

Original Article

Effect of TGFBR2 on apoptosis, invasion, migration, and signal transduction in SUDHL8 diffuse large B-cell lymphoma cell line

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Abstract: Transforming growth factor (TGF) β signaling plays a tumor suppressive role in many cell types. Although reduced expression or loss of the TGF- β type II receptors (TGFBR2) is reported in several human carcinoma cell lines, its role in lymphoma progression has not been fully clarified. In this study, we sought to determine the roles of TGFBR2 in the progression of lymphoma apoptosis, invasion, migration, and signaling transduction using SUDHL8 human lymphoma cell lines. Real-time PCR and Western blot analysis of samples from lymphoma patients as well as SUDHL8 cells revealed lowly detectable endogenous TGFBR2. TGFBR2 constructs were stably expressed in the SUDHL8 cells showing significantly cell viability inhibition, cell cycle arrest and apoptosis, accompanied by increases in the ratio of Bax/Bcl-2 and Fas/FasL. In addition, overexpression of TGFBR2 was also associated with decreased cell invasion and migration in SUDHL8 cells, which dependent on the decreased expression of MMP2 and MMP9, and inactivation of TGF- β /Smad-3 signaling pathway as well. Taken together, overexpression of TGFBR2 suppresses processes favorable to lymphoma cell viability, invasion and migration.

Keywords: Lymphoma, apoptosis, invasion, TGFBR2, TGF- β , Smad-3

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common non-Hodgkin's lymphoma making up approximately 40% newly diagnosed non-Hodgkin's lymphoma cases worldwide [1]. Although improvement in treatment protocols, a large number of DLBCL cases remain refractory to treatment. The cause of most DLBCL remains unknown, however, dysregulation of apoptosis or defective repair plays a role in its pathogenesis [2, 3].

Transforming growth factor beta (TGF- β) is a multifunctional cytokine that regulates cell proliferation, differentiation, apoptosis and extracellular matrix production [4]. TGF- β signaling has a dual role in the progression and metastasis of tumors. Under physiological conditions TGF- β inhibits tumor cell proliferation [5]. At the advanced stages of cancer development TGF- β contributes to cell proliferation, induces epithe-

lial mesenchymal transitions (EMT) and enhances invasion and migration of cancer cells, resulting in promotion of metastasis [6].

Signaling mediated by the TGF- β family cytokines is transduced through two distinct serine-threonine kinase receptors the type I and type II receptors [7]. The Type I receptor (TGFBR1) requires activation by Type II (TGFBR2) to be functional and able to bind the ligand by itself. Mutational inactivation of TGFBR2 is the most common genetic event affecting the TGF- β signaling pathway and occurs in approximately 20-30% of all colon cancers [8]. Using a mouse model that is null for TGFBR2 in the colonic epithelium, the authors demonstrated that a loss of TGFBR2 expression promotes the progression of azoxymethane-induced colon neoplasmas, suggesting that TGFBR2 is a tumor suppressor gene in colon cancer. TGFBR2-inactivating mutations are frequently also found in gastroin-

testinal, lung, and gliomas cancers associated with microsatellite instability [9, 10]. TGFBR2 is required for activation of JNK1 and the resulting upregulation of receptor of urokinase-type plasminogen activator (uPAR) expression [11].

In addition to the mutations of the TGF- β receptors, the downstream transducers of the TGF- β pathway, the Smads, have also been altered in cancer progression. Smad-2/3 is phosphorylated by an active form of TGFBR1 and associated with the common Smad-4 to form a hetero-oligomeric complex which translocates to the nucleus [7, 12]. Injured skin that lacks Smad-3 expression is shown to heal faster with a rapid rate of keratinocyte proliferation and migration [13]. In hematopoietic cells, TGF- β -induced apoptosis relies on Smad-dependent upregulation of SHIP expression, which inhibits signaling by the survival protein kinase AKT [14]. The adaptor protein DAXX has been shown to be required for TGF- β -induced apoptosis and is thought to physically associate with TGFBR2, providing an example of a Smad-independent mechanism [15]. The TGF- β signaling ultimately changes in the expression and activation of both pro- and anti-apoptotic members including Bax, Bcl-2, Fas, and FasL [16].

In the current study, we use SUDHL8 cells to examine the role of TGFBR2 in signaling. TGFBR2 was expressed in SUDHL8 cells and characterized. Effects on cell viability, apoptosis, invasion and migration were also investigated. The results show that overexpressed TGFBR2 inhibits viability, invasion and migration, and promotes apoptosis. Overexpressed TGFBR2 also activates Bax/Bcl-2 and Fas/FasL signaling, and inactivates MMP2/MMP9 and TGF- β /Smad-3 signaling in SUDHL8 cells.

