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Suppressed CCL2 expression inhibits the proliferation of leukemia cells via the cell cycle protein Cyclin D1: preliminary *in vitro* data

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Abstract. – OBJECTIVE: Chemokine (C-C motif) ligand 2 (CCL2) is a member of the CC subfamily, which displays chemotactic activity for monocytes and basophils. This molecule plays a very important role in many solid tumors and shows changes in the bone marrow microenvironment. However, its role in acute myeloid leukaemia (AML) is still unclear.

MATERIALS AND METHODS: In this study, we established a HL-60 cell line with CCL2 knockdown to explore its effect on leukemogenesis. Lentivirus with CCL2-knockdown was successfully constructed after screening effective CCL2 short hairpin RNA (shRNA) sequences and was transfected into HL-60 cells, which was further validated at the mRNA and protein levels by real-time polymerase chain reaction (PCR) and Western blotting, respectively.

RESULTS: Low expression of CCL2 significantly decreased HL-60 cell growth by increasing the cell arrest at G1 phase by 12% more than controls. We applied RNA sequencing technology to discriminate the gene expression profiles between the cells with CCL2 knockdown and the controls, and Cyclin D1 was selected for further experiments as its expression level was significantly downregulated, which was validated at the mRNA and protein levels. Cyclin D1 knockdown experiments showed that the cell proliferation rate was evidently decelerated, and cell cycle analysis also indicated a similar pattern for CCL2.

CONCLUSIONS: Our study revealed that Cyclin D1 is an effector that mediates CCL2's function in cell proliferation by blocking cells at G1 phase.

Key Words:
CCL2, Proliferation, Cell cycle, Cyclin D1.

Introduction

Chemokine (C-C motif) ligand 2 (CCL2), also known as monocyte chemotactic protein-1 (MCP-1), a key member of the CCβ chemokine superfamily, is a potent chemotactic factor for monocytes. Both CCL2 and its receptor C-C chemokine receptor type 2 (CCR2) have been demonstrated to be induced and involved in various diseases[1]. In many solid tumors, CCL2 signaling has been shown to correlate with tumor growth, invasion and metastasis. In prostate cancer, elevated secretion of CCL2 recruits prostate cancer epithelial cells to the bone microenvironment, promotes their proliferation via activating the phosphoinositide 3 (PI3) kinase/Akt signaling pathway, and increases cancer growth and bone metastasis[2,3]. CCL2 expression and macrophage infiltration are also linked to poor prognosis and metastatic disease in human breast cancer[4-7]. Moreover, CCL2 is expressed at high levels in many other solid tumors, including lung, esophageal, ovarian, and pancreatic cancers[8-12]. In addition, CCL2 can act as a direct mediator of angiogenesis, interacting with CCR2 on the endothelial cell surface, leading to increased vessel sprout formation and tumor progression[13]. Thus, elevated CCL2 level is associated with active angiogenesis, enhanced tumor proliferation, and poor prognosis in many solid tumors.

However, in hematologic neoplasms, the role of CCL2 has not yet been determined. There is evidence suggesting that the level of CCL2 has

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increased in the bone marrow environment of acute lymphoblastic leukaemia (ALL) patients and in the serum of untreated patients with acute myeloid leukaemia (AML)[14,15]. Therefore, we determined the level of CCL2 in leukaemia cell lines and found that CCL2 expression varies among cell lines, which indicated the complexity and heterogeneity of this gene. As the level of CCL2 is much higher in HL-60 and comparatively lower in THP-1, we constructed a cell line with CCL2 knockdown and a cell line with CCL2 overexpression to investigate the biological functions of this gene and try to explore the potential effect of CCL2 on leukemogenesis.

Materials and Methods

Cell Lines and Culture Methods

Leukemia cell lines HL-60, NB4, U937, THP-1, K562, MV4-11, Jurkat, and SHI-1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were maintained at 37°C with 5 % CO_2 incubation and cultured in RPMI 1640 (Gibco, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA). All the researches were approved by the Ethics Committee of Soochow University.

