

# Knockdown of long non-coding RNA PVT1 inhibits the proliferation of Raji cells through cell cycle regulation

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**Abstract.** Long non-coding RNA plasmacytoma variant translocation 1 (*PVT1*) has been reported to be associated with oncogenesis. However, the functional role of *PVT1* in Burkitt lymphoma has not yet been addressed. The purpose of the present study was to investigate the effect of *PVT1* knockdown by small interfering RNA (siRNA) on the proliferation of Burkitt lymphoma Raji cells and to explore its possible mechanism of action. An effective siRNA targeting *PVT1* was screened and the corresponding short hairpin RNA (shRNA) was reconstructed into a lentiviral vector. Cell proliferation and cell cycle distribution were assessed by Cell Counting kit-8 assay and flow cytometry, respectively. Protein expression levels of c-Myc, cyclin-dependent kinase inhibitor1A (*CDKN1A*, P21) and cyclin E1 (*CCNE1*) were detected by western blotting. A polymerase chain reaction (PCR) array was used to analyse the expression of genes associated with the cell cycle. *PVT1* knockdown markedly suppressed proliferation, and induced cell cycle arrest at the G<sub>0</sub>/G<sub>1</sub> phase in Raji cells. Protein expression levels of c-Myc and *CCNE1* were reduced, whereas P21 protein expression was markedly increased following downregulation of *PVT1* in Raji cells. The cell cycle PCR array revealed that 54 genes were upregulated and 26 genes were downregulated in Raji cells following *PVT1* knockdown. Reverse transcription-quantitative PCR demonstrated that cyclin G2 (*CCNG2*), *CDKN1A*, Retinoblastoma-like 2 (*RBL2*, p130), HUS1 checkpoint homolog, cyclin dependent kinase inhibitor 3 (*CDKN3*) and cyclin dependent kinase inhibitor 1B (*CDKN1B*) expression were upregulated, whereas the expression levels of *CCNE1*, cyclin D1 (*CCND1*) and cell division cycle 20 (*CDC20*) were downregulated in Raji cells with *PVT1* knockdown. In conclusion, *PVT1* knockdown may inhibit the proliferation of

Raji cells by arresting cells in G<sub>0</sub>/G<sub>1</sub> phase. Furthermore, inhibition of cell proliferation may be associated with a reduction in c-Myc expression and alterations in the expression levels of cell cycle-associated genes.

## Introduction

Burkitt lymphoma (BL) is an aggressive form of B-cell lymphoma that mostly affects children and adolescents. In BL, dysregulation of the oncogenic transcription factor Myc is considered to be the major driving force of lymphoma development (1,2). However, the molecular mechanisms of the pathogenesis of BL have not been fully elucidated.

Long non-coding RNAs (lncRNAs) are defined as cellular RNA molecules, >200 base pairs in length, without protein-coding capacity, which act as key regulators at transcriptional and post-transcriptional levels (3,4). Increasing evidence indicates that lncRNAs are involved in various biological processes, including DNA replication, stem cell pluripotency, proliferation and apoptosis (4,5). LncRNAs serve a critical role in the development of various types of human cancer, including haematopoietic malignancies, and may be potential targets for tumour treatment (5).

Human plasmacytoma variant translocation 1 (*PVT1*), also known as the Pvt1 oncogene, is a lncRNA that is homologous to the mouse plasmacytoma variant translocation gene. *PVT1* is located on chromosome 8, ~55 kb distal to the MYC proto-oncogene bHLH transcription factor (*c-Myc*) gene, and is frequently involved in translocations that occur in variant BL (6,7). The overexpression of *PVT1* is one of the most frequent events in a variety of malignant diseases, including melanoma (8), hepatocellular carcinoma (9,10), thyroid cancer and colorectal cancer (11,12). A number of studies have demonstrated that lncRNA *PVT1* interacts with the proliferation-associated nucleolar proteins NOP2 or c-Myc, stabilizes these proteins against degradation, and negatively modulates microRNA (miRNA) as a competing endogenous RNA or a molecular sponge, in order to exert a tumour-promoting effect (8,10,13,14). A large genome-wide association study identified one high-risk single nucleotide polymorphism (SNP; rs2608053) for classic Hodgkin lymphoma at 8q24 near the *Myc/PVT1* locus, which is associated with patient outcome (15). In a meta-analysis, two independent SNPs, rs13255292 and rs4733601, at 8q24.21 were identified for

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diffuse large B cell lymphoma (16). However, the functional role and molecular mechanism of *PVT1* in BL remain unclear.

In the present study, knockdown of *PVT1* was able to inhibit Raji cell growth by regulating cell cycle progression. Furthermore, it was revealed that *PVT1* may serve an important role in G<sub>0</sub>/G<sub>1</sub> arrest, which may be associated with the expression of *c-Myc* and cell cycle-associated genes. Together, these results indicated that lncRNA *PVT1* may serve a critical role in Raji cell proliferation, and may be considered a candidate target for novel treatment of human BL.

## Materials and methods

**Cell culture and transfection.** The Raji cell line was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China. <http://www.cellbank.org.cn/index.asp>). Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% heat-inactivated foetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified incubator with 5% CO<sub>2</sub>. Four small interfering RNA (siRNA) sequences targeting *PVT1* (siRNA54, siRNA176, siRNA845, siRNA1055) and a scrambled control (SC) siRNA were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequences of the siRNA are as follows: *PVT1*-siRNA54: 5'-CCUGAUGGAUUUACA GUGATT-3', *PVT1*-siRNA176: 5'-GCUGAAUGCCUCAUG GAUUTT-3', *PVT1*-siRNA845: 5'-CCUGUUACACCUGGG AUUUTT-3', *PVT1*-siRNA1055: 5'-GCUUCUCCUGUUGCU GCUATT-3', SC-siRNA: 5'-GCUACGAUCUGCCUAAGA UTT-3'. Raji cells (3–4 × 10<sup>5</sup> cells/ml) in the exponential growth phase were grown for 24 h, then *PVT1*-siRNA54, -siRNA176, -siRNA845 and -siRNA1055 were transfected into Raji cells using HiPerfect (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol. In addition to non-silencing SC siRNA control, cells in the mock transfection group were treated with HiPerfect agents only. The total concentration of siRNA applied in each case was 100 nM. At 24 and 48 h post-transfection, silencing of *PVT1* RNA was examined.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA from the Raji cells of post-transfection was isolated using a TRIzol® total RNA isolation system (Invitrogen; Thermo Fisher Scientific, Inc.). RNA purity and concentration were measured using a spectrophotometer, and RNA was reverse transcribed into first-strand cDNA using random hexamer primers and the reverse transcriptase Superscript II kit (Toyobo Life Science, Osaka, Japan), according to the manufacturer's protocol. The 2<sup>-ΔCt</sup> method (17) was used to analyse the relative changes in gene expression in RT-qPCR experiments with SYBR Green (Toyobo Life Science, Japan). The primers were designed and synthesized by Shanghai GeneChem Co., Ltd. (Shanghai, China). The primer sequences are listed in Table I. *GAPDH* was used as a reference gene. The total PCR reaction volume was 20 μl and reaction conditions were as follows: Enzyme activation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec, 60°C for 15 sec and 72°C for 32 sec. At the end of each run a melting curve was performed, starting at 65°C and reaching 95°C with an increase of 1°C/2 sec, to verify primer specificities,