Materials and methods

Patient and tissue samples

A total of 30 lymphoma tissues and paired normal human lymphocyte specimens were obtained from lymphoma patients who underwent surgery at the Shenzhen People's Hospital between February 2013 and December 2014. None of these patients had received any radiotherapy or chemotherapy. The percentage of tumor cells in the lymphoma tissues was at least 80% as determined by pathologi-

cal examination of histological slides. Subsequent to surgery, lymphoma and normal tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C until further analysis. The protocol of the present study was approved by the ethics committee of the Shenzhen People's Hospital. Written informed consent was obtained from all participants of this study.

Cell culture

Two lymphoma cell lines including SUDHL8 human diffuse large B-cell lymphoma (DLBCL) cells and EL-4 mouse T-lymphoma cells, and a normal lymphocyte were obtained from the Cell Bank of Academia Sinica (Shanghai, China) and cultured in RPMI 1640 medium with 20% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin (both from Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in a humidified atmosphere containing 5% CO₂.

Vector construction and transfections

TGFBR2 was cloned from total RNA extracted from PC-3 human prostate cancer cells by real-time PCR. The resulting full-length construct was subcloned into a lentivirus vector pLVX-AcGFP-C1 (Addgene, Cambridge, MA, USA). The constructs delivered into DLBCL cells by using the lentiviral transfection system. Briefly, 239T cells were seeded in 60 mm dishes and after 24 h were co-transfected with 2 μ g of the plasmid vector, 1 μ g pLVX-AcGFP-C1-TGFBR2, 0.1 μ g psPAX2 and 0.9 μ g pMD2G by using lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instruction. The recombinant lentivirus pLVX-AcGFP-C1-TGFBR2 was collected 48 h after transfection and used to infect SUDHL8 cells. The black pLVX-AcGFP-C1 was used as the negative control (NC).

Cell viability

Cell viability was measured by a Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Beijing, China). Briefly, SUDHL8 cells were infected either with pLVX-AcGFP-C1-TGFBR2 or with pLVX-AcGFP-C1 plated at a density of 5×10^3 cells per well into a 6-well plate. Cell viability was examined at 24, 48 and 72 h after seeding. Culture medium, 100 μ l,