Quantitative Reverse Transcription PCR (Qrt-PCR)

Total RNA was isolated from bone marrow mononuclear cells (BMMCs) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA concentrations and purities were measured with a spectrophotometer at A260 and A260/280, respectively. 2 mg of total RNA were reverse transcribed into cDNA. The primers were designed according to the sequences of CCL2 mRNAs (GenBank No: NM_002982). The sense primer for CCL2 was 5'-CCCTCGAGAGCAAACTGAA GCTCGCACT-3', and the antisense primer was 5'-GTTAACTCTTGGGTTGTGGA GT GA-3'. The PCR amplification was carried out in a 20 µl reaction volume containing 2 µl cDNA template, 1 µl 20× EvaGreen Dye (Biotium, Waltham, MA, USA), 10 µl 2× Fast-Plus EvaGreen Mix (Biotium, Waltham, MA, USA), 5 µM forward and reverse primer and 6 µl nuclease-free water. After a 5 min denaturation at 94°C, PCR was performed for 40 cycles. Each cycle was 94°C for 30 s, 60°C for 30 s, a 72°C elongation for 30 s, and a final hold at 72°C for 10 min. ABL, the housekeeping gene, was amplified at the same time as the control with the same templates. The upstream/downstream primers of ABL were 5'-TGGAGATAACACTCTAAGCATA ACTAAAGGT-3' and 5'-GATGTAGTTG CTTGGGACCCA-3', respectively. In all samples, amplification of ABL reached the threshold within 30 cycles. Positive and negative controls were included in all assays. CCL2 transcription level was normalized to ABL transcription level. $^{\Delta}$CT(CCL2) = CT (CCL2) – CT (ABL). All the primers were Invitrogen products. The expected PCR products of CCL2 and ABL were 57 bp and 156 bp, respectively. qRT-PCR detection was performed by using an Applied Biosystems system (7500 Real-Time PCR System) (Thermo Fisher Scientific, Waltham, MA, USA). In addition, we applied this method to estimate the mRNA expression levels of other target genes, including PCNA, Cyclin D1, c-jun, survivin, ERK1 and ERK2.

Western Blot Analysis

Whole cell extracts were obtained according to the standard protocol using radio immunoprecipitation assay buffer, followed by sonication for 5 min. Protein concentrations were measured using the Pierce BCA Protein Assay Kit. Equal amounts of protein extracts (20 µg) were subjected to 10 or 12 % sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and then blocked for 1 h in 5 % non-fat powdered milk in TBST. The membrane was incubated with primary antibodies for CCL2 (Abcam, Cambridge, MA, USA), Cyclin D1 (Beyotime, Shanghai, China), and GAPDH (Beyotime, Shanghai, China). Blots were then incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit antibody (Beyotime, Shanghai, China). Signals were detected using ECL reagents (Thermo Scientific, Waltham, MA, USA).

Transfecting CCL2 Into THP-1 Cells

The human CCL2 gene was cloned from U937 and into the pMD19 vector (TaKaRa, Otsu, Shiga, Japan) before sequencing. The CCL2 gene was recombined with the transfer plasmid-PLVX (supplied by the Hematology Center, Cyrus Tang Medical Institute in Suzhou, China) and transfected into 293T cells by DNA-calcium phosphate. The virus particles were collected and transfected into THP-1 cells. Successful transfection of CCL2 into THP-1 cells was validated by RT-PCR and Western blot.

Target Gene Silencing by Lentiviral-Delivered RNA Interference

For shRNAs, CCL2-specific target sequence was chosen according to online shRNA tools provided by Invitrogen, using the CCL2 reference sequence (GenBank Accession No. NM_002982). Double-stranded DNA containing the interference sequences were synthesized according to the structure of a pGV248 viral vector (Gikai Gene Company, Shanghai, China) and then inserted into vector. The most efficient target sequence, from 5' to 3', was TCGCGAGCTATAGAAGAAT. In addition, we also used this method to knock down Cyclin D1, which was targeted at the efficient sequence[16] "gatccccCAAACAGATCATCCGCAAAttcaagagaTTTGCGGATGATCTGTTTGtttttggaaa".