specificity of amplification and absence of primer dimers. RT-qPCR was repeated in at least three separate experiments.

**Western blot analysis.** Cells were washed with PBS (10 mM, pH 7.4) and incubated in 200 μl cell lysis buffer (Beyotime Institute of Biotechnology) on ice for 30 min, and centrifuged at 13,000 × g for 15 min at 4°C. The protein content of the cell lysate was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's protocol. Whole cell extracts equivalent to 100 μg total protein were separated by 8% SDS-PAGE and transferred to nitrocellulose membranes (Gibco; Thermo Fisher Scientific, Inc.) at 18 V for 10–15 min. The blots were immersed in blocking buffer (10% non-fat dry milk, 1% Tween-20, 20 mM Tris-buffered saline, pH 7.5) for 1 h at room temperature, and were then incubated with appropriate anti-human primary antibodies [rabbit immunoglobulin G (IgG) anti-c-Myc (1:1,000 dilution; cat. no., sc-40, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-P21 (1:1,000 dilution; cat. no., ab109520, Abcam, Cambridge, MA, USA) or anti-cyclin E1 (CCNE1) (1:1,000 dilution; cat. no., ab33911, Abcam, Cambridge, MA, USA USA), or mouse anti-GAPDH/IgG (1:1,000 dilution; cat. no., ab8245, Abcam, Cambridge, MA, USA) USA] in blocking buffer overnight at 4°C. Blots were then incubated with anti-rabbit (cat. no., sc-2357) or anti-mouse (cat. no., sc-516102) horseradish peroxidase-conjugated secondary antibodies (1:2,000 dilution; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature and bands were detected by chemiluminescence using Enhanced Chemiluminescence Hyperfilm (EMD, Millipore, USA).

***PVT1* short hairpinRNA (shRNA)-expressing plasmid construction and cloning screen.** To stably knockdown the expression of *PVT1*, a shRNA sequence targeting *PVT1* (siRNA1055) was cloned into the pGV248-lentivirus vector (Shanghai GenePharma Co., Ltd.). Subsequently, *PVT1* knockdown vectors were reconstructed and sequenced. pGV248 vector containing the negative control (NC) shRNA was used as a control. Subsequently, 293T cells from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (Gibco; ThermoFisher Scientific, Inc., Waltham, MA, USA) containing 10% FBS, maintained at 37°C in a humidified incubator with 5% CO<sub>2</sub> and transfected with pGV248-shRNAs, Helper 1.0 and Helper 2.0 (Shanghai GenePharma Co., Ltd.) using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The media were replaced with 10 ml fresh medium after incubation overnight. The virus-containing supernatants (LV-*PVT1*-shRNA and LV-NC-shRNA) were collected after 48 h. Raji cells were infected, respectively, with LV-*PVT1*-shRNA and LV-NC-shRNA (multiplicity of infection=250) at 37°C for 72 h, and were then selected using 4 μg/ml puromycin to screen single cell clones for 2 weeks and expand the culture for 4 weeks. Green fluorescence of cells was observed by inverted fluorescence microscopy. The knockdown efficiency was measured using RT-qPCR. Cell proliferation and cell cycle distribution were analysed in positive clone Raji cells. In subsequent assays, Raji cells were divided into three groups: Blank control group (cells without infection), NC group (cells with LV-NC-shRNA) and *PVT1* knockdown group (cells with LV-*PVT1*-shRNA).

Table I. Sequences of primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>PVT1</i>	GTCTTGGTGCTCTGTGTTC	CCCGTTATTCTGTCCTTCT
<i>CCNG2</i>	GGTTTCACCTTCATAAGAGCC	GCTGAGTTTGATTGAGGCTAC
<i>CDKN1A</i>	AGCGACCTTCCTCATCCACC	AAGACAACACTACTCCCAGCCCCATA
<i>HUS1</i>	ATGGGTCACAATGCGGCTACT	GCTAACATCGGAAACTTATCTCG
<i>CDKN1B</i>	GGGCAAGTACGAGTGGCAAGAG	CAAATGCGTGTCCTCAGAGTTAGC
<i>CDKN3</i>	AGTCCCAAACCTTCTGGATCTCTAC	CTCCCAAGTCCTCCATAGCAGTG
<i>RBL2</i>	TTCTGGTAGTGCTGGCTGGTG	GGGTGACTGAAGTTCGTGCTG
<i>CCNE1</i>	AAAGGTTTCAGGGTATCAGTGGTG	TCTCTGTGGGTCTGTATGTTGTGTG
<i>CCND1</i>	CCCTCGGTGTCCTACTTCAAATGT	GGAAGCGGTCCAGGTAGTTCAT
<i>CDC20</i>	TCACCAGAGCTTGCACTCCAC	ACCTGCCGTTACATTCCTTCC
<i>GAPDH</i>	GGACCTGACCTGCCGTCTAG	GTAGCCAGGATGCCCTTGA

**Cell proliferation assay.** For the quantitative determination of cellular proliferation, a Cell Counting kit-8 (CCK8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) assay was performed. The assay was performed for Raji cells carrying LV-*PVT1*-shRNA and LV-NC-shRNA. Cells were seeded at a density of  $5 \times 10^4$  cells/well in 96-well plates and cultured at 37°C with 5% CO<sub>2</sub> in a humidified incubator. According to the manufacturer's protocols 10 µl CCK8 solution was added 4 h prior to the end of incubation at 37°C at 24, 48, 72 and 96 h. Cell proliferation was measured using a spectrophotometer (Bio-Rad Laboratories, Inc.) at an absorbance wavelength of 450 nm. All experiments were repeated three times.

