

DJ-1 Activates the Noncanonical NF- κ B Pathway via Interaction with Cezanne to Inhibit the Apoptosis and Promote the Proliferation of Ishikawa Cells

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Abstract

Endometrial cancer is generally one of the most evident malignant tumours of the female reproductive system, and the mechanisms underlying its cell proliferation and apoptosis are key to research in gynaecological oncology. In the paper, the in-depth molecular mechanism by which DJ-1 protein regulates the proliferation and apoptosis of Ishikawa cells was investigated. DJ-1 knockdown and overexpressing Ishikawa stable cell lines were established by lentiviral transduction. The levels of DJ-1 and noncanonical NF- κ B signaling proteins were evaluated by Western blotting. Cell counting kit-8 (CCK-8) and flow cytometry were applied to analyze the cell viability and apoptosis. Co-immunoprecipitation experiment was utilized to assess the DJ-1-Cezanne interaction. The results showed that DJ-1 overexpression conferred apoptosis resistance and high proliferation on Ishikawa cells, while DJ-1 knockdown in Ishikawa cells produced the opposite results. These findings again suggested that DJ-1 inhibits the apoptosis and promotes the proliferation of Ishikawa cells. More crucially, further data showed that the noncanonical NF- κ B activation was required for the regulation of Ishikawa cell proliferation and apoptosis by DJ-1. Meanwhile, it was found that noncanonical NF- κ B pathway may be activated by DJ-1 interacting with and negatively regulating Cezanne in Ishikawa cells. Overall, this work revealed that DJ-1 associates with and negatively regulates Cezanne and consequently triggers the noncanonical NF- κ B activation, thereby regulating Ishikawa cell proliferation and apoptosis.

Introduction

Endometrial cancer (EC) is one of the three most common female genital cancers, accounting for approximately 20% ~ 30% of female genital tract malignancies[1]. In recent years, EC morbidity has been rising consistently with a marked youth-oriented tendency. The main reasons for treatment failure are distant metastasis and local recurrence[2]. Therefore, it is critical to understand the mechanisms underlying EC proliferation and apoptosis processes.

The DJ-1 gene was first confirmed by Nagakubo in 1997 as a new mitogen-dependent oncogene, and its encoded protein can cooperate with Ras to induce the transformation of mouse NIH-3T3 cells[3]. The DJ-1 protein participates in various cellular processes in the form of dimers, such as gene transcription regulation, anti-oxidative stress, inhibition of apoptosis, molecular chaperone, and promoting cell proliferation[4–7]. According to a recent study[8], the expression of DJ-1 protein in EC, which is markedly higher than in normal endometrial tissue, is related to the degree of differentiation, invasion depth, and lymph node metastasis of EC. In addition, silencing DJ-1 expression by RNAi in EC cells could clearly promote cell apoptosis and inhibit cell proliferation. These results demonstrated that DJ-1 may regulate the apoptosis and proliferation of EC cells. However, its molecular regulation mechanism remains to be clearly explicated.

At present, it has been confirmed that the noncanonical NF- κ B is one of the most significant signalling pathways related to many carcinomas, including EC[9]. Research has shown that the noncanonical NF- κ B signalling pathway is abnormally activated in EC[10], yet the molecular mechanism that mediates the

abnormal activation has not been fully elucidated. Previous reports have suggested that the noncanonical NF- κ B activation mainly depends on the accumulation of NF- κ B-inducing kinase (NIK) and the protein degradation of tumour necrosis factor receptor-associated factor 3 (TRAF3)[11]. Normally, NIK binds to a protein complex comprising TRAF2, TRAF3, and cellular inhibitor of apoptosis 1/2 (cIAP1/2) and continues to degrade through ubiquitination to keep a low level. During the tumorigenesis process of some cancers, TRAF3 degrades and contributes to the dissociation of the complex. Thus, NIK accumulates in the cytoplasm, resulting in phosphorylation of the downstream target IKK α , which promotes proteasome cleavage of the inactive NF- κ B precursor protein p100 into active p52. Subsequently, p52 forms a heterodimer with RelB and enters the nucleus to initiate the transcription of downstream cancer-related target genes, including anti-apoptotic genes (eg, Bcl-xL, Bcl2, XIAP, IAP, etc.) and cell proliferation regulators (eg, Cyclin D1, VCAM1, ICAM1, E Selectin, etc.)[11, 12].

Cezanne (cellular zinc finger anti-NF- κ B, also known as OTUD7B) is a key regulator of the noncanonical NF- κ B signaling pathway[13]. Cezanne is classified as part of the A20 protein family and possesses K48-specific deubiquitinase activity. It can bind and deubiquitinate TRAF3, thereby suppressing TRAF3 degradation and subsequent noncanonical NF- κ B activation. Recent studies have found that the occurrence and progression of glioma, non-small cell lung cancer, hepatocellular carcinoma, and other tumours are related to Cezanne inhibition and noncanonical NF- κ B activation [14, 15, 13]. Furthermore, notably, McNally et al.[16] lately found that DJ-1 directly binds to Cezanne and inhibits its deubiquitinase activity in H157 non-small cell lung cancer cells. It was suggested that DJ-1 may be a key negative regulator of Cezanne. On this basis, we presumed that DJ-1 inhibits the apoptosis and promotes the proliferation of Ishikawa cells probably by activating the noncanonical NF- κ B signaling pathway by interacting with Cezanne in Ishikawa cells. Accordingly, this research was conducted to clarify this possibility by linking small interfering RNA (siRNA) technology and biochemical analysis in DJ-1-knockdown and -overexpressing Ishikawa cells.