Cell Cycle Analysis

For cell cycle analysis, cells (1 x 10^6 cells/well) were grown in regular medium for 24 h, washed, trypsinized, and fixed with 70% ethanol overnight at 4°C. Cell pellets were suspended in a 1 mL solution containing 100 mg/mL RNase (Sigma-Aldrich, St. Louis, MO, USA) and 50 mg/mL propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 30 min at 37°C. Cell cycle analysis was performed on a Beckman Coulter FC500 Cytometer and analyzed by a Multicycle AV programme (Beckman Coulter, Brea, CA, USA).

Cell Viability Assay

Cells (1×10^4/well) were plated in 96-well plates at a total volume of 100 µl per well. According to the manufacturer's instructions, cell viability was detected by Cell Counting Kit-8 (Dojindo Laboratories, Shanghai, China), which relies on tetrazolium-based technology, and the OD value for each well was read at a wavelength of 450 nm on a microplate reader (Bio-Rad, Hercules, CA, USA) to determine the cell viability.

RNA Sequencing

The complementary DNA (cDNA) libraries for single-end sequencing were prepared using an Ion Total RNA-Seq Kit v2.0 (Life Technologies, Waltham, MA, USA) according to the manufacturer's instructions. The cDNA libraries were then processed for the Proton Sequencing process according to the commercially available protocols. Samples were diluted and mixed, and the mixture was processed on a OneTouch 2 instrument (Life Technologies, Waltham, MA, USA) and enriched on a OneTouch 2 ES station (Life Technologies, Waltham, MA, USA) to prepare the template-positive Ion PI™ Ion Sphere™ Particles (Life Technologies, Waltham, MA, USA) according to the Ion PI™ Template OT2 200 Kit v2.0 (Life Technologies, Waltham, MA, USA). After enrichment, the mixed template-positive Ion PI™ Ion Sphere™ Particles of the samples were loaded onto 1 P1v2 Proton Chip (Life Technologies, Waltham, MA, USA) and sequenced on Proton Sequencers according to the Ion PI Sequencing 200 Kit v2.0 (Life Technologies, Waltham, MA, USA).

Statistical Analysis

Statistical comparisons of mean values in cell line study were performed using Unpaired, two-tailed Student's *t*-test. One-way ANOVA including post hoc Tukey multiple comparison tests was used to study the difference. All data were exported and calculated into Microsoft Excel, and statistical analyses were performed using the statistic software SPSS 17.0 version (SPSS Inc., Chicago, IL, USA) and GraphPad Prism software version 5 (GraphPad Software, La Jolla, CA, USA). A p-value of <0.05 was considered statistically significant.

Results

Knocking Down CCL2 in HL60 Decreased the Cell Growth Rate and Influenced Cell Cycle Progression, While CCL2 Overexpression Did Not Change These Biological Functions of THP-1 Cells

We examined the expression levels of CCL2 in leukemia cell lines and found that CCL2 mRNA levels were significantly higher in HL-60 and comparatively lower in THP-1 (Figure 1A, 1B). First, we overexpressed CCL2 in THP-1, which was validated at the mRNA and protein levels (Figure 1C, 1D). However, CCL2 up-regulation did not affect cell proliferation and cell cycle progression (Figure 1E, 1F). Then, we knocked down the expression of CCL2 in HL-60 cells using lentivirus-delivered shRNA with the most efficient sequence (Figure 2A). CCL2 mRNA and protein levels were significantly inhibited in HL-60 cells (Figure 2B, 2C). In the stably transfected cells, after culture for 72 h, the proliferation of downregulated CCL2 cells was slower than control cells by 44.44% (p =0.012). The cell cycle progression indicated that cells with downregulated CCL2 had a decrease in cells in S phase by 12% compared to control cells after culturing for 24 h (Figure