**Cell cycle assay.** Raji cells containing LV-*PVT1*-shRNA or LV-NC-shRNA were harvested. The cells were fixed with 70% ethanol at -20°C overnight and stained with propidium iodide (5 µg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in the presence of ribonuclease A (1 mg/ml; Sigma-Aldrich; Merck KGaA) for 30 min at room temperature. The cell cycle distribution was analysed via flow cytometry (BD Biosciences, San Jose, CA, USA). All experiments were repeated three times.

**RT-PCR-based array analysis.** Human Cell Cycle RT<sup>2</sup> RNA QC PCR arrays (Qiagen, Inc.) were used to screen a panel of 84 representative cell cycle-associated genes in Raji cells infected with LV-*PVT1*-shRNA. Total RNA was isolated from the LV-*PVT1*-shRNA and LV-NC-shRNA cells using the Qiagen RNeasy Mini kit (Qiagen, Inc.) according to the manufacturer's protocol. RNA was quantified by the NanoDrop<sup>®</sup> ND-1000 (NanoDrop; Thermo Scientific, Inc., Wilmington, DE, USA), and quality was assessed by visualizing 18S and 28S ribosomal RNA bands separated by 1% agarose gel electrophoresis with ethidium bromide staining (Sigma-Aldrich; Merck KGaA). According to manufacturer's protocol, the first-strand cDNA was obtained using an RT<sup>2</sup> First Strand kit (Qiagen, Inc.). qPCR was conducted using 2X RT<sup>2</sup> SYBR Green qPCR Master Mix (Qiagen, Inc.) on a Bio-Rad Real-Time PCR system (Bio-Rad Laboratories, Inc.) according to the RT<sup>2</sup> Profiler PCR array protocols under the following conditions: 95°C for 10 min, then 40 cycles at 95°C for 15 sec and 60°C for 1 min. At the end of each run a melting curve was performed, extending at 60°C

for 1 min. Microarray data were normalized for housekeeping genes (*ACTB*, *B2M*, *GAPDH*, *HPRT1*, *RPLP0*) by calculating the  $\Delta C_q$  and  $2^{-\Delta\Delta C_q}$  (17) for each gene of interest in the plate. Fold changes of each gene between the LV-*PVT1*-shRNA and LV-NC-shRNA groups were calculated as  $2^{-\Delta\Delta C_q}$ , and scatter plots were analysed.

**Statistical analysis.** All data are expressed as the means  $\pm$  standard deviation. Differences among three or more groups were compared using one-way analysis of variance (ANOVA), followed by SNK post hoc test. All data were analysed using SPSS v13.0 software (SPSS, Inc., Chicago, IL, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

***PVT1* siRNAs or shRNA suppress *PVT1* RNA and c-Myc protein expression in Raji cells.** To determine the efficiency of *PVT1* inhibition following siRNA (siRNA54, siRNA176, siRNA845, siRNA1055) transfection, *PVT1* RNA expression was analysed. RT-qPCR data were obtained from at least three independent experiments. The relative qPCR formula:  $2^{-\Delta C_t} \times 100\%$  was used. As shown in Fig. 1A, at 24 and 48 h after transient transfection, the relative expression levels of *PVT1* RNA in Raji cells transfected with *PVT1* siRNA (siRNA1055) were significantly lower than in cells transfected with the control SC siRNA ( $P < 0.05$ ). Conversely, siRNA54, siRNA176, siRNA845 had no significant effect on the expression of *PVT1* RNA when compared respectively with the control SC siRNA. A specific siRNA (siRNA1055) against *PVT1* was used to stably knockdown *PVT1* expression. Subsequently, lentiviral vectors carrying LV-*PVT1*-shRNA or LV-NC-shRNA were successfully constructed. Raji cells stably carrying *PVT1*-shRNA or NC-shRNA were screened. Raji cells expressing LV-*PVT1*-shRNA were observed to exhibit green fluorescence under an inverted fluorescence microscope (data not shown). As shown in Fig. 1B, following stable infection with the LV-*PVT1*-shRNA expression vector, the expression levels of *PVT1* RNA were significantly reduced compared with in the LV-NC-shRNA and blank control cell groups