TGFBR2 inhibits TGF- β /Smad-3 signaling in lymphoma

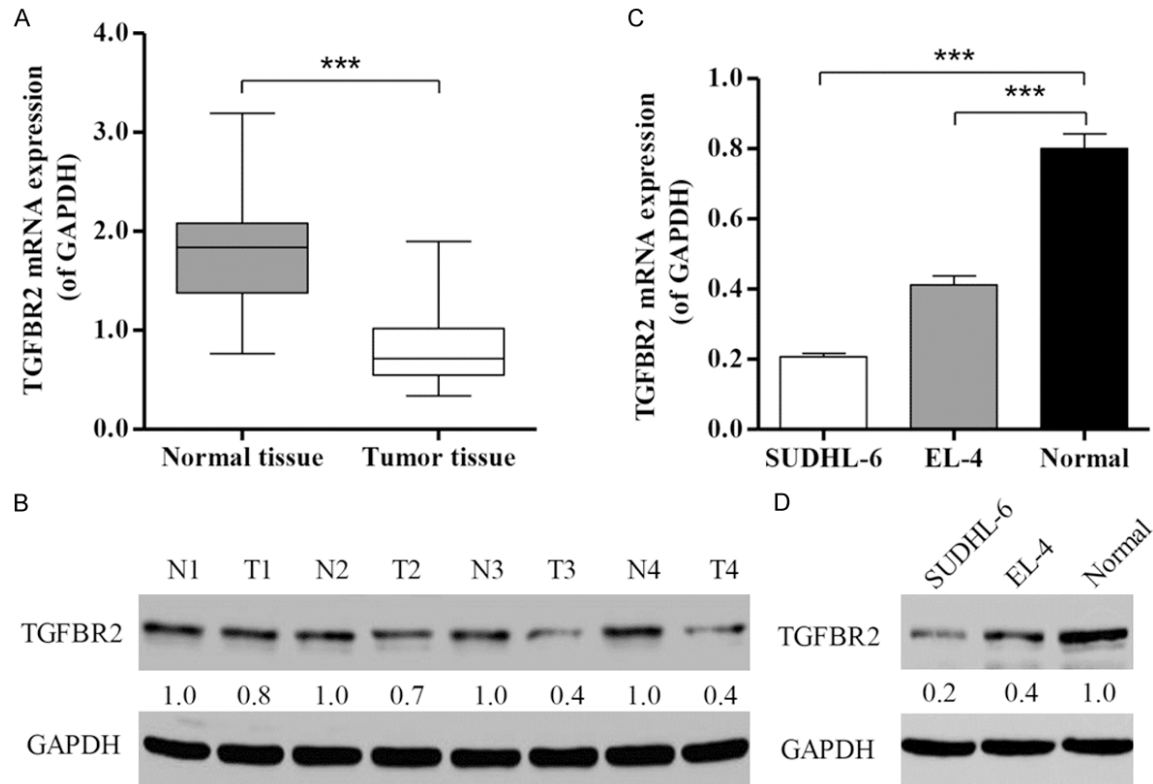


Figure 1. TGFBR2 expression is downregulated in lymphoma tissues and cell lines. A. TGFBR2 mRNA level were assessed by Real-time PCR in 30 paired lymphoma tissues and corresponding normal tissues. The boxes indicate the value range. B. Western blot analysis of protein levels of TGFBR2 in random selected four paired lymphoma tissues and corresponding normal tissues. C. TGFBR2 mRNA level were assessed by Real-time PCR in two lymphoma cell lines and a normal lymphocyte. D. Western blot analysis of protein levels of TGFBR2 in two lymphoma cell lines and a normal lymphocyte. Samples N and T1-4: Four paired normal and tumor tissues. N, normal tissue; T: tumor tissue. *** $P < 0.001$.

was added together with 20 μ l of Cell Titer 96 Aqueous One Solution and then incubated at 37°C for 2 h. Optical density was measured at 490 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell cycle assay

Cell cycle was measured by propidium iodide (PI; Sigma-Aldrich, St. Louis, MO, USA) staining and flow cytometer (BD Biosciences, San Diego, CA, USA). Briefly, SUDHL8 cells were infected either with pLVX-AcGFP-C1-TGFBR2 or with pLVX-AcGFP-C1 for 48 h and were washed once with PBS, resuspended in 500 μ l hypotonic staining buffer (sodium citrate 250 mg, Triton X 0.75 ml, PI 25 μ g, ribonuclease A 5 μ g, and 250 ml water), and analyzed by flow cytometry.

Cell apoptosis assay

Cell apoptosis was measured by flow cytometry after staining with fluorescein-conjugated

annexin V (annexin V-FITC; Beyotime Institute of Biotechnology, Haimen, China) and PI. Briefly, SUDHL8 cells were infected either with pLVX-AcGFP-C1-TGFBR2 or with pLVX-AcGFP-C1 for 48 h and were collected and re-suspended in 500 μ l binding buffer containing 5 μ l annexin V-FITC and 5 μ l PI, followed by incubation for 5 min at room temperature in the dark. Analysis was immediately performed using a flow cytometer.

Invasion and migration assays

Cell invasion was assessed using a Boyden chamber method with some modifications. The infected SUDHL8 cells were serum-starved for 24 h, following which 5×10^4 cells were seeded in 500 μ l serum-free medium in the upper chamber of an 8- μ m transwell insert (BD Biosciences) coated with 20 μ g of Matrigel (BD Biosciences). Culture medium supplemented with 10% FBS (750 μ l) was added to the lower

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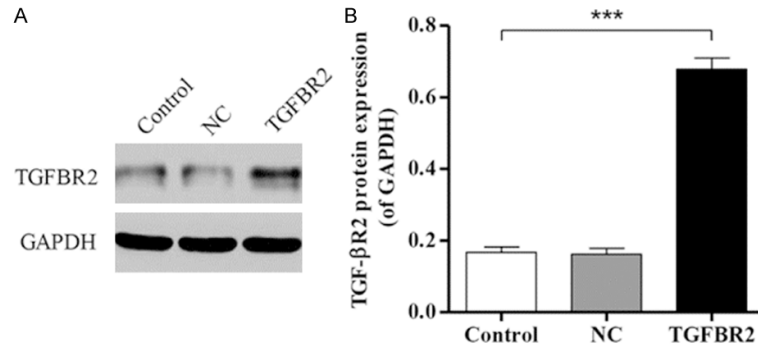


Figure 2. TGFBR2 overexpression by infection with pLVX-AcGFP-C1-TGFBR2 constructs in SUDHL8 cells. After treatment of SUDHL8 cells with pLVX-AcGFP-C1-TGFBR2, expression of TGFBR2 was analyzed by Real-time PCR (A) and Western blot (B), as described in Materials and Methods. *** $P < 0.001$.

well of the chamber. Cells that invaded into the lower chambers were counted by microscopy (Olympus Corporation, Tokyo, Japan). Migration assays were conducted using the same protocol, except that the insert was not coated with Matrigel.