Materials And Methods

Chemicals and reagents

Puromycin was provided by Yisheng Biotechnology Co. (Shanghai, CHN). Anti-GAPDH, anti-TRAF3, anti-Cezanne, and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Sanying Biotechnology Co., Ltd. (Wuhan, CHN). Anti-NIK and anti-P52 were purchased from Zhengneng Biotechnology Co. (Chengdu, CHN). Anti-DJ-1 was purchased from Abcam (Cambridge, MA, USA). All other chemicals were of reagent grade and obtained from Sigma (St. Louis, MO, USA) unless otherwise stated.

Cell culture

The human EC cell line Ishikawa was purchased from Jikai Gene Technology Co, Shanghai, China. Cells were cultured at 37 °C in DMEM/F-12 added with 10% heat-inactivated FBS in a humidified incubator with 5% CO₂ and 95% air (Sanyo, Japan).

Cell line construction

DJ-1 knockdown and overexpressing Ishikawa stable cell lines were constructed according to our previously published study [17, 18]. Briefly, lentiviral vectors either expressing DJ-1-specific shRNA (LV-shDJ-1) or overexpressing DJ-1 (LV-DJ-1) were commercially constructed by GeneChem Corporation (Shanghai, China). To obtain Ishikawa cells with stable knockdown of DJ-1 (Ishikawa/shDJ-1) and with stable overexpression of DJ-1 (Ishikawa/LV-DJ-1), LV-shDJ-1 and LV-DJ-1 were separately used to infect Ishikawa cells according to the protocols of the manufacturer. The stable transfectants were screened by puromycin according to protocols and identified by western blotting.

Transfection of short interfering RNA (siRNA)

Cezanne siRNA and NIK siRNA were constructed by Ruibo Biotechnology Co., Ltd. (Guangzhou, CHN). These siRNAs were transfected into Ishikawa, Ishikawa/LV-DJ-1, or Ishikawa/shDJ-1 cells for 48 h using Lipofectamine™ 2000 siRNA Transfection Reagent (Thermo Fisher Scientific, USA) following the manufacturer's specifications.

Western blotting analysis

The whole-cell lysates and cytosolic and nuclear fractions were analyzed by Western blotting analysis as reported previously [18]. Antibodies against the nuclear marker protein Lamin B1 and the cytosol marker tubulin were used to evaluate the purity of nuclear or cytosol fractions to ensure that there is no obvious contamination in the cytosolic or nuclear fraction. GAPDH was used as the whole-cell loading control. Blots were incubated with primary antibodies at 1:1000 dilution, except for NIK (1:800) and GAPDH (1:5000), and secondary antibodies at 1:2000 dilution.

Co-immunoprecipitation assay

Co-immunoprecipitation (IP) experiments were performed as reported previously [18]. The pre-cleared cell lysates were incubated with normal IgG (used as negative control) or the indicated primary antibody at 4°C overnight. The antigen-antibody complexes were captured for 2 h at 4°C with A/G-agarose. The beads were washed with IP lysis buffer twice for 15 min each, and the proteins on the beads and input sample were boiled for 10 min in SDS sample loading buffer. The samples were resolved by SDS-PAGE and analyzed by probing with various antibodies.

Cezanne deubiquitinase activity assay

Cezanne deubiquitinase activity was measured with the Deubiquitinase Assay Kit (#ab241002, Abcam, Cambridge, UK) according to the protocols of the manufacturer. Briefly, Cells were lysed in ice-cold DUB assay buffer with 1 mM DTT at 4°C. After centrifuging at 10,000 × g for 15 min, the supernatant was collected and immunoprecipitated with anti-Cezanne antibody overnight at 4°C, followed by adding protein A/G-agarose at 4°C for 2 h. After the addition of substrate mix, microplate reader (Bio-Rad, USA) was used to measure the fluorescence intensity at 350 nm excitation and 440 nm emission in order to analyze the activity of precipitated Cezanne deubiquitinase.

Cell proliferation assay

A modified cell counting kit-8 (CCK-8) (TransGen Biotech, Beijing, CHN) assay was applied to evaluate cell proliferation. Briefly, cells were digested and routinely inoculated into a 96-well plate at a density of 5×10^3 cells per well. After adding 10 μ l of CCK-8 reagent to each well followed by incubation for 2 h at 37°C in 5% CO₂, the optical density was detected at 490 nm using a microplate reader.

Cell apoptosis assay

The evaluation of cell apoptosis was performed by the Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences) assay. The cells were collected by centrifugation and suspended in 400 μ l of Annexin V Binding Solution. After incubation with 5 μ l Annexin V-FITC for 20 min at 4°C, the cells were then incubated with 10 μ l PI for 5 min at 4°C and immediately detected by flow cytometry.

Statistical analysis

The results were represented as the means \pm SEM and analysed by GraphPad Prism5. One-way variance (ANOVA) and Student's t-test were carried out to analyse discrepancies between experimental groups. *P* values < 0.05 were supposed to demonstrate statistical significance.