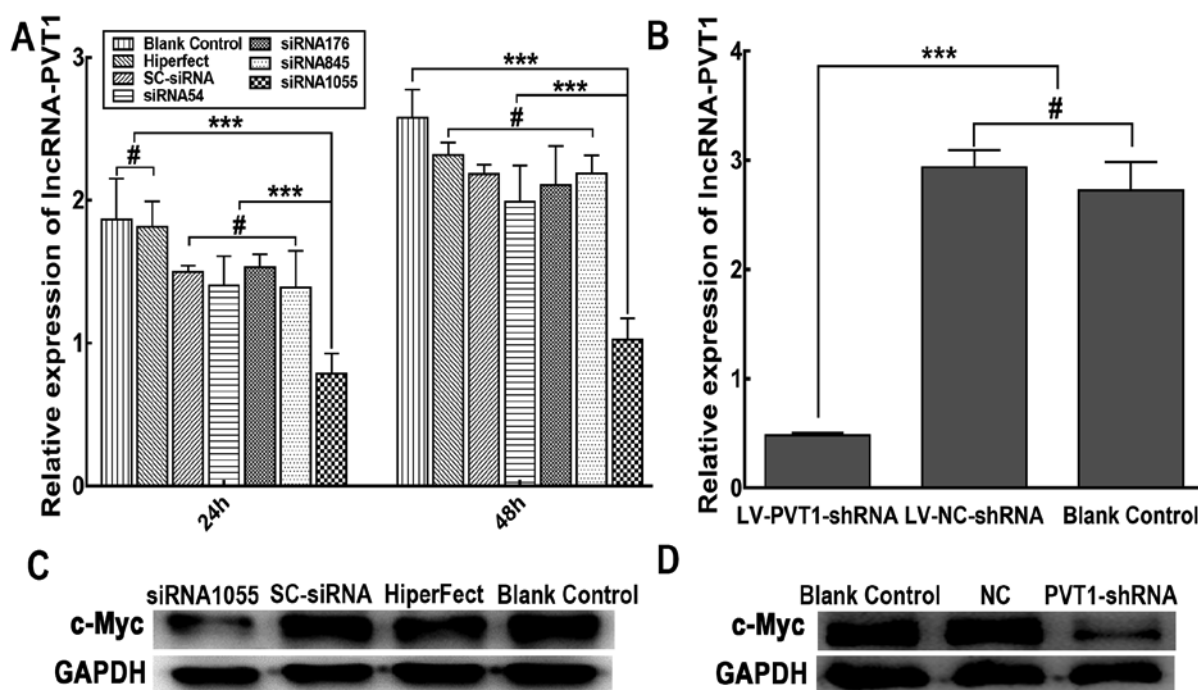


Figure 1. Effect of *PVT1* siRNAs or shRNA on *PVT1* RNA and c-Myc protein expression in Raji cells. (A) Suppression of *PVT1* RNA expression was measured by reverse transcription-quantitative polymerase chain reaction at 24 and 48 h after transient transfection with *PVT1* siRNAs (siRNA54, siRNA176, siRNA845 and siRNA1055). Non-silencing SC siRNA-transfected, mock-transfected (HiPerfect reagents only) and non-treated (blank control) cells were used as controls. *GAPDH* was used as the reference gene. (B) Expression levels of *PVT1* RNA in Raji cells stably infected with LV-*PVT1*-shRNA and LV-NC-shRNA. (C) Expression of c-Myc protein in Raji cells at 48 h after *PVT1*-siRNA transfection, assessed by western blotting. (D) Protein expression levels of c-Myc in Raji cells stably infected with LV-*PVT1*-shRNA. Representative images are shown. The results are expressed as the mean values of three independent experiments  $\pm$  standard deviation. \*\*\* $P < 0.01$ , # $P > 0.05$ . c-Myc, MYC proto-oncogene bHLH transcription factor; lncRNA, long non-coding RNA; LV, lentiviral vector; NC, negative control; *PVT1*, plasmacytoma variant translocation 1; SC, scrambled; shRNA, short hairpin RNA; siRNA, small interfering RNA.

( $P < 0.05$ ). Additionally, no significant differences between the LV-NC-shRNA group and the blank control cell group were identified ( $P > 0.05$ ).

The protein expression levels of c-Myc were assessed by western blotting. As shown in Fig. 1C, western blot analysis confirmed that c-Myc protein expression was decreased in the Raji cells transfected with *PVT1* siRNA1055 after 48 h compared with in the SC siRNA and blank control cell groups. There were no clear differences in c-Myc expression among the SC siRNA group, mock-transfected cells and blank control cells. Similarly, c-Myc protein expression was decreased in Raji cells stably carrying LV-*PVT1*-shRNA compared with either the LV-NC-shRNA group or blank control group (Fig. 1D). There was no difference identified between c-Myc expression in the LV-NC-shRNA group and blank control group. These findings indicated that *PVT1* siRNA and shRNA were effective at reducing c-Myc protein expression.

**Effect of *PVT1* knockdown on the proliferation and cell cycle distribution of Raji cells.** A CCK8 assay was performed to quantify cell proliferation. As shown in Fig. 2A, the proliferative activity of Raji cells carrying the LV-*PVT1*-shRNA was significantly inhibited ( $P < 0.01$ ) compared with Raji cells carrying LV-NC-shRNA and blank control cells ( $P < 0.01$ ). No significant difference in proliferation of LV-NC-shRNA group and blank control cells was identified. *PVT1* siRNA had a similar effect on the proliferative activity of Raji cells (data not shown).

Proliferation of Raji cells was inhibited following *PVT1* knockdown. Additionally, the effect of decreased *PVT1* expression on the cell cycle was examined. According to flow cytometric analysis, the percentages of cells in  $G_0/G_1$ , S and  $G_2/M$  phases were  $54.20 \pm 0.61$ ,  $34.07 \pm 0.64$  and  $11.70 \pm 0.00\%$  in Raji cells carrying LV-*PVT1*-shRNA, respectively. In Raji cells carrying LV-NC-shRNA, the percentages of cells in  $G_0/G_1$ , S and  $G_2/M$  phases were  $47.37 \pm 0.60$ ,  $43.27 \pm 0.55$  and  $9.37 \pm 0.51\%$ , respectively. There was a significant decrease in the percentage of cells in the S phase and a marked accumulation of cells in the  $G_0/G_1$  phase in Raji cells carrying LV-*PVT1*-shRNA compared with those carrying LV-NC-shRNA and blank control cells (Fig. 2B and C). There was no significant difference identified between the Raji cells carrying LV-NC-shRNA and blank control cells. These results indicated that knockdown of *PVT1* expression in Raji cells induced  $G_0/G_1$  phase arrest.

**Alteration in expression pattern of cell cycle-associated genes in Raji cells as a result of *PVT1* knockdown.** Differential mRNA expression levels of 84 genes involved in the cell cycle were assessed. The results of the PCR array revealed that 54 genes were upregulated and 26 genes were downregulated in the LV-*PVT1*-shRNA group compared with the LV-NC-shRNA group. The other four genes exhibited too low an expression level. There were more upregulated genes than downregulated genes in Raji cells carrying LV-*PVT1*-shRNA (Fig. 3A; Table II). The