Real-time PCR assay

Total RNA was isolated by TRIzol Reagent and reverse transcribed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies). The DyNAmo Flash SYBR Green qPCR kit (Finnzymes Oy, Espoo, Finland) was used according to the manufacturer's instructions. Real-time PCR was performed to detect the mRNA levels of the indicated genes. The primer sequences (sense/antisense) used were as follows: TGFBR2, 5'-ACGCCAAGGGCAACCTACAG-3' and 5'-CGCAGGGAAAGCCCAAAGTC-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-AATCCCATCACCATCTTC-3' and 5'-AGGCTGTGTCATACTTC-3' (Sangon Biotech Co., Ltd.). Data collection was performed using an ABI 7500 (Thermo Fisher Scientific) and relative quantification of gene expression was performed using the $2^{-\Delta\Delta Ct}$ method [17] with normalization to GAPDH.

Western blotting

Total protein was extracted from lymphoma cell lines or from homogenized tumor samples and corresponding normal tissues using radioimmunoprecipitation buffer (JRDUN Biotechnology Co., Ltd. Shanghai, China). The

protein concentration was assessed using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific). 50 μ g protein lysates was separated by 10-15% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Sigma-Aldrich), followed by incubated with primary antibodies and secondary antibody labeled with horseradish peroxidase. The blots were developed using enhanced chemiluminescence (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and

determined using Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistics

Values are expressed as the mean value \pm standard deviation. The software used for statistical analysis was GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Differences between the groups were analyzed using unpaired two-tailed Student's t-test. The limit of significance for all analyses was defined as a P value of 0.05.

Results

TGFBR2 is downregulated in lymphoma tissues and SUDHL8 cells

Real-time PCR and Western blot analysis were used to assess the mRNA and protein levels of TGFBR2 in 30 paired lymphoma and normal tissues. As shown in **Figure 1A**, the mRNA expression of TGFBR2 was significantly decreased in lymphoma tissues when compared with that in paired normal tissues. Similarly, TGFBR2 protein expression levels in randomly selected four lymphoma tissues were decreased compared with those in their paired normal tissues (**Figure 1B**). These results indicated that TGFBR2 may suppress lymphoma carcinogenesis.

Next, we compared gene and protein expression of TGFBR2 in two lymphoma cell lines, EL-4 and SUDHL8 cells, and a normal lymphocyte. We found that EL-4 and SUDHL8 cells had a lower level of TGFBR2 expression compared

TGFBR2 inhibits TGF- β /Smad-3 signaling in lymphoma

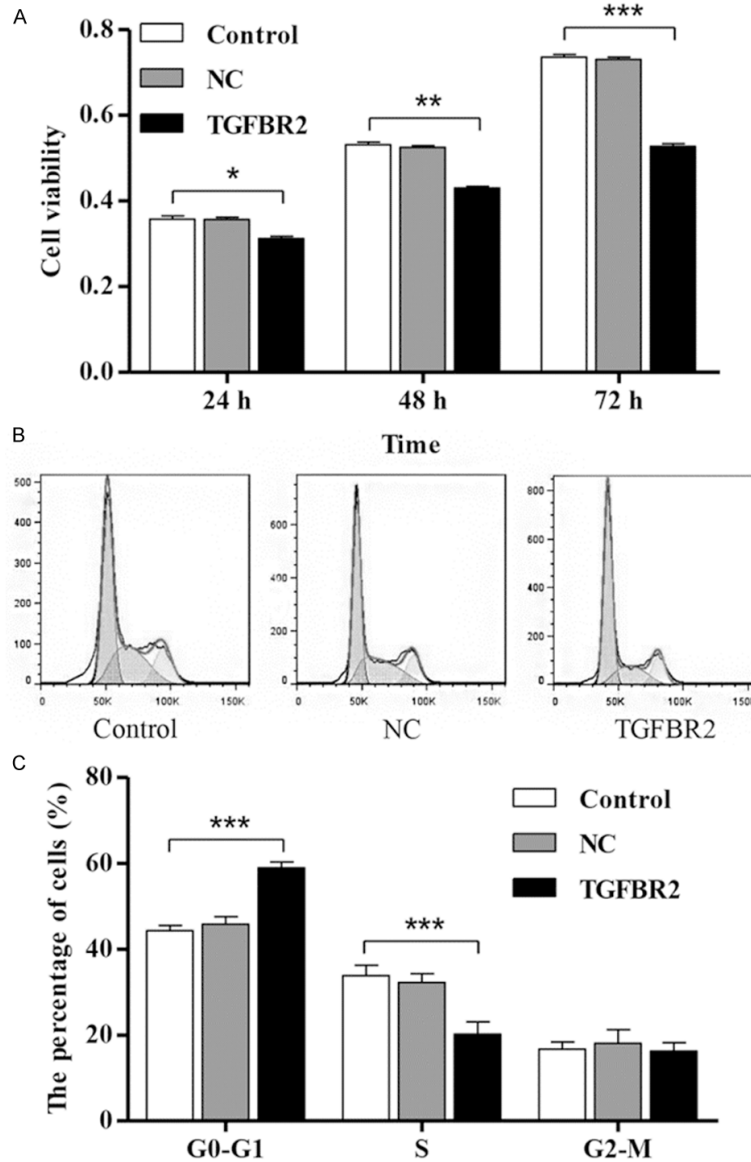


Figure 3. Effect of TGFBR2 overexpression on viability and cell cycle of SUDHL8 cells. After treatment of SUDHL8 cells with pLVX-AcGFP-C1-TGFBR2, cell viability (A) and cell cycle (B, C) were measured, as described in Materials and Methods. *** $P < 0.001$.