Results

DJ-1 regulates Ishikawa cell apoptosis and proliferation

First, the effect of DJ-1 on the apoptosis and proliferation of Ishikawa cells was detected in DJ-1-overexpressing Ishikawa cells (Ishikawa/LV-DJ-1) and DJ-1-knockdown Ishikawa cells (Ishikawa/shDJ-1). As shown in Fig. 1a, the percentage of apoptosis in Ishikawa/LV-DJ-1 cells measured by flow cytometry was significantly decreased compared to that in Ishikawa/LV-NC (negative control) cells. However, the increased percentage of apoptosis emerged in Ishikawa/shDJ-1 cells. Moreover, CCK-8 assays demonstrated that downregulating DJ-1 in Ishikawa cells significantly inhibited cell proliferation, whereas overexpressing DJ-1 in Ishikawa cells exerted the opposite result (Fig. 1b). These data again showed that DJ-1 can inhibit cell apoptosis and promote cell proliferation in Ishikawa cells.

DJ-1 interacts with and inhibits Cezanne and activates noncanonical NF- κ B signalling pathway in Ishikawa cells

Subsequently, to analyze whether the proliferation-promoting and apoptosis-inhibitory effects of DJ-1 is related to Cezanne/noncanonical NF- κ B signalling pathway in Ishikawa cells, we further examined the impact of DJ-1 on Cezanne activity and the noncanonical NF- κ B pathway. As shown in Fig. 2, DJ-1 could associate with Cezanne in Ishikawa cells (Fig. 2a). Importantly, DJ-1 overexpression resulted in notable increase in the association of DJ-1 with Cezanne (Fig. 2a) accompanied by a decrease in Cezanne deubiquitinase activity (Fig. 2b) in Ishikawa/LV-DJ-1 cells. In contrast, DJ-1 knockdown had the opposite

effects on the connection of DJ-1 with Cezanne and Cezanne activity in Ishikawa/shDJ-1 cells. The results implied that DJ-1 interacts with Cezanne and represses its deubiquitinase activity in Ishikawa cells. Moreover, the data shown in Fig. 2c and d demonstrated that overexpression of DJ-1 decreased TRAF3 but increased NIK and p52 levels (Fig. 2c), leading to translocation of p52 into the nucleus (Fig. 2d) in Ishikawa/LV-DJ-1 cells, while knockdown of DJ-1 increased TRAF3 levels but decreased NIK and p52 levels (Fig. 2c) and inhibited p52 nuclear translocation (Fig. 2d) in Ishikawa/shDJ-1 cells. On the basis of the results, we proposed that DJ-1 activates the noncanonical NF- κ B signalling pathway in Ishikawa cells.

Knockdown of Cezanne restores the growth-promoting and apoptosis-inhibitory effects of DJ-1 in Ishikawa/shDJ-1 cells

Next, to further validate the causal role of the Cezanne inhibition in the proliferation-promoting and apoptosis-inhibitory effects of DJ-1 in Ishikawa cells, we observed the effect of Cezanne knockdown on DJ-1 expression, noncanonical NF- κ B signalling activation, cell apoptosis and proliferation in Ishikawa and Ishikawa/shDJ-1 cells. As shown in Fig. 3, knockdown of Cezanne by siRNA in Ishikawa cells did not affect DJ-1 expression but mimicked the effects of DJ-1 overexpression and caused an obvious decrease in TRAF3 expression and a marked increase in NIK and p52 levels (Fig. 3a), accompanied by proliferation increase (Fig. 3b) and apoptosis inhibition (Fig. 3c). More importantly, Cezanne knockdown also reversed the inactivation of noncanonical NF- κ B (Fig. 3a), inhibition of cell proliferation (Fig. 3b), and increase of cell apoptosis (Fig. 3c) by DJ-1 knockdown in Ishikawa/shDJ-1 cells. This result suggested that Cezanne inhibition is essential for DJ-1 to activate the noncanonical NF- κ B signalling pathway and to modulate the cell proliferation and apoptosis in Ishikawa cells.

Knockdown of NIK impaired the ability of DJ-1 overexpression to promote proliferation and inhibit apoptosis in Ishikawa/LV-DJ-1 cells

Similarly, to clarify the causal role of noncanonical NF- κ B signalling pathway in the proliferation-promoting and apoptosis-inhibitory effects of DJ-1 in Ishikawa cells, we further investigated the effect of noncanonical NF- κ B signalling inactivation by NIK knockdown on DJ-1 and Cezanne expression, and cell proliferation and apoptosis in Ishikawa and Ishikawa/LV-DJ-1 cells. As shown in Fig. 4, knockdown of NIK by siRNA in Ishikawa cells did not affect DJ-1 and Cezanne expression, but significantly decreased p52 levels (Fig. 4a), inhibited cell proliferation (Fig. 4b), and facilitated cell apoptosis (Fig. 4c) compared with the control groups. These results implied that DJ-1 and Cezanne are upstream molecules of the noncanonical NF- κ B pathway, whose inactivation inhibits Ishikawa cell proliferation and promotes apoptosis. In addition, NIK knockdown significantly abrogates DJ-1 overexpression-induced p52 protein increment in Ishikawa/LV-DJ-1 cells (Fig. 4a). At the same time, it also abrogates DJ-1 overexpression-induced the proliferation-promoting and apoptosis-inhibitory effects (Fig. 4b, c). Collectively, these

findings indicated that the noncanonical NF- κ B activation is a prerequisite for DJ-1 to inhibit the apoptosis and promote the proliferation of Ishikawa cells.