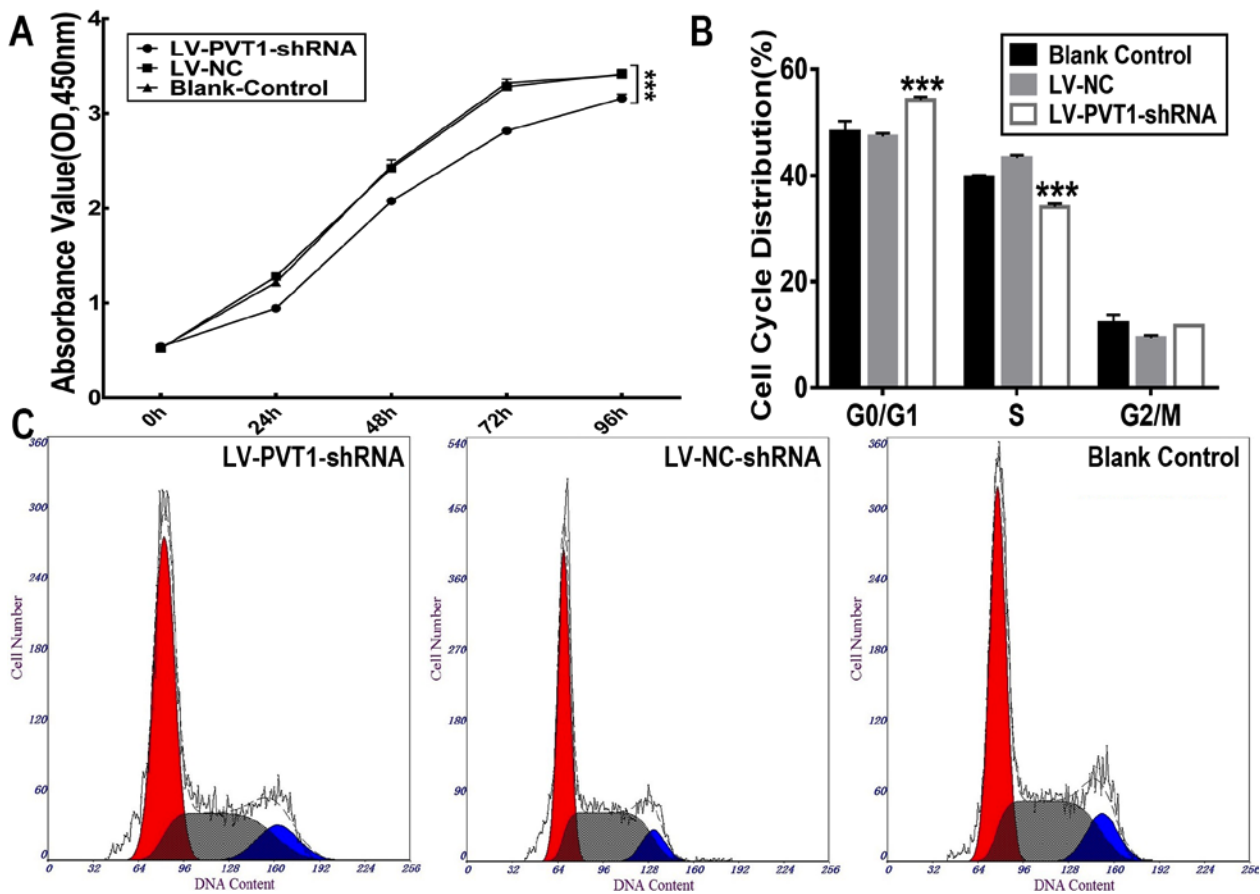


Figure 2. Effect of PVT1 knockdown on the proliferation and cell cycle distribution of Raji cells. (A) Raji cell proliferation was determined by Cell Counting kit-8 assay. Raji cells stably infected with LV-*PVT1*-shRNA and LV-NC-shRNA were incubated for different time intervals (24, 48, 72 and 96 h). Absorbance values were plotted. (B) Cell cycle distribution of Raji cells stably infected with LV-*PVT1*-shRNA was analysed by flow cytometry. The results represent the average values of three independent experiments  $\pm$  standard deviation. There was a significant decrease in the percentage of cells in the S phase and a marked accumulation of cells in the G<sub>0</sub>/G<sub>1</sub> phase in Raji cells carrying LV-*PVT1*-shRNA compared with those carrying LV-NC-shRNA and blank control cells. (C) Representative fluorescence-activated cell sorting images of cell cycle distribution analysis of Raji cells. \*\*\* $P < 0.01$  vs. LV-NC-shRNA. LV, lentiviral vector; NC, negative control; OD, optical density; *PVT1*, plasmacytoma variant translocation 1; shRNA, short hairpin RNA.

present study demonstrated that 16/84 examined genes were upregulated at least two-fold in the LV-*PVT1*-shRNA group.

Candidate gene selected from PCR microarray analyses was validated. As shown in Fig. 3B, western blotting revealed that P21, encoded by cyclin-dependent kinase inhibitor 1A (*CDKN1A*) was increased and *CCNE1* was decreased in the LV-*PVT1*-shRNA group compared within the LV-NC-shRNA group and in blank control cells. As shown in Fig. 3C, the RT-qPCR assay demonstrated that the expression levels of cyclin G2 (*CCNG2*), Retinoblastoma-like 2 (*RBL2*, p130), *CDKN1A*, HUS1 checkpoint homolog (*HUS1*), cyclin dependent kinase inhibitor 3 (*CDKN3*) and cyclin dependent kinase inhibitor 1B (*CDKN1B*) were upregulated in Raji cells infected with LV-*PVT1*-shRNA compared with the LV-NC-shRNA group and blank control cells. Although the expression of *CDKN1B* was very low in the three groups, there was statistically significant difference between the LV-*PVT1*-shRNA group and the LV-NC-shRNA group ( $P < 0.05$ ). In addition, *CCNE1*, *CCND1* and cell division cycle 20 (*CDC20*) were significantly downregulated in the LV-*PVT1*-shRNA group compared with in the LV-NC-shRNA group and blank control cells ( $P < 0.05$ ). The RT-qPCR assay results of the 9 selected differentially expressed genes did not change notably compared with the PCR array results.

## Discussion

*PVT1* is aberrantly expressed in a variety of tumour types and acts as a potential oncogene that promotes cancer cell proliferation (8-12). Gain of *PVT1* lncRNA expression is required for high MYC protein levels in 8q24-amplified human cancer cells (13). *PVT1* RNA and MYC protein expression are correlated in primary human tumours, and copy number of *PVT1* co-increases in >98% of MYC-copy-increase types of cancer (13). Although it was reported in 1990 that the *PVT1* locus may be a site of variant translocations, including in human BL (6), to the best of our knowledge, there are no reports about the role of *PVT1* in BL. To investigate the role of *PVT1* in BL cell proliferation, RNA interference was used to inhibit its expression in the human BL-derived Raji cell line. The results of the present study revealed that the expression levels of *PVT1* RNA and c-Myc protein decreased following transfection of Raji cells with *PVT1* siRNA targeting the 1,055-1,074 nt region of the *PVT1* sequence. No significant effect of the other three siRNAs targeting *PVT1* (siRNA54, siRNA176 and siRNA845) on the expression levels of *PVT1* RNA was identified. The specific siRNA against *PVT1*, siRNA1055, was successfully incorporated into a lentiviral vector. Raji cells carrying the *PVT1*-shRNA lentiviral vector were screened. Once Raji cells