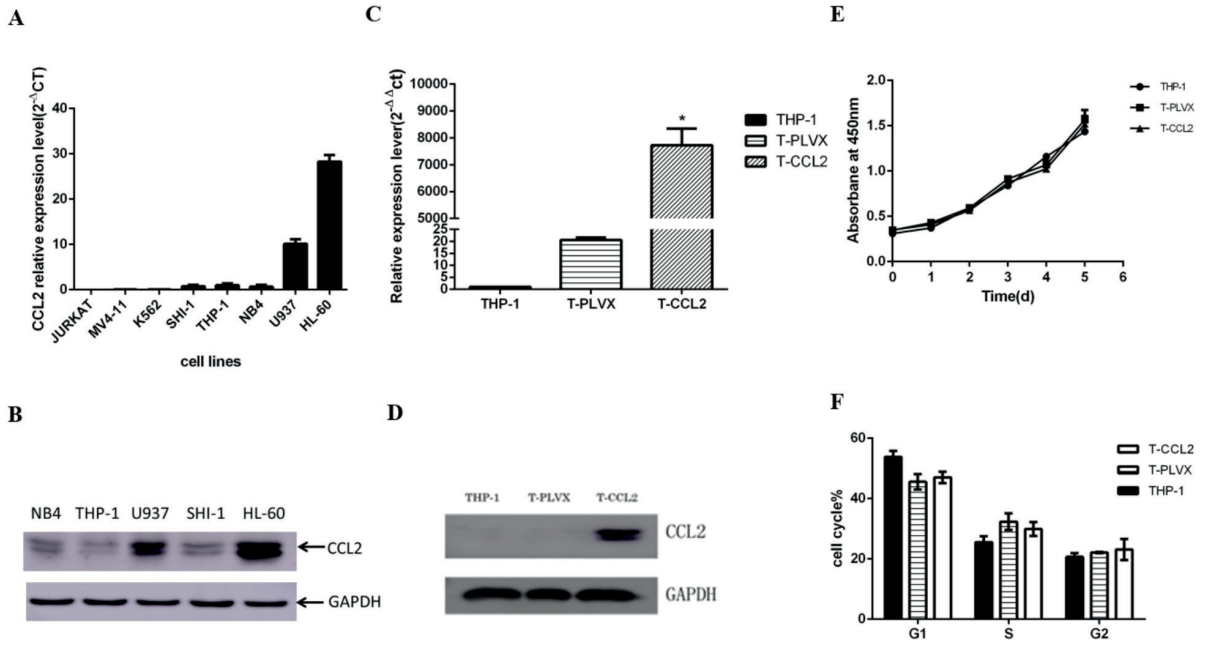

Figure 1. CCL2 overexpression does not change the biological functions of THP-1 cells. ***(A)*** The mRNA level of CCL2 in leukemia cell lines was determined by quantitative reverse transcription PCR (qRT-PCR). ***(B)*** The protein level of CCL2 in leukemia cells was determined by Western blot. ***(C)*** The overexpressed CCL2 in THP-1 was validated at the mRNA level. ***(D)*** The overexpressed CCL2 in THP-1 was validated at the protein level. (***E)*** CCL2 up-regulation was not involved in cell proliferation. ***(F)*** CCL2 up-regulation was not involved in cell cycle progression.

3A, 3B). We also evaluated cell apoptosis by flow cytometry between the CCL2 silencing group and the control group, and no significant difference was identified (data not shown).

Gene Expression Profiling of CCL2-Silenced HL-60 Cells Identified Cyclin D1 As A Potential Downstream Target

To investigate the potential mechanism of how CCL2 silencing affects the cell proliferation rate and cell cycle, we applied RNA sequencing to compare the gene expression profiles between CCL2-knockdown cells and those transfected with non-silencing control shRNAs. The data identified 159 differentially expressed genes in total, including 58 up-regulated genes and 101 genes downregulated genes (Figure 4A). Pathway analysis indicated that 56 pathways were significantly different, including the cell cycle, NOD-like receptor signaling pathway, TNF signaling pathway, NF-κB signaling pathway, etc. The most