Discussion

EC is one of the most common female genital malignant tumours, whose morbidity and mortality are increasing across the world, especially in China[19, 20]. The primary reasons for its treatment failure are distant metastasis and local recurrence[21]. Therefore, discovering the molecular mechanisms underlying the cell proliferation and apoptosis of EC will be particularly helpful in providing an experimental basis for the development of valid targeted curative drugs. As far as we know, this study is the first report that DJ-1 regulates the apoptosis and proliferation of Ishikawa cells by activating the Cezanne/noncanonical NF- κ B pathway.

Recently, Shu K[8] showed that DJ-1 is an oncogene with abnormally high expression in EC and is closely associated with lymph node metastasis, the degree of differentiation and the invasion depth of EC. Notably, silencing DJ-1 expression by RNAi in EC inhibited cell viability and promoted cell apoptosis. These results demonstrated that DJ-1 regulates the apoptosis and proliferation of EC cells. Consistent with above-mentioned reports, we also revealed here that DJ-1 overexpression produced a lower percentage of apoptosis as well as higher proliferation in Ishikawa cells, whereas these effects disappeared in DJ-1-knockdown Ishikawa cells. The results again indicated that DJ-1 protein can inhibit cell apoptosis and promote cell proliferation in Ishikawa cells. However, its underlying mechanism remains unclear. Hence, in this study, we focus on investigating the signalling pathway by which DJ-1 suppresses the apoptosis and promotes the proliferation of Ishikawa cells.

It was known that the non-canonical NF- κ B pathway is a key cell survival pathway, whose activation enhances cell survival by stimulating cell proliferation and inhibiting apoptosis[22, 11]. Several studies have observed that noncanonical NF- κ B is aberrantly activated in many carcinomas, including EC, and affects hallmarks of cancer via the transcriptional activation of genes related to cell proliferation and apoptosis suppression [10, 9]. Moreover, it has been reported that Cezanne, which is classified as a member of the A20 protein family and possesses K48-specific deubiquitinase activity, is associated with the occurrence and progression of malignant tumours as a negative regulator of the noncanonical NF- κ B signalling pathway[13–15]. Notably, an elegant study has shown that DJ-1 protein can interact with Cezanne and negatively regulate its activity in HEK293T cells[16]. On this basis, we speculated that DJ-1 could inhibit the apoptosis and promote the proliferation of Ishikawa cells through inhibition of Cezanne and subsequent activation of noncanonical NF- κ B signalling pathway. To clarify the question, we first investigated the impact of altered expression of DJ-1 on Cezanne activity and the key modulators of noncanonical NF- κ B signalling pathway in Ishikawa cells. Interestingly, our study discovered that the association DJ-1 with Cezanne was increased, accompanied by the decreased Cezanne activity in DJ-1-overexpression Ishikawa cells. Meanwhile, TRAF3 expression was decreased, whereas NIK level was elevated and p52 expression and its nuclear translocation were increased. However, the aforementioned effects were reversed in DJ-1-knockdown Ishikawa cells. These findings suggested that DJ-1 not only

interacts with and inhibits Cezanne, but also activates the noncanonical NF- κ B signalling pathway in Ishikawa cells.

Moreover, to clarify the causal roles of Cezanne inhibition and noncanonical NF- κ B pathway activation in the proliferation-promoting and apoptosis-inhibitory effects of DJ-1 and their upstream and downstream relationship, we further observed the effect of Cezanne or NIK knockdown on DJ-1 and Cezanne expression, noncanonical NF- κ B signalling pathway activation, and cell apoptosis and proliferation. As expected, we observed that Cezanne knockdown reversed DJ-1 knockdown-induced proliferation inhibition and apoptosis increase, whereas the inhibition of the noncanonical NF- κ B signalling pathway by NIK siRNA abrogated the proliferation-promoting and apoptosis-inhibitory effects of DJ-1 overexpression in Ishikawa cells. The above data convincingly demonstrated that the inhibition of Cezanne and the activation of noncanonical NF- κ B signalling pathway are the critical mechanisms by which DJ-1 inhibits the apoptosis and promotes the proliferation of Ishikawa cells. Importantly, we found that Cezanne knockdown activated noncanonical NF- κ B signalling pathway in Ishikawa cells and reversed noncanonical NF- κ B inactivation in DJ-1-knockdown Ishikawa cells, but had no effect on DJ-1 expression. Moreover, NIK knockdown did not affect DJ-1 and Cezanne expression in Ishikawa cells but abolished the activation effect of DJ-1 overexpression on the noncanonical NF- κ B signalling pathway. The above results indicated that DJ-1 associates with and negatively regulates Cezanne to activate the noncanonical NF- κ B signalling pathway, resulting in inhibiting cell apoptosis and promoting cell proliferation in Ishikawa cells. Nevertheless, it is worth noting that the precise mechanism by which DJ-1-mediated the activation of noncanonical NF- κ B pathway inhibits the apoptosis and promotes the proliferation of Ishikawa cells has yet to be determined. It is now documented that the noncanonical NF- κ B activation can regulate transcriptional expression of multiple proliferation-promoting genes (eg, Cyclin D1, VCAM1, ICAM1, E Selectin, etc.) and anti-apoptotic genes (eg, Bcl-xL, Bcl2, IAP, XIAP, etc.) [11, 12, 23, 24]. Obviously, after activation of NF- κ B signaling pathway by DJ-1, which specific proliferation- and apoptosis-related effectors are regulated thereby affecting Ishikawa cell proliferation and apoptosis is an important question for future research.