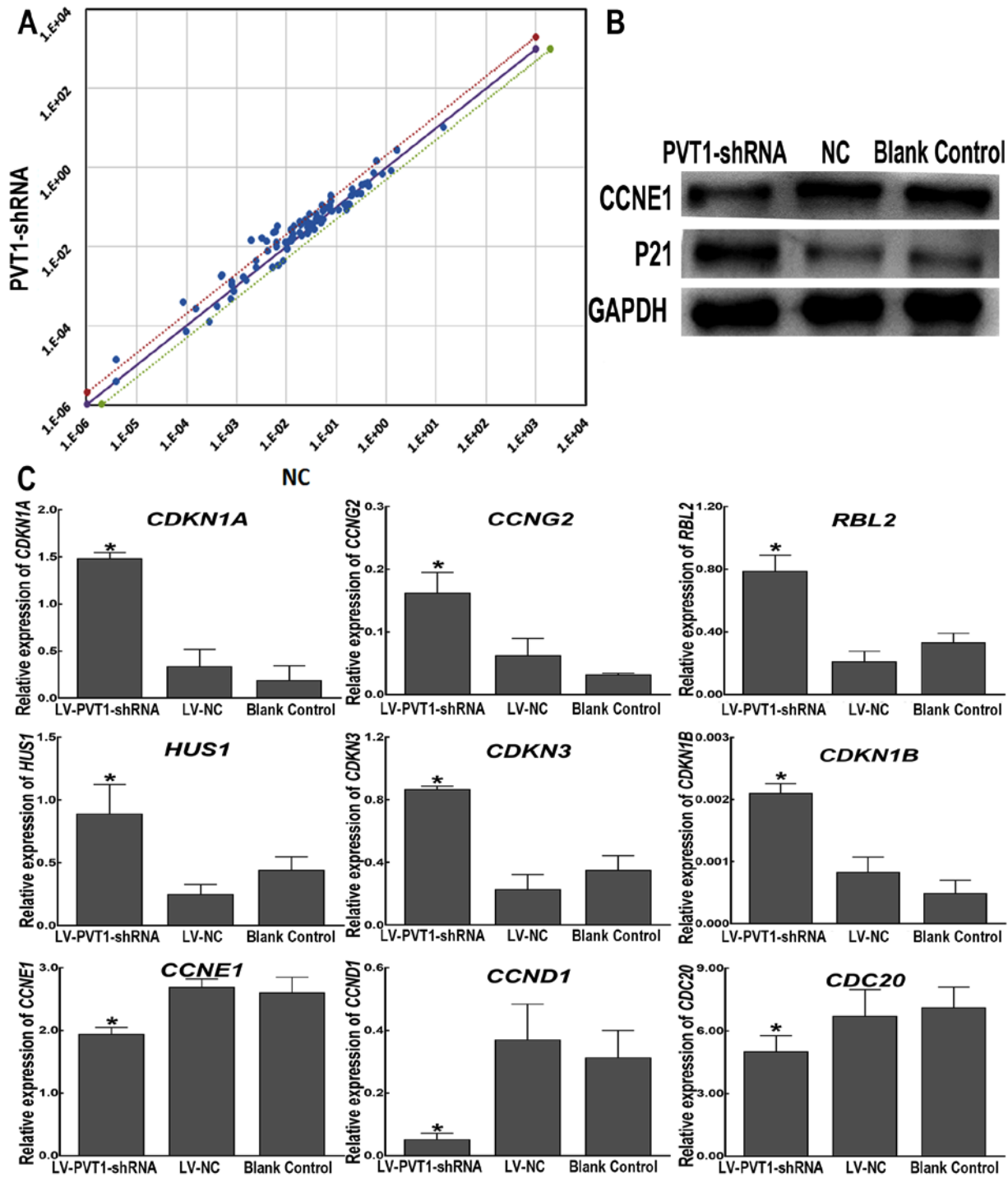


Figure 3. Differential expression of cell cycle-associated genes in Raji cells with *PVT1* knockdown. (A) There are three straight lines in the coordinate system, and the black line represents the fold changes ( $2^{-\Delta\Delta C_t}$ ) of 1. Dots above the black line represent genes that are upregulated, and dots below the black line indicate genes that are downregulated in the LV-*PVT1*-shRNA group compared with the LV-NC-shRNA group. A point within the range of the two dotted lines signifies a differential expression of >1-fold but <2-fold in Raji cells infected with LV-*PVT1*-shRNA compared with cells infected with LV-NC-shRNA. Points outside the two dotted lines are >2-fold differentially expressed. (B) Protein expression levels of P21 and CCNE1 in Raji cells were determined using western blot analysis following *PVT1* knockdown. GAPDH served as a loading control. (C) Reverse transcription-quantitative polymerase chain reaction analysis of nine genes in Raji cells stably infected with LV-*PVT1*-shRNA compared with the genes in the negative control cells infected with LV-NC-shRNA. GAPDH was used as a reference gene. The graphs depict the mean mRNA expression changes  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$  vs. LV-NC-shRNA. CCNE1, cyclin E1; CCND1, cyclin D1; CCNG2, cyclin G2; CDC20, cell division cycle 20 homolog (*S. cerevisiae*); CDKN1A, cyclin-dependent kinase inhibitor 1A (P21, Cip1); CDKN1B, cyclin-dependent kinase inhibitor 1B (P27, Kip1); CDKN3, cyclin-dependent kinase inhibitor 3; HUS1, HUS1 checkpoint homolog (*S. pombe*); LV, lentiviral vector; NC, negative control; *PVT1*, plasmacytoma variant translocation 1; RBL2, Retinoblastoma-like 2 (p130); shRNA, short hairpin RNA.

stably expressed *PVT1*-shRNA, the levels of *PVT1* RNA and c-Myc protein were markedly reduced, which is consistent with the effect of *PVT1* siRNA1055.

In the present study, the proliferation of Raji cells carrying *PVT1*-shRNA was significantly decreased compared with in control cells. However, downregulation of *PVT1* expression

Table II. Differentially expressed genes associated with the cell cycle in Raji cells following PVT1 knockdown.