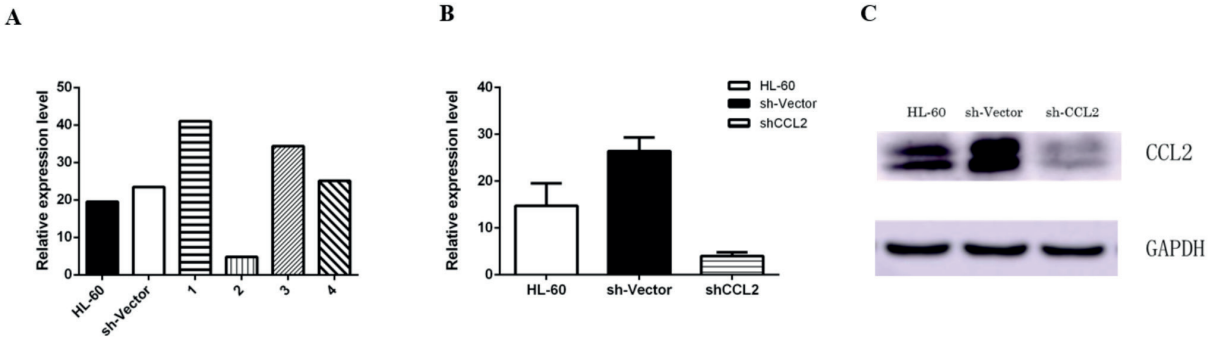

Figure 2. CCL2 was successfully downregulated in HL-60 cells. ***(A)*** Optimizing the efficient CCL2 interference sequence. ***(B)*** qRT-PCR analysis confirmed that the mRNA levels of CCL2 were downregulated in HL-60 cells. ***(C)*** Western blot analysis confirmed that the CCL2 knockdown cell line was successfully constructed.

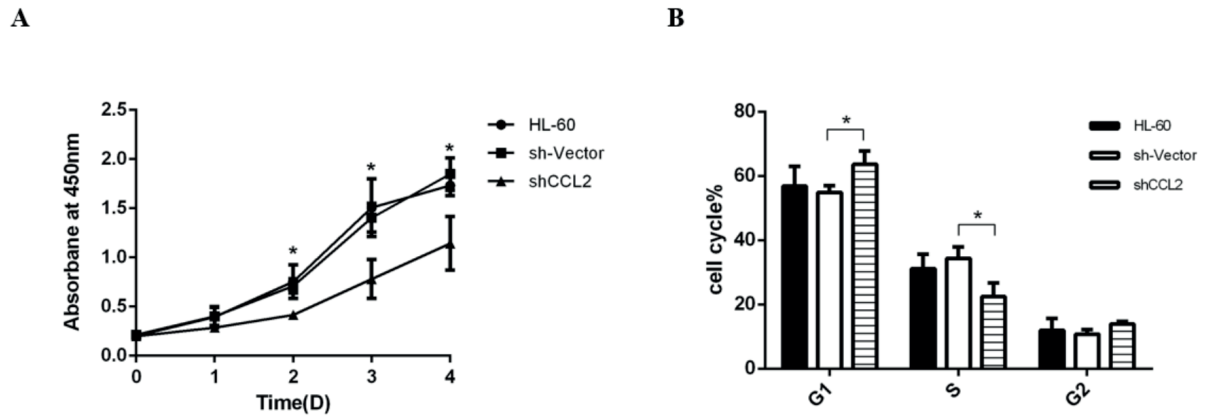

Figure 3. Stable shRNA-mediated knockdown of CCL2 leads to growth inhibition of HL-60 and affects the cell cycle process *in vitro*. **(A)** Downregulation of CCL2 decelerated the cell proliferation speed. ***(B)*** More cells transfected with shCCL2 were arrested at G1 phase compared with the control group. *, $p < 0.05$ compared with the control.

important top ten pathways are shown in Table I. Six downregulated genes related to the cell cycle were CCNA2, CCNB2, CCND1, CDC20, PLK1, and GADD45A, and only the TFDP2 gene was significantly up-regulated (Table II). We detected the mRNA expression levels of proliferation-associated genes, and only Cyclin D1 expression was downregulated (Figure 4B). Western blot analysis showed that the protein expression of Cyclin D1 was downregulated (Figure 4C), which further confirmed the gene profiling results. Based on the above information and previous references[16-19], we selected Cyclin D1 as a target gene for further experiments.

ShRNA-Mediated Knockdown of Cyclin D1 In HL60 Decelerated Cell Proliferation Speed and Blocked Cells At G1 Phase, Suggesting A Similar Pattern to That of CCL2

To further reveal the relationship between CCL2 and Cyclin D1, we interfered with the expression of Cyclin D1 in HL-60 cells with directed shRNAs. Cyclin D1 mRNA and protein expression was significantly suppressed in HL-60 cells after stable transfection (Figure 5A, 5B). In addition, similar to CCL2 inhibition, the suppression of Cyclin D1 expression inhibited cell proliferation, with the highest suppression rate being 69.42 %, and arrested 14.5 % more cells at G1 phase (Figure 5C, 5D).