In conclusion, this study uncovered the potential mechanism by which DJ-1 regulates Ishikawa cell apoptosis and proliferation and found that DJ-1 associates with and inhibits Cezanne to activate the noncanonical NF- κ B signalling pathway, thereby affecting the biological behaviours of Ishikawa cell proliferation and apoptosis. These findings may contribute to illuminate the mechanisms of the occurrence and progression of EC and provide important insights in the search for biomarkers for the development of EC targeted drugs.

Declarations

Funding

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Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Author contributions

QZZ: Protocol development, data collection, data analysis, and manuscript writing. HYL: Data collection, data analysis, and manuscript writing. XYZ, LJQ, TTZ, and XYW: Data collection. HPC and ZQX: Project development, manuscript editing. All authors approved final manuscript.

Compliance with ethical standards

Conflict of interest

The authors state that they have no conflict of interest.

Ethical approval

This article does not contain any research conducted by any author on human participants or animals.

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Figures

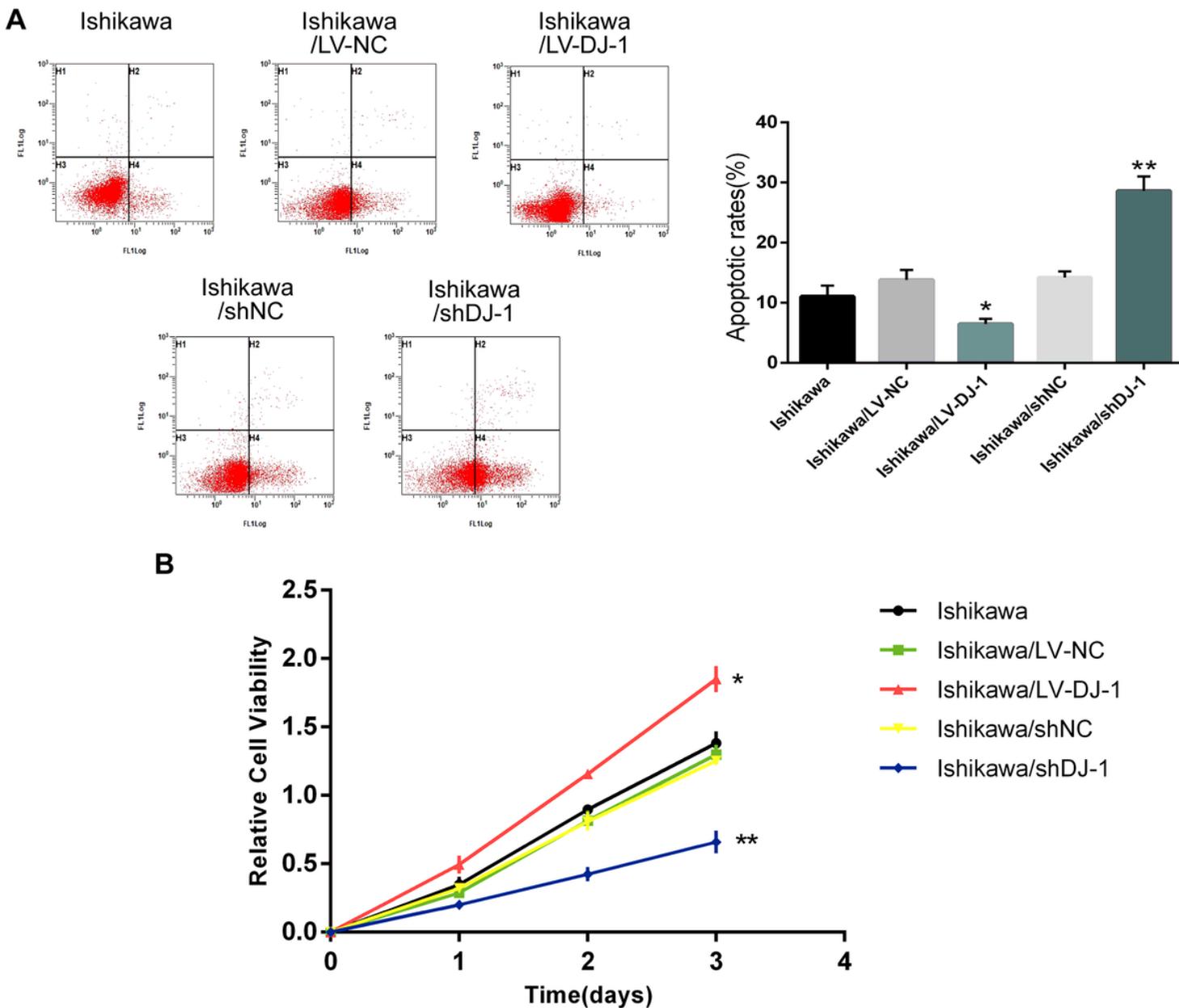


Figure 1

Effect of altered expression of DJ-1 on the proliferation and apoptosis of Ishikawa cells. a Cell apoptosis was assessed by flow cytometry with Annexin-V-FITC/PI staining, and b cell viability was evaluated by CCK-8 assay at the indicated times in Ishikawa, Ishikawa/LV-NC, Ishikawa/LV-DJ-1, Ishikawa/shNC, and