Gene symbol	Accession no.	Gene description	Fold change
<i>CCNG2</i>	NM_004354	Cyclin G2	7.03
<i>RBL2</i>	NM_005611	Retinoblastoma-like 2 (p130)	5.26
<i>CDKN1A</i>	NM_000389	Cyclin-dependent kinase inhibitor 1A (P21, Cip1)	4.78
<i>CCNT1</i>	NM_001240	Cyclin T1	4.17
<i>CASP3</i>	NM_004346	Caspase 3, apoptosis-related cysteine peptidase	3.96
<i>RBL1</i>	NM_002895	Retinoblastoma-like 1 (p107)	3.70
<i>RB1</i>	NM_000321	Retinoblastoma 1	3.68
<i>HUS1</i>	NM_004507	HUS1 checkpoint homolog (S. pombe)	3.38
<i>CCND2</i>	NM_001759	Cyclin D2	2.51
<i>CDC6</i>	NM_001254	Cell division cycle 6 homolog (S. cerevisiae)	2.34
<i>CDKN1B</i>	NM_004064	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	2.31
<i>TFDP2</i>	NM_006286	Transcription factor Dp-2 (E2F dimerization partner 2)	2.23
<i>CDC16</i>	NM_003903	Cell division cycle 16 homolog (S. cerevisiae)	2.21
<i>GADD45A</i>	NM_001924	Growth arrest and DNA-damage-inducible, alpha	2.08
<i>CDKN3</i>	NM_005192	Cyclin-dependent kinase inhibitor 3	2.07
<i>ABL1</i>	NM_005157	C-abl oncogene 1, non-receptor tyrosine kinase	2.04
<i>BRCA2</i>	NM_000059	Breast cancer 2, early onset	-2.22
<i>CCNE1</i>	NM_001238	Cyclin E1	-2.05
<i>CCND1</i>	NM_053056	Cyclin D1	-1.98
<i>CDK6</i>	NM_001259	Cyclin-dependent kinase 6	-1.85
<i>CDC20</i>	NM_001255	Cell division cycle 20 homolog (S. cerevisiae)	-1.81
<i>ATM</i>	NM_000051	Ataxia telangiectasia mutated	-1.67
<i>CKS2</i>	NM_001827	CDC28 protein kinase regulatory subunit 2	-1.56
<i>CDK4</i>	NM_000075	Cyclin-dependent kinase 4	-1.52

Positive and negative values represent upregulation or downregulation of genes in the LV-PVT1-shRNA group compared with the LV-NC-shRNA group, respectively.

by shRNA could not induce apoptosis of Raji cells (data not shown). Cell cycle distribution analysis indicated that knockdown of *PVT1* in Raji cells resulted in notable G<sub>0</sub>/G<sub>1</sub> phase arrest. Previous studies have reported that *PVT1* can promote the proliferation of cells, including hepatocellular carcinoma (10) and thyroid cancer cells (11). The results of the present study were consistent with those of previous studies. Taken together, the results suggested that the *PVT1* shRNA-mediated suppression of Raji cell proliferation may occur via cell cycle arrest.

*PVT1* may be associated with a series of signalling pathways and genes in tumour development and progression (18-20). According to topological measures, Paci *et al* (20) revealed that the lncRNA *PVT1* is connected to 753 different mRNAs (~50% of total mRNAs in the network), and the miR-200 family members mediate >80% of these interactions. However, the mechanism by which knockdown of *PVT1* causes cell cycle alteration in Raji cells remains largely unexplained. It has been confirmed that *PVT1* is located adjacent to the proto-oncogene *c-Myc*, and the expression of *PVT1-Myc* in the majority of tumours is positively correlated with the copy number of 8q24 (13,15,21). Tseng *et al* (13) demonstrated that *c-Myc* protein expression is dependent on the expression levels of *PVT1*; therefore, this lncRNA may promote cell proliferation

and tumourigenesis by regulating the expression of *c-Myc* protein. Following transfection of Raji cells with *PVT1*-siRNA or shRNA, the expression of *c-Myc* protein decreased, which was consistent with previous reports (13). It has been demonstrated that *c-Myc* is involved in regulation of the cell cycle and proliferation (22). Yang *et al* (23) revealed that *c-Myc* regulates the cyclin-dependent kinase1 (CDK1)/cyclin B1-dependent G<sub>2</sub>/M cell cycle progression by controlling histone H4 acetylation. Additionally, a study has reported that loss of *c-Myc* function impedes G<sub>1</sub>-phase progression (22). The CDK inhibitors P27 and P21 appear to be critical targets of *c-Myc*, which cooperates with the zinc finger MIZ-type containing 1 to repress the transcription of cell-cycle inhibitors, including P15, P21 and P27 (24). Yang *et al* (23) also reported that suppression of *c-Myc* induces the upregulation of P21 and the downregulation of P27 in Raji cells. Recently, Wang *et al* (25) reported that the epigenetically induced lncRNA *EPIC1* promotes cell cycle progression by interacting with *c-Myc* via the 129-283 nt region of *EPIC1*. *EPIC1* knockdown reduces the affinity of *c-Myc* to its target genes, including *CDKN1A*, *CCNA2*, *CDC20* and *CDC45* (25). The results of the present study suggested that the regulation of G<sub>0</sub>/G<sub>1</sub> cell cycle progression by *PVT1* may be associated with *c-Myc* expression or *c-Myc* regulation of cell cycle-associated genes.

To further evaluate the alterations in cell cycle-associated gene expression in Raji cells with *PVT1* knockdown, a cell cycle PCR microarray was used to detect alterations in cell cycle-associated genes in Raji cells stably expressing *PVT1*-shRNA. The results revealed that cell cycle-associated genes, including *CCNG2*, *RBL2*, *CDKN1A*, *HUS1*, *CDKN3* and *CDKN1B*, were upregulated in Raji cells with *PVT1* knockdown. The majority of the aforementioned genes inhibit cell cycle progression. *CDKN1A* encodes a cell cycle-dependent kinase inhibitor, which binds and inhibits the activity of cell cycle-associated genes with cyclin and CDK complexes, including *CCND1-CDK4* and *CCNE-CDK2*. P21 is a member of the cell cycle regulator CIP/KIP family, which is involved in the regulation of numerous important biological behaviours, the most important of which is the regulation of cell cycle progression, particularly cell cycle arrest at the G<sub>1</sub> phase (26,27). Therefore, P21 serves a key role in cell quiescence, senescence and differentiation (28). In addition, a recent study identified a positive correlation between *PVT1* and miR-1207-5P, and a negative correlation between miR-1207-5P and signal transducer and activator of transcription 6 (*STAT6*) in breast cancer (29). miR-1207-5P, produced by *PVT1* transcription and targeting *STAT6*, promotes the proliferation of breast cancer cells by regulating P21 and *CDKN1B* (29). In nasopharyngeal cancer cells, *PVT1* promotes cancer stem cell-like properties by inhibiting miR-1207 and activating the phosphoinositide 3-kinase/protein kinase B signalling pathway (30). In the present study, following the downregulation of *PVT1* expression in Raji cells, the RNA expression levels of *CDKN1A* and P21 protein expression were increased, and the cell cycle was blocked in G<sub>0</sub>/G<sub>1</sub> phase, suggesting that *PVT1* may promote cell cycle progression by inhibiting the expression of P21. In pancreatic cancer cell lines, *PVT1* can promote epithelial-to-mesenchymal transition by downregulating the expression of P21, thus promoting cell proliferation and migration (31). In addition, a study by Cui *et al* (32) suggested that *PVT1* promotes cell proliferation and migration by downregulating P21 in pancreatic cancer cells. Therefore, the results of the present study were consistent with the literature.

