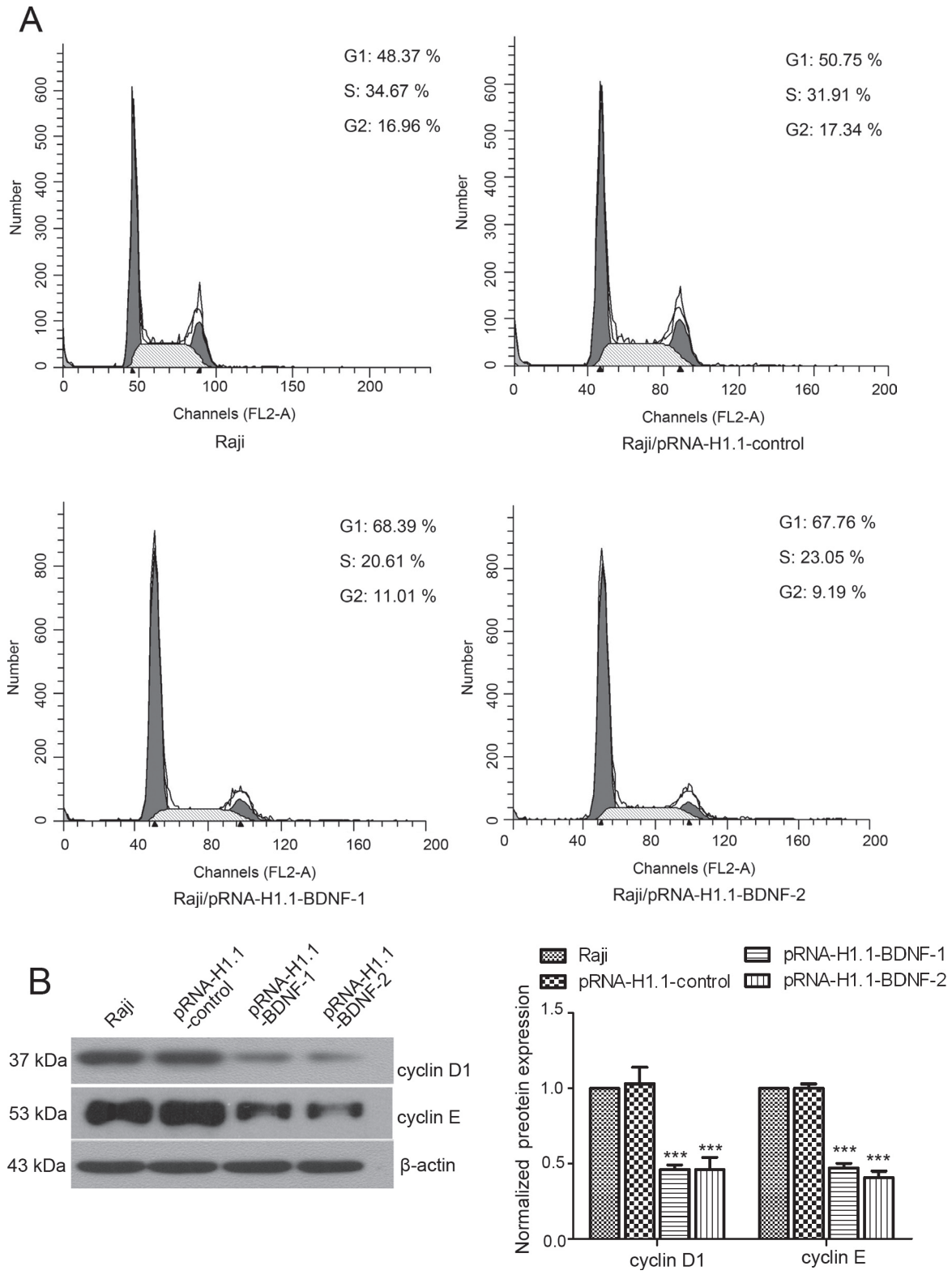

Figure 3. Inhibition of BDNF induced G0/G1 phase arrest and decreased cyclin D1 and cyclin E expression in Raji cells.
(A) Representative flow cytometric results showing the distribution of cell cycle. Knockdown of BDNF via shRNA in Raji cells resulted in cell cycle arrest in G1 phase at 48 h after transduction. (B) Protein expression levels of cyclin D1 and cyclin E in Raji cells determined by Western blot. BDNF silencing suppressed cyclin D1 and cyclin E expression. Data were presented as mean ± standard deviation (SD) (n = 3 per group). β-actin served as endogenous control. ***$P < 0.001$ versus control shRNA cells.