Ishikawa/shDJ-1 cells. Data are presented as the mean \pm SEM from four independent experiments. * $P < 0.05$, ** $P < 0.01$ vs Ishikawa cells.

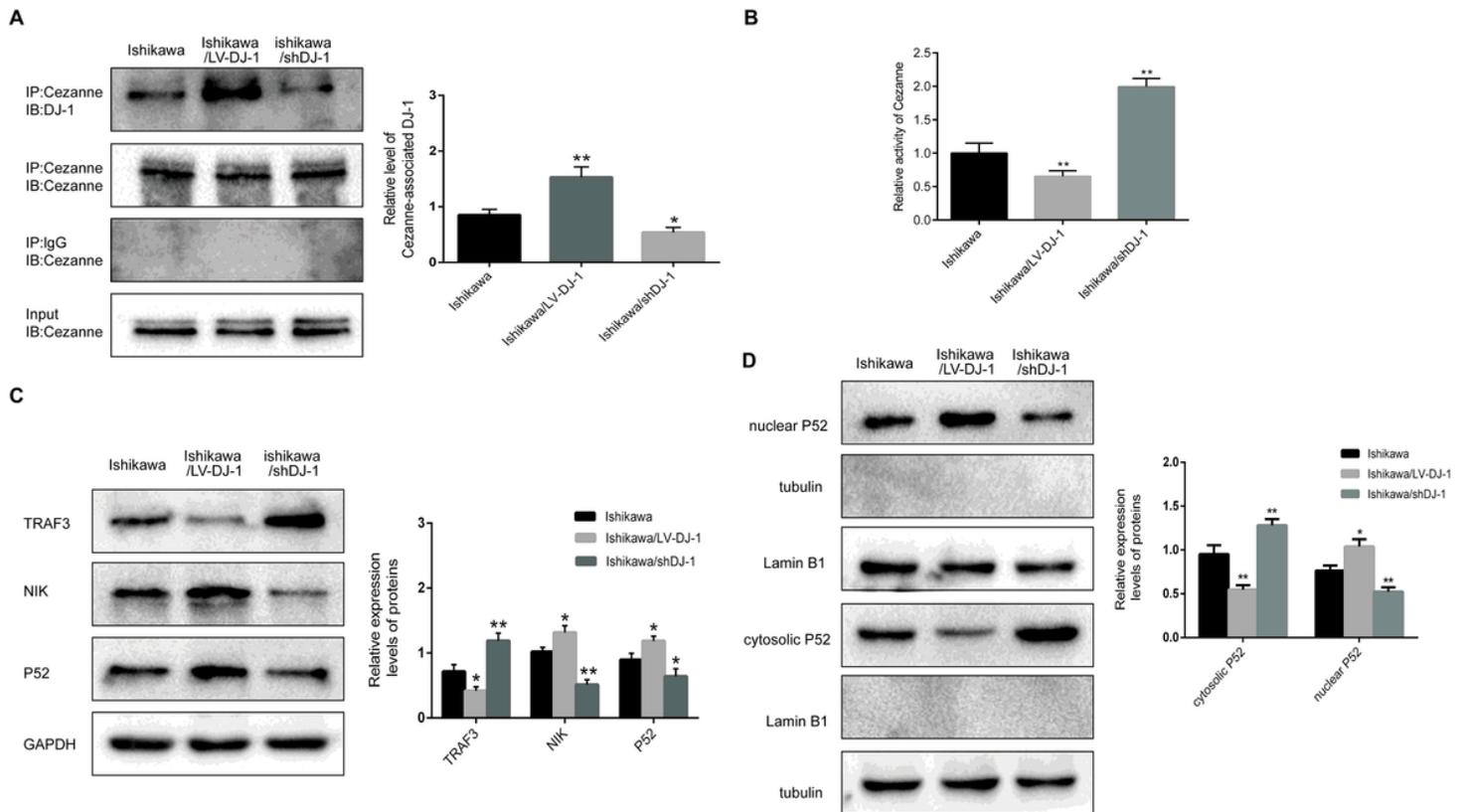


Figure 2

Effect of altered expression of DJ-1 on the association of DJ-1 with Cezanne, Cezanne activity, the expressions of TRAF3, NIK, and P52, and the nuclear translocation of P52 in Ishikawa cells. a The interaction of DJ-1 and Cezanne was determined by using co-immunoprecipitation (IP) assay in Ishikawa, Ishikawa/shDJ-1, and Ishikawa/LV-DJ-1 cells. Cell lysates were immunoprecipitated with anti-Cezanne antibody or control IgG. Immunoprecipitates were immunoblotted (IB) with anti-Cezanne or anti-DJ-1 antibody. Total protein extracts (prior to IP) were used as controls (input). b Cezanne activity was detected using the Deubiquitinase Assay Kit in Ishikawa, Ishikawa/shDJ-1, and Ishikawa/LV-DJ-1 cells. c The expressions of TRAF3, NIK, and P52 were evaluated by Western blotting analysis in Ishikawa, Ishikawa/shDJ-1, and Ishikawa/LV-DJ-1 cells. d