with the normal lymphocyte, with the lower expression detected in SUDHL8 cells compared with the EL-4 cells (Figure 1C and 1D). The SUDHL8 cells were therefore used for subsequent experiments. Taken together, these results support the notion that TGFBR2 may act as a tumor suppressor in lymphoma.

Characterization of pLVX-AcGFP-C1-TGFBR2-infected SUDHL8 cells

For this study, a human TGFBR2 construct was engineered in pLVX-AcGFP-C1 lentivirus vector.

Full-length TGFBR2 was generated by real-time PCR from RNA prepared from PC-3 cells. The results demonstrated that SUDHL8 cells with pLVX-AcGFP-C1-TGFBR2 infection showed highly expressed level of TGFBR2 compared with SUDHL8 cells without treatment (Figure 2A and 2B). TGFBR2 expression was increased by 2.8-fold in SUDHL8 cells infected with pLVX-AcGFP-C1-TGFBR2. SUDHL8 cells infected with black pLVX-AcGFP-C1 vector (NC) had no effect on the protein expression of TGFBR2. These findings suggest that SUDHL8 cells with TGFBR2 overexpression was successful construction.

Effect of TGFBR2 overexpression on SUDHL8 cell viability and cell cycle

Turning to longer-term cellular responses, we examined cell viability for the SUDHL8 cells of interest. As shown in Figure 3A, the cell viability for SUDHL8 cells was significantly reduced by $12.7\% \pm 0.1\%$, $19.1\% \pm 0.1\%$, and $28.3\% \pm 0.2\%$ at 24, 48 and 72 h after injected with pLVX-AcGFP-C1-TGFBR2, respectively, compared with that for cells without treatment. Additional study indicated that cell cycle was significantly increased the G0-G1-phase population by $24.7\% \pm 0.11\%$ by infection with pLVX-AcGFP-C1-TGFBR2 in SUDHL8 cells (Figure 3B and 3C), indicating G0-G1-phase arrest. In contrast, the SUDHL8 cells infected with black pLVX-AcGFP-C1 vector (NC) had no effect on the cell viability and cell cycle.

Effect of TGFBR2 overexpression on SUDHL8 cell apoptosis

In addition, the apoptotic rate of SUDHL8 cells infected with pLVX-AcGFP-C1-TGFBR2 was examined by flow cytometry. The results