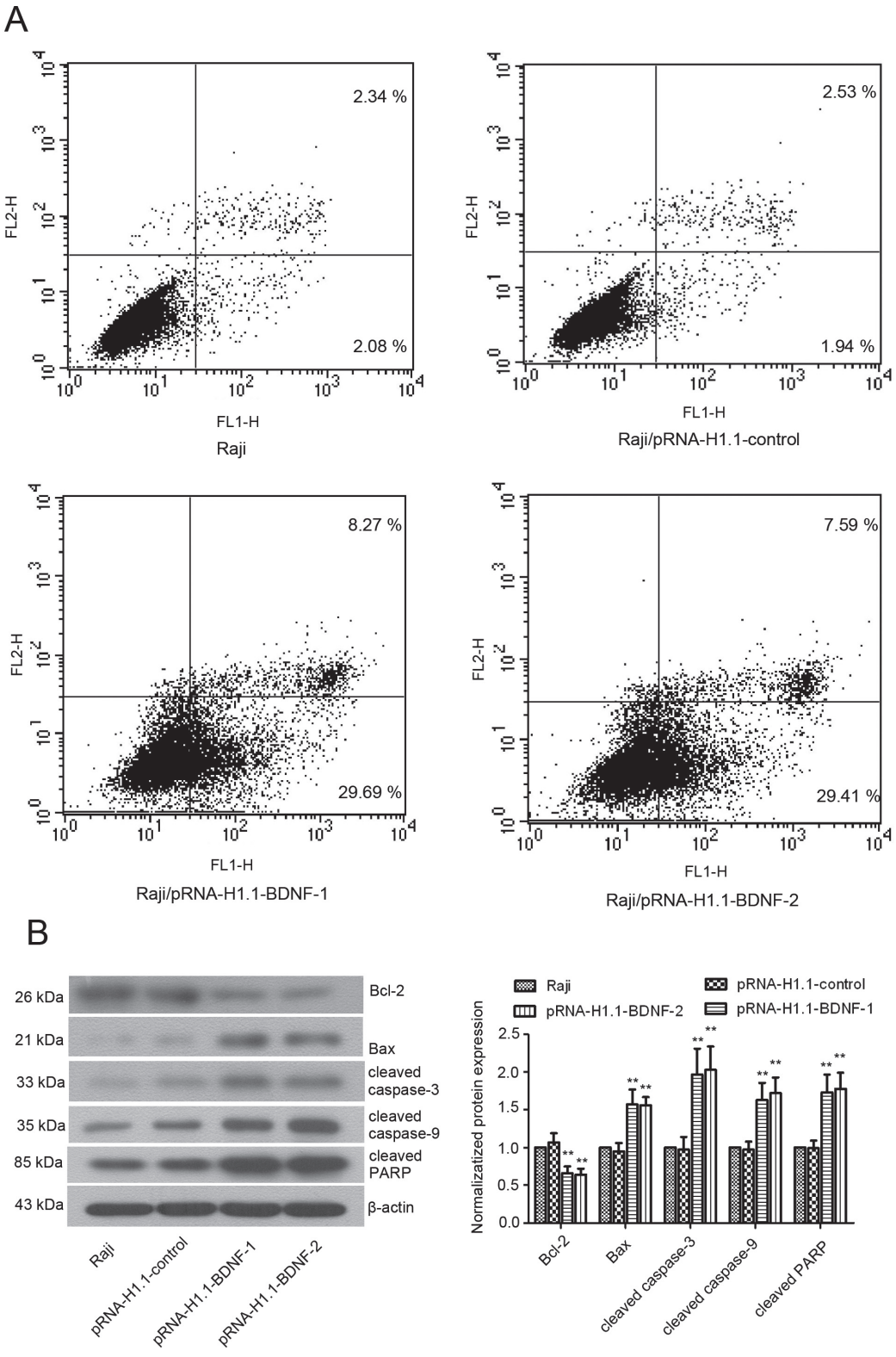

Figure 4. Knockdown of BDNF induced apoptosis in Raji cells.
(A) Flow cytometry analysis with Annexin V-PI staining was performed to determine the percentage of apoptotic cells. BDNF inhibition induced a significant apoptosis and death in cells. (B) Protein expression levels of Bcl-2, Bax, cleaved caspase-3, cleaved caspase-9 and cleaved PARP were determined by Western blot assay. Data were presented as mean ± standard deviation (SD) (n = 3 per group). β-actin served as endogenous control. **$P < 0.01$ versus control shRNA cells.