The nuclear translocation of P52 was examined by western blotting in Ishikawa, Ishikawa/shDJ-1, and Ishikawa/LV-DJ-1 cells. Lamin B1 served as the nuclear marker, tubulin as the cytosolic marker. Values are expressed as the mean \pm SEM from four independent experiments. * $P < 0.05$, ** $P < 0.01$ vs Ishikawa cells.

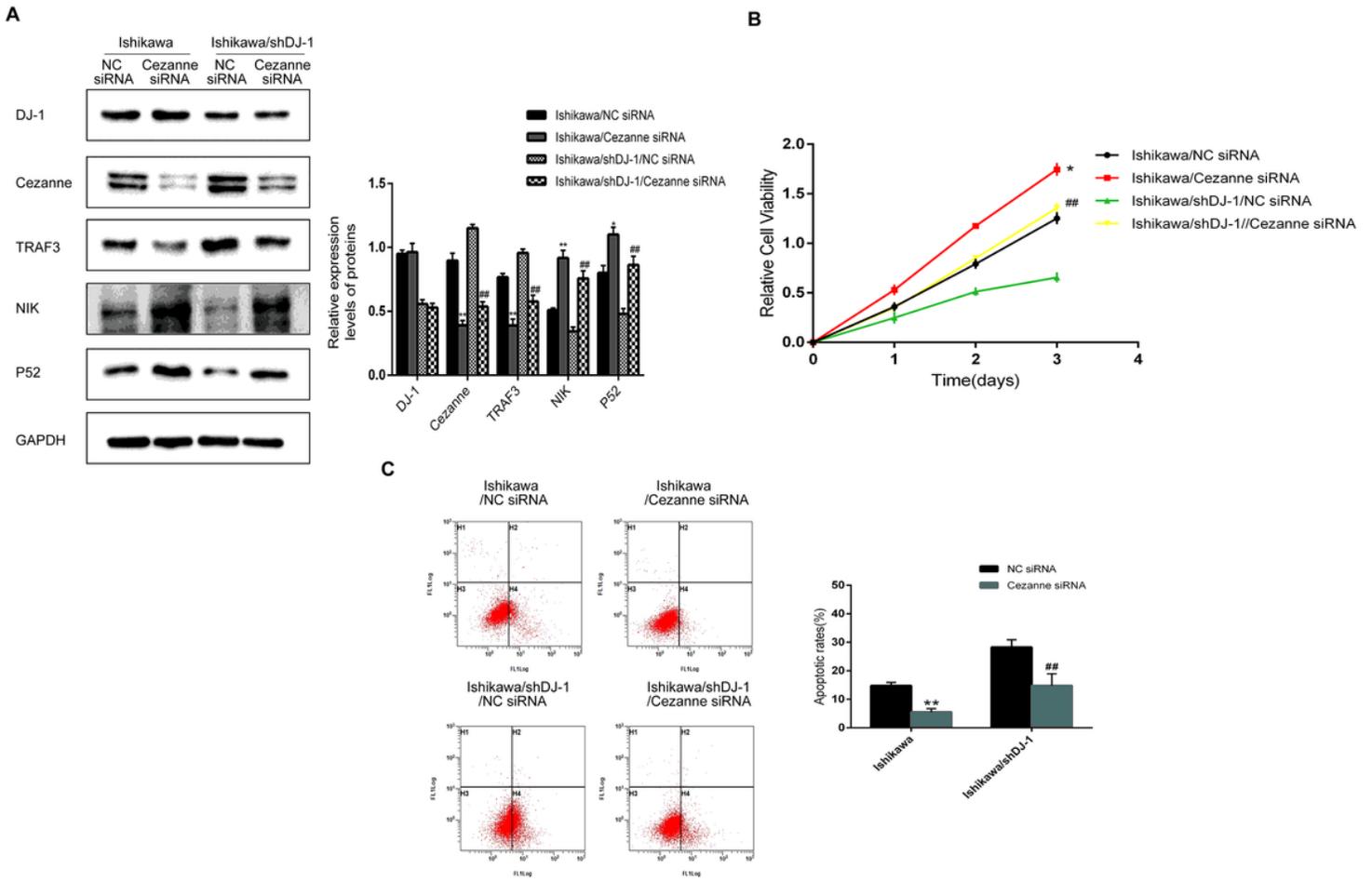


Figure 3

Effect of Cezanne knockdown on DJ-1, TRAF3, NIK, and P52 expressions and cell proliferation and apoptosis in Ishikawa and Ishikawa/shDJ-1 cells. Ishikawa and Ishikawa/shDJ-1 cells were respectively transfected for 48 h with 50 nM Cezanne siRNA or negative control (NC) siRNA. Subsequently, the levels of DJ-1, Cezanne, TRAF3, NIK and P52 were analysed by western blotting. b Cell viability was determined by CCK-8 assay. c Cell apoptosis was assessed by flow cytometry with Annexin-V-FITC/PI staining. Values are shown as the mean \pm SEM from four independent experiments. * $P < 0.05$, ** $P < 0.01$ vs Ishikawa/NC siRNA; ### $P < 0.01$ vs Ishikawa/shDJ-1/NC siRNA.