TGFR2 inhibits TGF- β /Smad-3 signaling in lymphoma

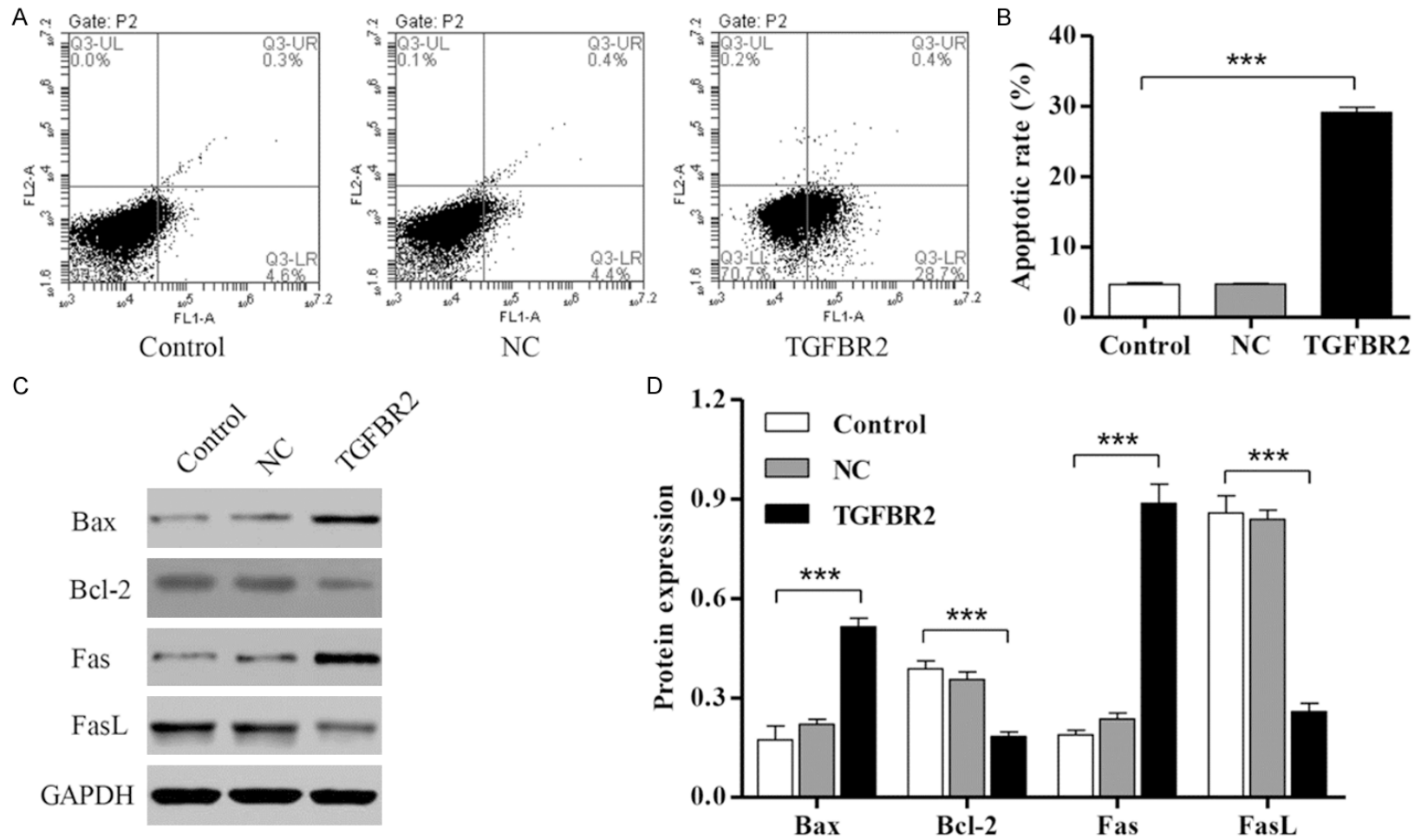


Figure 4. Effect of TGFR2 overexpression on apoptosis of SUDHL8 cells. After treatment of SUDHL8 cells with pLVX-AcGFP-C1-TGFR2, cell apoptosis (A, B) and the expression of apoptosis-related markers (C, D) were measured, as described in Materials and Methods. *** $P < 0.001$.