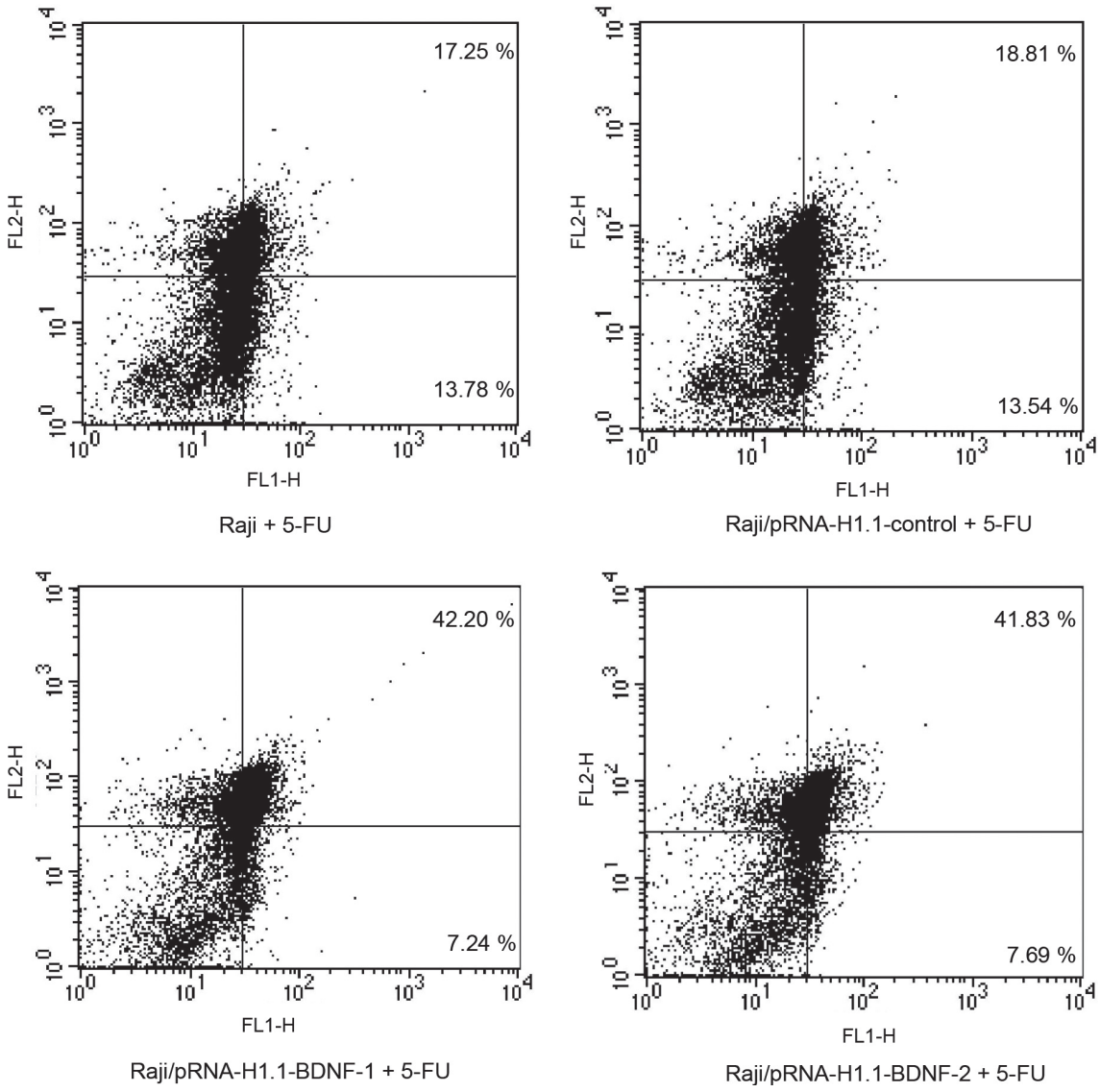

Figure 5. Inhibition of BDNF enhanced 5-FU-induced apoptosis in Raji cells.
Cell apoptosis measured by annexin-V/PI staining and analyzed by flow cytometry. BDNF enhanced the chemotherapeutic effect of 5-FU in cells.

number (percentage) increased in Raji cells in which BDNF was inhibited. High expression level of Bcl-2 has been shown to contribute to prolonged survival of malignant B cells [27], whereas reduction of Bcl-2 expression increases cellular death dramatically [28]. Contrarily, elevation of Bax in Namalwa cells accelerates cell death induced by chemotherapeutic drugs such as camptothecin and etoposide [29]. These previous studies indicate an anti-apoptotic effect of Bcl-2 and pro-apoptotic effect of Bax in neoplastic cells. In our research, knockdown of BDNF was found to decrease Bcl-2 expression but increase Bax in Raji cells. Such result suggested that BDNF affected the cell apoptotic process by altering the expression these two apoptosis related proteins. Besides, Schrantz et al. have demonstrated that inhibition Manganese-induced caspase-3 activation via Zinc enhances the apoptosis of human Burkitt lymphoma B cell lines (Ramos) [30]. Georgakis et al. have proven that activation of caspase-9 results in apoptosis of SP35 mantle cell lymphoma cell lines [31]. These two researches indicate that the activation of caspases leads to apoptosis in lymphoma cells. Our present data illustrated that the activation of caspase-3 and caspase-9 was induced in Raji cells when BDNF was suppressed by shRNA. These findings suggested that BDNF mediated caspase related cell apoptosis in B-cell lymphoma *in vitro*. Notably, we also found that inhibition of BDNF could enhance the sensitivity of Raji cells to 5-FU-induced apoptosis. Since 5-FU exerts antitumor activity by inducing apoptosis in a wide range of tumors [32-33], our findings that the knockdown of BDNF enhanced 5-FU chemotherapeutic

effects provided a novel therapeutic approach for patients of B-cell lymphoma.

In conclusion, we report that the specific shRNA-mediated knockdown of BDNF expression suppresses the tumorigenesis of B-cell lymphoma *in vitro*, probably by inducing the G0/G1 cell cycle arrest and subsequent cell apoptosis. Our research provides a promising therapeutic strategy for human B-cell lymphoma by targeting BDNF.

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