Table II. Knocking down CCL2 affects the expression of cell cycle-related genes.

Gene name	κ^2 (differential folds)	*p*
CCNA2	-1.18	1.0909E-03
CCNB2	-1.32	9.6326E-04
CCND1	-3.63	3.0711E-05
CDC20	-1.30	1.5777E-08
PLK1	-1.16	3.9519E-08
GADD45A	-1.44	4.1520E-04
TFDP2	1.90	2.7563E-04

Table I. Pathways: the first ten pathways in KEGG.

Term	Number	*p*	Q-value
MicroRNAs in cancer	11	1.02E-08	1.36E-06
Cell cycle	7	2.73E-07	1.83E-05
NOD-like receptor signaling pathway	5	1.57E-06	7.02E-05
HTLV-I infection	8	3.94E-06	1.32E-04
TNF signaling pathway	5	3.95E-05	1.06E-03
p53 signaling pathway	4	8.73E-05	1.93E-03
FoxO signaling pathway	5	1.01E-04	1.93E-03
NF-κB signaling pathway	4	2.69E-04	4.01E-03
Rheumatoid arthritis	4	2.69E-04	4.01E-03
Cytokine-cytokine receptor interaction	6	3.18E-04	4.26E-03

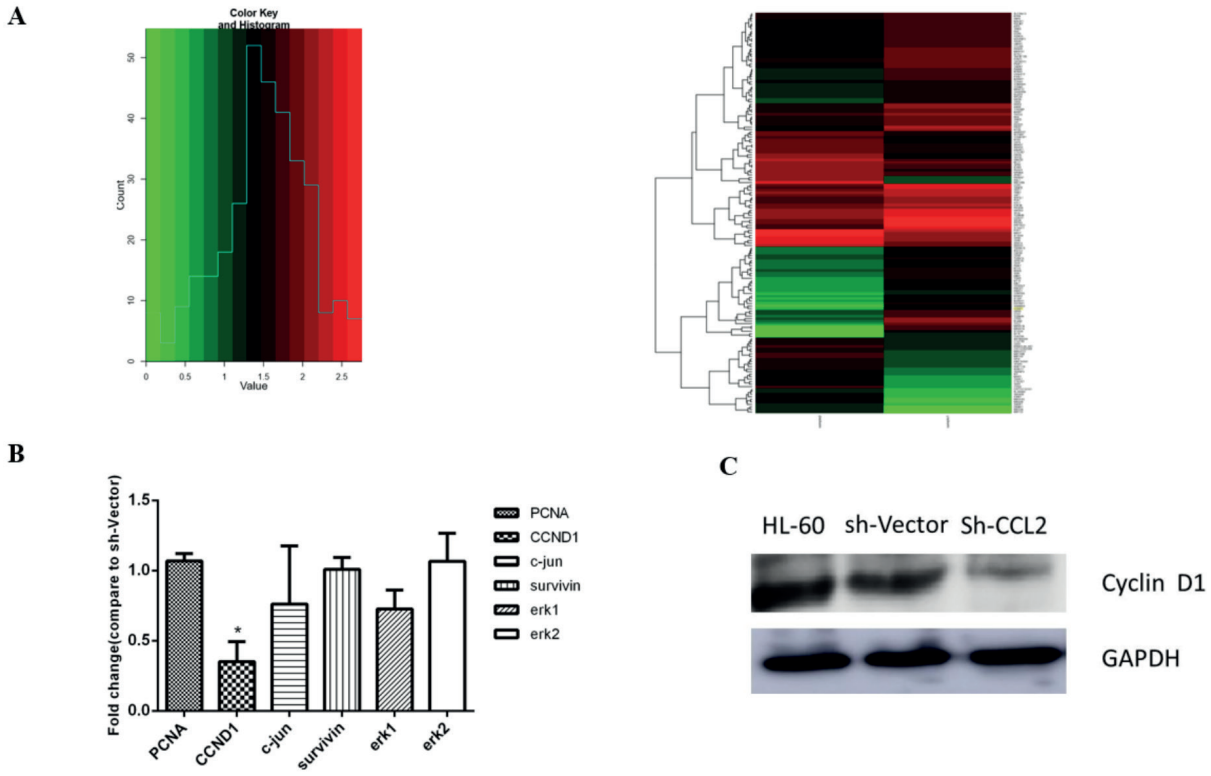

Figure 4. The potential mechanisms of CCL2 knockdown influencing the cell proliferation and cell cycle of HL-60 cells after stable transfection. ***(A)*** Thermal analysis of differential genes by gene expression profiling. ***(B)*** qRT-PCR analysis showed the mRNA levels of proliferation-related genes: PCNA, Cyclin D1, c-jun, survivin, erk1, and erk2. Among them, Cyclin D1 expression was the lowest. ***(C)*** Western blot analysis confirmed the protein expression of total Cyclin D1. *, p <0.05 compared with the control. Each bar represents the mean ± SD of 3 experiments.

Discussion

In some solid tumors, CCL2 has been proposed as a therapeutic target, and a human monoclonal antibody against CCL2, Carlumab, is at different stages of clinical trials[20-23]. However, reports investigating CCL2 in leukemia are relatively rare, especially reports on leukemia proliferation. In our study, we investigated the expression level of CCL2 in leukemia cell lines and found that the expression of CCL2 varies among leukemia cell lines, which indicated the leukemia cell lines' heterogeneity. In addition, we overexpressed CCL2 in THP-1 cells and knocked down CCL2 in HL-60 cells to investigate the function of CCL2. Our study showed that after suppressing CCL2 expression in HL-60 cells, the cell proliferation rate decreased a maximum of 44.44 % compared to control cells, and 12% more cells were arrested at G1 phase. Gene expression profiling data identified 159 differentially expressed genes in total related to the cell cycle, NOD-like receptor signaling pathway, TNF signaling pathway and NF-κB signaling pathway, including Cyclin D1, which was downregulated by 60 %; meanwhile, Cyclin D1 was decreased at the mRNA and protein levels. Moreover, we found that the shRNA-mediated inhibition of