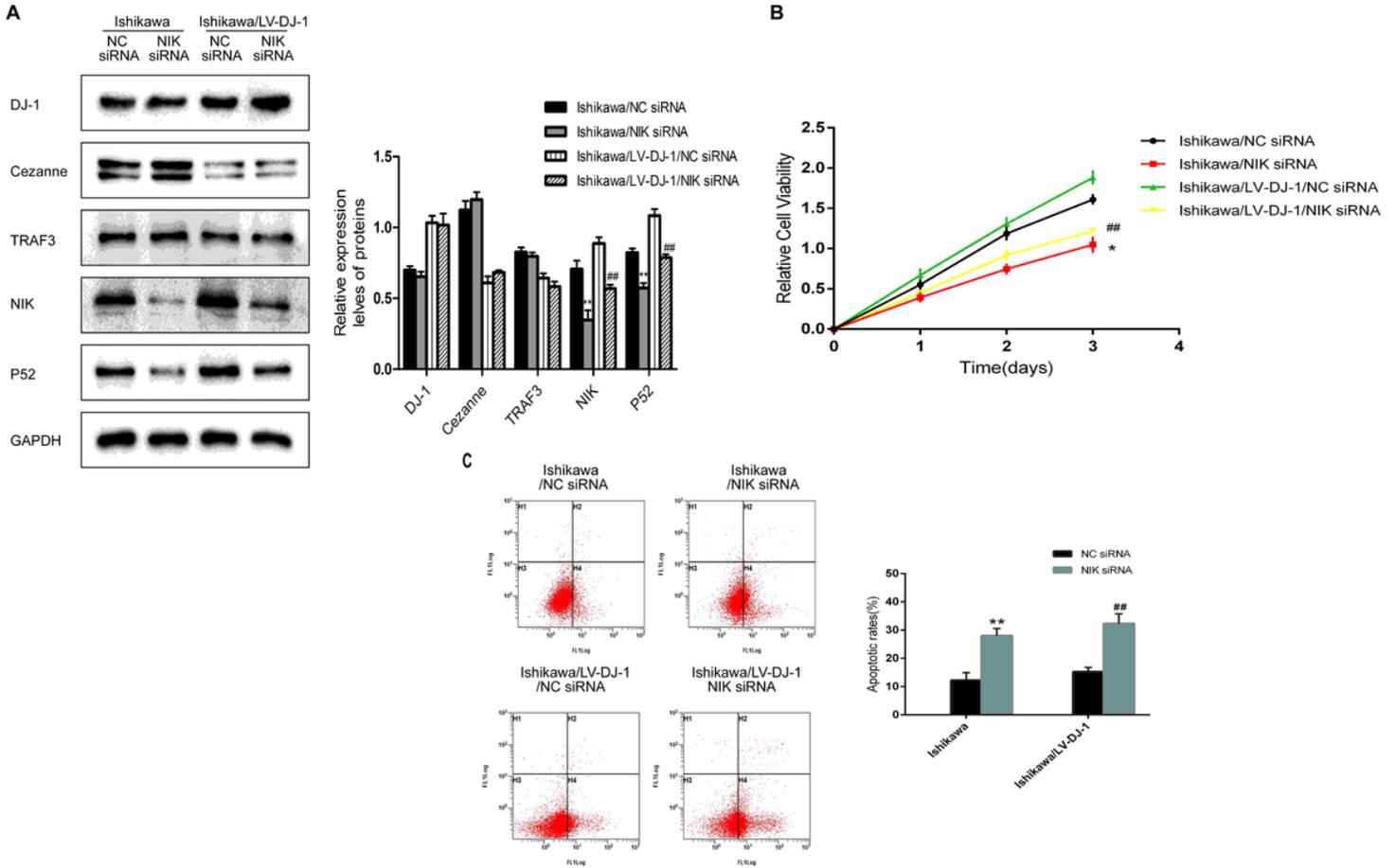


Figure 4

Effect of NIK knockdown on DJ-1, Cezanne, TRAF3, and P52 expressions and cell proliferation and apoptosis in Ishikawa and Ishikawa/LV-DJ-1 cells. Ishikawa and Ishikawa/LV-DJ-1 cells were respectively transfected for 48 h with 50 nM NIK siRNA or negative control (NC) siRNA. Subsequently, a the levels of DJ-1, Cezanne, TRAF3, NIK, and P52 were assessed by western blotting. b Cell viability was measured by CCK-8 assay. c Cell apoptosis assessed by flow cytometry with Annexin-V-FITC/PI staining. Values are shown as the mean \pm SEM from f four independent experiments. *P < 0.05, **P < 0.01 vs Ishikawa/NC siRNA; ##P < 0.01 vs Ishikawa/LV-DJ-1/NC siRNA.