TGFBR2 inhibits TGF- β /Smad-3 signaling in lymphoma

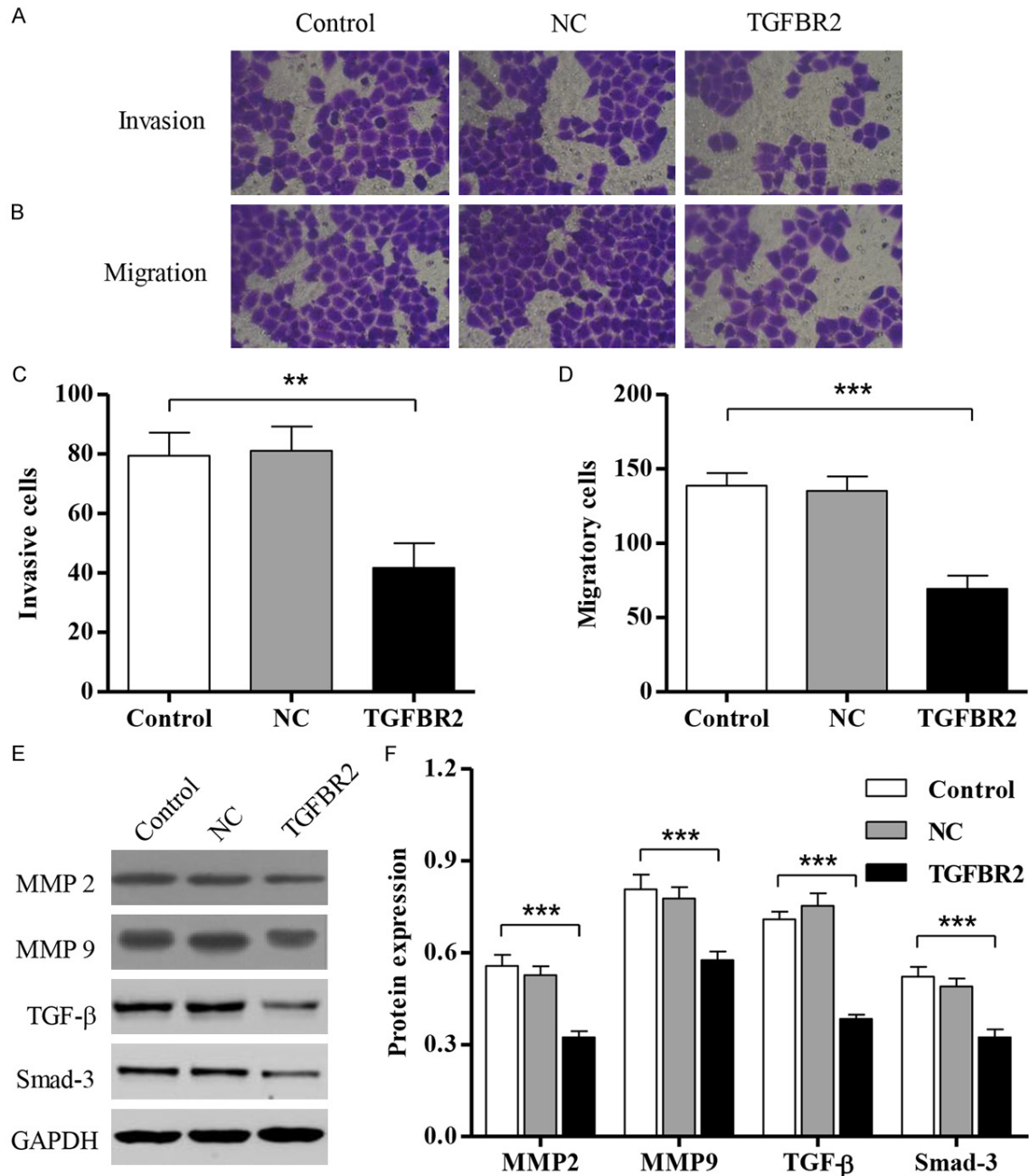


Figure 5. Effect of TGFBR2 overexpression on invasion and migration of SUDHL8 cells. After treatment of SUDHL8 cells with pLVX-AcGFP-C1-TGFBR2, cell invasion (A, C), migration (B, D) and the expression of metastasis-related markers (E, F) were measured, as described in Materials and Methods. ** $P < 0.01$, *** $P < 0.001$.

showed that the apoptotic rate was significantly increased in the TGFBR2 overexpression groups (>4.9-fold) compared with that in the SUDHL8 cells without treatment (Figure 4A and 4B). However, the SUDHL8 cells infected with blank pLVX-AcGFP-C1 vector (NC) had no effect on the cell apoptosis. These data suggest that TGFBR2 may promote apoptosis in SUDHL8 cells.

The proteins of the Bcl-2 family perform critical roles in the regulation of apoptosis by functioning as promoters (e.g. Bax) or as inhibitors (e.g. Bcl-2) of cell death processes [18, 19]. Western blotting was performed to detect the protein expression levels of Bcl-2 and Bax in the pLVX-AcGFP-C1-TGFBR2-infected SUDHL8 cells (Figure 4C and 4D). TGFBR2 overexpression resulted in a marked reduction in the level

of the antiapoptotic protein, Bcl-2, with a concomitant increase in the level of proapoptotic protein Bax, compared with the SUDHL8 cells without treatment. The Fas/FasL system is another important apoptosis signal transduction pathway in which a ligand-receptor interaction activates the cell death pathway [20]. As shown in **Figure 3C** and **3D**, the expression ratio of Fas/FasL was significantly increased in pLVX-AcGFP-C1-TGFBR2-infected SUDHL8 cells compared with the SUDHL8 cells without treatment. However, the SUDHL8 cells with blank pLVX-AcGFP-C1 vector (NC) infection had no effect on the expression of these apoptosis-related proteins. These data suggest that the overexpression of TGFBR2 expression may increase the ratio of Bax/Bcl-2 and Fas/FasL, which may contribute to the increase in cell apoptosis.

Effect of TGFBR2 overexpression on SUDHL8 cell invasion and migration

To examine whether TGFBR2 affected the invasive ability of SUDHL8 cells, a Matrigel-coated membrane chamber invasion assay was performed. As shown in **Figure 5A** and **5C**, a markedly reduced invasive ability was observed in TGFBR2 overexpression cells compared with the cells without treatment. The number of invading pLVX-AcGFP-C1-TGFBR2-infected cells was $41.3\% \pm 3.3\%$ compared with the control cells (Control, 171.3 ± 9.6 ; NC, 168.7 ± 22.7 ; TGFBR2 overexpression, 100.7 ± 15.5). Additionally, the effect of TGFBR2 overexpression on cell migration was also analyzed. As shown in **Figure 5B** and **5D**, TGFBR2 overexpression significantly decreases migration of SUDHL8 cells by 22.9% compared with the SUDHL8 cells without treatment (Control, 264.7 ± 33.6 ; NC, 258.7 ± 37.9 ; TGFBR2 overexpression, 204.0 ± 10.8). However, the SUDHL8 cells with blank pLVX-AcGFP-C1 vector (NC) infection had no effect on cell invasion and migration.