Cyclin D1 suppressed cell growth and blocked more cells at G1 phase, consistent with CCL2 silencing. Many studies have demonstrated that the CCL2-CCR2 axis played an important role in enhancing tumor cell proliferation through activating several signaling pathways in solid tumors. Activation of the PI3 kinase/Akt pathway was found to be vital for the proliferative effects of prostate cancer epithelial cells[2]. Moreover, CCL2 can promote the viability, proliferation and invasiveness of endometrial stromal cells through the Akt and MAPK/Erk1/2 signal pathways[24]. In addition, IL-33 enhances proliferation and invasiveness of decidual stromal cells by up-regulation of CCL2/

CCR2 via NF-κB and ERK1/2 signaling[25]. Here, we demonstrated that in leukemia cells, CCL2 silencing resulted in growth retardation, and Cyclin D1 interference with cell cycle progression might contribute to this change. Cyclin D1, which functions as a mitogenic sensor, is one of the more frequently altered cell cycle regulators in cancers[26,27]. It is frequently overexpressed in many tumours, including mantle cell lymphoma (MCL), non-small cell lung cancer and carcinomas of the breast, head and neck, and esophagus[28-32]. The role Cyclin D1 protein plays in regulating cell progress during the G1 phase of the cell cycle has been well characterized. D-type cyclins bind to and activate the cyclin-dependent kinases Cdk4 and Cdk6, which in turn phosphorylate the retinoblastoma protein Rb. Upon Rb phosphorylation, the E2F transcription factors activate the expression of S-phase genes and thereby induce cell cycle progression[17]. A previous work[18] showed that 8-chloroadenosine inhibits growth in human promyelocytic leukemia HL-60 cells by a G0/G1 phase arrest, with suppressed expressions of cyclin D1 and c-myc. Knocking down Cyclin D1 in MCL cell lines with specific lentiviral shRNA led to a moderate growth retardation and a 15% shift from S phase to G1 phase of the cell cycle[16]. These findings are consistent to our results. In addition, there is also emerging evidence revealing the association between CCL2 and Cyclin D1 and that MCP-1 induced the growth of human aortic smooth muscle cells (HASMCs), which required NFATc1-dependent cyclin D1 expression and CDK4/6 activity[19]. Here, in our study, with the help of second-generation sequencing technology, we further demonstrated that in leukemia cell lines, loss of Cyclin D1 affects cell proliferation through the effect of Cyclin D1 on cell cycle progression. We identified Cyclin D1 as a potential downstream target of CCL2 silencing that affects cell proliferation in HL60 cells.