*CCNG2*, which has been shown to be associated with various types of tumour, can cause cell cycle arrest in the G<sub>1</sub> phase (33-37). A study by Cui *et al* (37) indicated that *CCNG2* expression is decreased in prostate cancer and that PC-3 cells transfected with *CCNG2* exhibit a lower survival rate, a higher percentage of cells in the G<sub>0</sub>/G<sub>1</sub> phases and lower CDK2 protein expression, suggesting that *CCNG2* may serve important roles as a negative regulator of prostate cancer cells. The results of the present study demonstrated that the downregulation of *PVT1* in Raji cells increased the expression of *CCNG2*, which suggested that *PVT1* may also promote cell cycle progression by inhibiting the expression of *CCNG2*. The *RBL2* gene, encoding the proline rich protein BstNI subfamily 2 (pRb2) protein, is most abundant in the G<sub>0</sub> phase. *RBL2* maintains G<sub>0</sub> arrest in quiescent or differentiated cells, controls the transition from G<sub>1</sub> to S phase, and is a key regulator of growth arrest in cellular senescence (38). AKT inhibition reduces cell viability, induces cell accumulation in G<sub>0</sub>/G<sub>1</sub> phase and triggers apoptosis, which proves to be largely dependent on *RBL2*/pRb2 itself, as shown by *RBL2*/pRb2 silencing (39). The *HUS1* protein, encoded by the *HUS1* gene, is a component

of an evolutionarily conserved and genotoxin-activated checkpoint complex that is involved in cell cycle arrest in response to DNA damage. In the present study, the expression levels of the cell cycle progression regulators *RBL2* and *HUS1* were increased in Raji cells with *PVT1* knockdown, suggesting that the involvement of *PVT1* in cell cycle progression may be associated with the expression of *RBL2* and *HUS1*. *CDKN3* (also called CDII or KAP), encoded by the *CDKN3* gene, is a member of the dual specificity protein phosphatase family, which is essential for mitosis and G<sub>1</sub>/S phase transition, due to its role in regulating the CDK1 (also called CDC2) signalling axis (40), and *CDKN3* serves an important role in regulating cell division. *CDKN3* may also function as an oncogene by inducing G<sub>0</sub>/G<sub>1</sub> phase progression, apoptosis and metastasis in ovarian cancer cell lines (41). In the present study, the expression levels of *CDKN3* were increased in Raji cells with *PVT1* knockdown, suggesting that *CDKN3* may be involved in the cell cycle arrest by the induction of *PVT1* downregulation.

In the present study, *CCNE1*, *CCND1* and *CDC20* were significantly downregulated in Raji cells following knockdown of *PVT1*. *CCNE1* is a positive regulator of the cell cycle that controls the transition of cells from the G<sub>1</sub> to the S phase, and serves an important role in cell proliferation and tumourigenesis. Overexpression of *CCNE1* can increase tumour incidence and susceptibility to multiple tumourigenesis in mice (42-44). *CCND1*, which belongs to the G<sub>1</sub> cyclins, serves an important role in cell cycle regulation and promotes cell cycle progression from the G<sub>1</sub> phase to S phase through its interaction with CDKs. *CCND1* is overexpressed and/or amplified in numerous human types of cancer (45,46). A previous study reported that *CCND1* allows for the progression from the G<sub>1</sub> to the S phase by binding and sequestering P21 (47). A study by Li *et al* (48) suggested that *PVT1* promotes cell cycle progression by increasing the expression of *CCND1*. The mechanism involved indicated that *PVT1* promotes the progression of clear cell renal cell carcinoma partly via activation of the epidermal growth factor receptor pathway (48). In melanoma cells, silencing of *PVT1* significantly inhibits cell proliferation, migration and invasion, and arrests the cell cycle at G<sub>0</sub>/G<sub>1</sub> stage by significantly decreasing *CCND1* expression (49). *CDC20*, which has important functions in chromosome segregation and mitotic exit, is abnormally expressed in a wide range of tumours, including human bladder carcinoma, pancreatic, colorectal breast and lung cell cancer (50). It has been reported that *CDC20* were significantly downregulated by lncRNA EPIC1 knockdown in both tumor samples and cell lines (25). Therefore, the results of the present study suggested that the role of *PVT1* in regulating G<sub>0</sub>/G<sub>1</sub> cell cycle progression was likely to be associated with the expression levels of *CCNE1*, *CCND1* and *CDC20*.

In conclusion, the results of the present study revealed that knockdown of *PVT1* may inhibit the proliferation of Raji cells by arresting cells in the G<sub>0</sub>/G<sub>1</sub> phase. The role of *PVT1* in regulating cell cycle progression may be associated with the expression of c-Myc and cell cycle-associated genes, including *CCNG2*, *RBL2*, *CDKN1A*, *HUS1*, *CDKN3*, *CDKN1B*, *CCNE1*, *CCND1* and *CDC20*. However, it remains unclear how *PVT1* influences the expression levels of cell cycle-associated genes. Furthermore, it is not clear which signalling pathways could be directly associated with *PVT1*. Therefore, further studies are required to understand the potential molecular mechanisms that underlie the regulation



of cell cycle-associated genes by *PVT1* in Raji cells. Certainly, the expression pattern of *PVT1* in BL should be investigated following the collection of clinical samples. It will be useful to develop a comprehensive understanding of the role of *PVT1* and its potential as an oncotarget in BL.

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## Availability of data and materials

The datasets used and/or analysed in the present study are available from the corresponding author on reasonable request.

## Authors' contributions

CZ and YX performed the experiments and interpreted results. DH and YL developed the original concept, designed the study and contributed to the writing of the manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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