To further investigate the signal transduction pathway involved in the invasion and migration of SUDHL8 stratified by the median of TGFBR2 expression, the MMP2, MMP9, TGF- β and Smad-3 expression analysis by Western blotting was performed. As shown in **Figure 5E** and **5F**, TGFBR2 overexpression resulted in a marked reduction in the level of MMP2, MMP9, TGF- β and Smad-3, compared with the SUDHL8

cells without treatment. However, the SUDHL8 cells with blank pLVX-AcGFP-C1 vector (NC) infection had no effect on these metastasis-related protein expressions. These data suggest that the overexpression of TGFBR2 expression may decrease the levels of MMP2, MMP9, TGF- β and Smad-3, which may contribute to the decrease in cell invasion and migration.

Discussion

The work described herein has examined several aspects of the role of TGFBR2 in lymphoma cells. Our studies address the regulation of TGFBR2, its effects on cell viability, apoptosis, invasion and migration, and its effects on signal transduction. In general, the major findings are that TGFBR2 enhances processes favorable to tumor formation in SUDHL8 cells. The cellular effects observed with TGFBR2 overexpression are associated with higher basal levels of Bax/Bcl-2 and Fas/FasL, and with lower basal levels of MMP2, MMP9, TGF- β and Smad-3.

We compared TGFBR2 expression between lymphoma tissues and adjacent-normal tissues in Shenzhen People's Hospital. TGFBR2 expression was significantly decreased in lymphoma tissues compared with the adjacent-normal tissues. Our results are consistent with previous reports that TGFBR2 is lowly expressed in prostate, colon, breast and gastric cancer [21-24]. Additionally, we assessed TGFBR2 expression in lymphoma cell lines and found that SUDHL8 cells highly expressed TGFBR2 mRNA and protein. Importantly, TGFBR2 overexpression in SUDHL8 cells by infection with pLVX-AcGFP-C1-TGFBR2 constructs showed decreases in cell viability, invasion and migration, and induction of cell cycle arrest and apoptosis. Previous studies showed that TGFBR2 was identified as direct target of miRNA-165 in pancreatic cancer cell lines, has been revealed to be major component of TGF- β signaling pathway and to induce EMT through repression of CDH1/E-cadherin [25, 26].

It has recently been shown that TGFBR2 is responsible for stimulating the MAPK-ERK pathway, which may mediate apoptosis. More specifically, high levels of TGFBR2 mediate this pro-apoptotic response to TGF- β signaling [27]. These findings are similar to our results that TGFBR2 overexpression promotes cell apopto-

sis of SUDHL8 cells, which accompanied with increased levels of Bax/Bcl-2 and Fas/FasL. Reduced expression of Fas and/or increased expression of FasL have been detected in many types of human cancer, including lung cancer, prostate cancer and lymphoma [28-30], suggesting that the Fas/FasL system may play an important role in many cancer formation. There is strong evidence demonstrating that increased expression of Fas may promote elimination of transformed cells, but decreased expression of FasL may suppress the ability of tumor cells to counterattack the immune system by killing Fas sensitive lymphocytes and therefore attenuate cancer development [31].

Invasion and migration are two of the most important marks of cancer and function as the lethal factors for malignant cancer in general and lymphoma in particular [32, 33]. Management of migration will therefore contribute to the improvement of prognosis for the lymphoma patient. In SUDHL8 cells, overexpression of TGFBR2 significantly inhibited cell invasion and migration, which accompanied with decreased levels of MMP2 and MMP9 and inactivation of TGF- β /Smad-3 signaling pathway. In agreement with our findings that down-regulation of TGFBR2 in glioblastoma cells effectively diminished the expression of TGFBR2 and abolished TGF- β -activated Smad signaling [34]. Retrovirus-mediated introduction of a dominant negative TGFBR2 to bone marrow cells led to generation of leukocytes capable of potent anti-tumor response and suppression of metastasis in melanoma and prostate cancer model [35].

In conclusion, our studies indicate that expression of TGFBR2 enhances Bax/Bcl-2 and Fas/FasL levels, and inactivates TGF- β /Smad-3 signaling in SUDHL8 cells. Overexpression of TGFBR2 exerts inhibitory effects on cell viability, invasion and migration, and exerts enhanced effects on cell apoptosis. TGFBR2 and related molecules may serve as a potential therapeutic target for metastatic lymphoma.

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Disclosure of conflict of interest

None.

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TGFBR2 inhibits TGF- β /Smad-3 signaling in lymphoma

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TGFBR2 inhibits TGF- β /Smad-3 signaling in lymphoma

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