Conclusions

We demonstrated an indispensed role for CCL2 in solid tumors and suggest it may be a therapeutic target during treatment regimens. Our study preliminarily demonstrated that in a leukemia cell line, interfering with the expression of CCL2 suppresses cell proliferation mediated by Cyclin D1

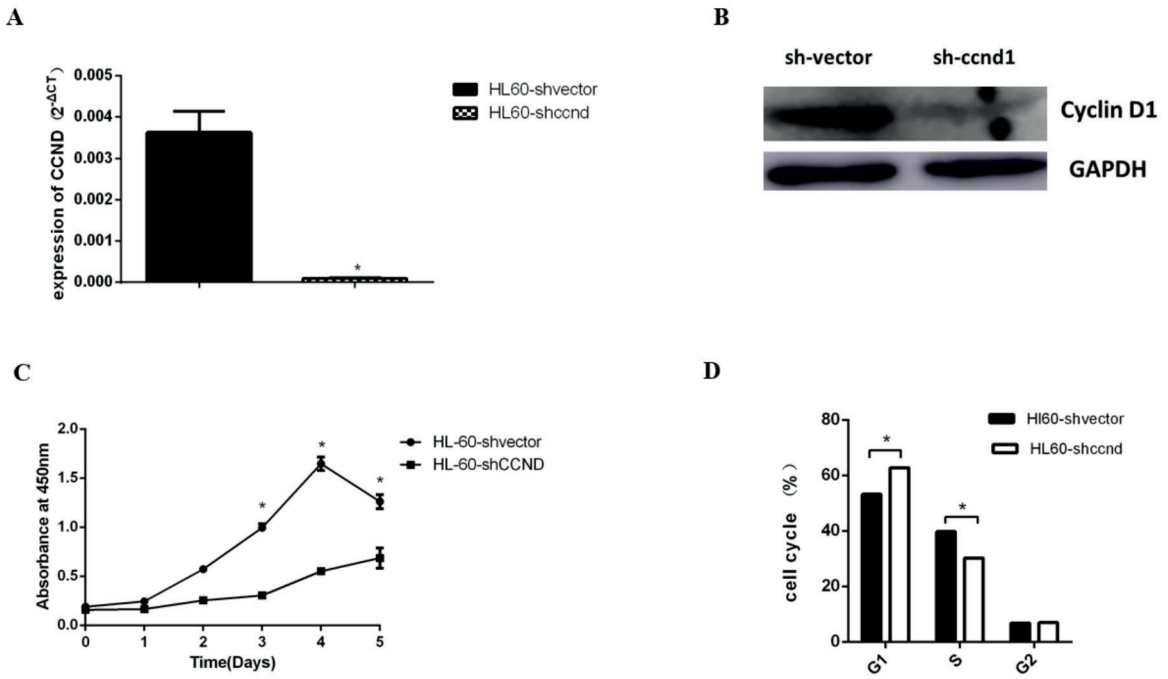

Figure 5. The effect of Cyclin D1 knockdown on cell proliferation and cell cycle *in vitro*. ***(A)*** qRT-PCR revealed that the mRNA expression level of Cyclin D1 was downregulated by shRNA-mediated interference. ***(B)*** Protein expression level showed the downregulation of Cyclin D1, which was analyzed by Western blot. ***(C)*** Knockdown of Cyclin D1 inhibits cell growth. ***(D)*** Knocking down Cyclin D1 blocked more cells at G1 phase compared with the control group.

via arresting cells at G1 phase, which was supported by gene expression profiling data. The role of CCL2 in leukemogenesis remains to be further investigated.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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