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Original Article

Downregulation of CDKL1 promotes gastric cell apoptosis through inhibiting cell growth and colony formation

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Abstract: Gastric cancer is the most common malignancy in digestive tract with rising incidence and younger trend. Cyclin-dependent kinase-like (CDKL1), a member of cell division cyclin (CDC-2) associated serine threonine protein kinase family, is found to be overexpressed in malignancy such as breast cancer and gastric cancer. The specific role and related mechanism in gastric cancer has not been reported. This study intends to investigate the impact of CDKL1 in gastric cancer cell growth, colony formation, and cell apoptosis. CDKL1 expression in human gastric cancer and adjacent tissues was tested by Western blot. CDKL1 level was regulated by lentivirus transfection. Cell proliferation was determined by MTT assay. Colony formation was evaluated by the colony forming assay. Cell cycle and apoptosis were detected by flow cytometry. CDKL1 significantly upregulated in gastric cancer tissue compared with adjacent tissue ($P < 0.05$). Downregulation of CDKL1 obviously restrained cell proliferation and colony formation, induced cell apoptosis, and blocked cell cycle in G1 phase. CDKL1 overexpression obtained the opposite results. CDKL1 was related to gastric cancer cell growth, colony formation, apoptosis, and cell cycle. It could be treated as a potential target for gastric cancer therapy.

Keywords: Gastric cancer, CDKL1, colony formation, apoptosis, apoptosis

Introduction

Gastric cancer is the most common type of malignancy in digestive tract [1]. In recent years, it shows rising incidence and younger trend. Except surgical treatment, inhibition of tumor growth and metastasis is stringent. Therefore, it is of great significance to explore the potential diagnosis and treatment target for gastric cancer.

The main characteristic of malignant tumor is uncontrolled excessive proliferation [2, 3]. It is mainly caused by the degradation of regulatory genes to check cell cycle, leading to the uncontrollable gene clone and amplification with genetic instability. Similarly, other than diet and stomach disease, genetic defects and variation also account for the pathogenesis of gastric cancer [4]. Checkpoint damage in the process of DNA replication is one of the important genetic defects affect the occurrence of gastric cancer [5]. Likewise, defective checkpoint may also be treated as a therapeutic target for the gastric cancer [6]. Cyclin-dependent kinase (CDK) protein family has confirmed to be the checkpoint of G1/S and G2/M [7, 8]. CDK forms active complex with specific cell cycle protein to involve in cell cycle downstream gene expression [9, 10]. Cyclin-dependent kinase-like (CDKL1), a member of cell division cyclin (CDC-2) associated serine threonine protein kinase family, is an important molecule to regulate cell cycle [11]. CDKL1 was found upregulated in breast cancer and gastric cancer [12, 13]. It was also reported that CDKL1 facilitated colony formation and inhibited cell apoptosis in melanoma [14]. However, the mechanism of CDKL1 in the occurrence and development of gastric cancer is still unclear.

Thus, we constructed lentivirus to knockdown or overexpress CDKL1 gene to regulate its level in gastric cancer cell line MGC-803. We aimed to investigate CDKL1 expression in gastric can-

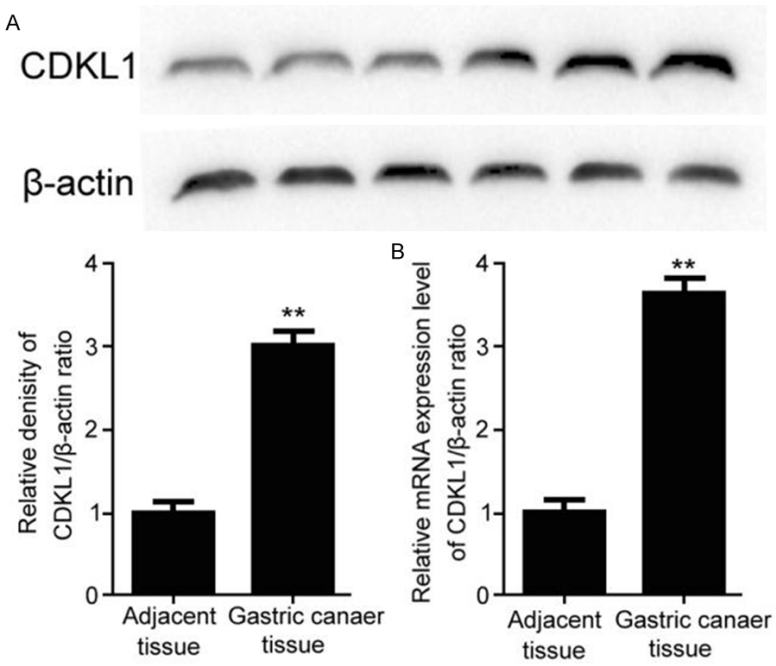

Figure 1. CDKL1 expression in human gastric cancer and adjacent tissues. A: CDKL1 protein expression. The left three samples were collected from adjacent tissues while the right three samples were from gastric cancer tissues. B: Relative expression folds of CDKL1 mRNA and protein in adjacent tissues and gastric cancer tissues. The level of CDKL1 was up-regulated in gastric cancer tissues. **P < 0.01, compared with adjacent tissue.

cer tissue and its role in gastric cancer cell proliferation, colony formation, and cell apoptosis.

Materials and methods

Main reagents and drugs

MTT reagent was purchased from Sigma (Shanghai, China). RT-qPCR one-step kit was bought from Transgen (Beijing, China). DMEM, FBS, and penicillin-streptomycin were got from Gibco (New York, NY). Human gastric cancer cell line MGC-803 and HEK293T cells were obtained from cell bank of Chinese academy of sciences (Shanghai, China). CDKL1 polyclonal antibody was derived from Abcam (Hong Kong, China). Goat anti-rabbit IgG (H+L) and β-actin antibodies were purchased from Proteintech (Wuhan, China). Lentivirus was bought from Genechem (Shanghai, China). AV-PI kit was got from Beyotime (Shanghai, China).

Sample collection

Tissue samples were derived from patients received gastrectomy in Shanxi Tumor Hospital. All subjects had signed informed consent. The tissue was stored at -80°C.

Cell culture and transfection

Human gastric cell line MGC-803 was cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained at 5% CO_2 and 37°C.

Lentivirus transfection

Lentivirus for CDKL1 knockdown and overexpression were synthetized by Genepharma (Shanghai, China) and cloned to pGCSIL-GFP plasmid vector with Age I/EcoRI. Then the lentivirus was used to transfect HEK-293T cells together with Lipofectamine 2000. After 72 h incubation, the CDKL1 knockdown or overexpressed lentivirus were collected and enriched by CentriconPlus-20 ultrafiltration centrifugation device.

The gastric cancer cells were seeded in 6-well plate and added with lentivirus according to the virus titer. The medium was changed on the second day and the transfection efficiency was evaluated under the fluorescent microscope.

Western blot

MGC-803 cells were digested by trypsin and lysed by 100 μl RIPA containing protease inhibitor, PMSF, and phosphatase inhibitor. After homogenization, the mixture was centrifuged at 14000 g for 10 min. Next, the supernatant was added with equal amount of loading buffer and degenerated by boiling for 5 min. The protein was separated by SDS-PAGE and transferred to PVDF membrane. After blocked by skim milk for 1 h, the membrane was incubated in primary antibody (CDKL1, 1:1000; β-actin, 1:1000) at 4°C overnight. Then the membrane was incubated in secondary antibody (1:1000) at 37°C for 1 h after washing for three times. At last, the membrane was imaged by ECL.

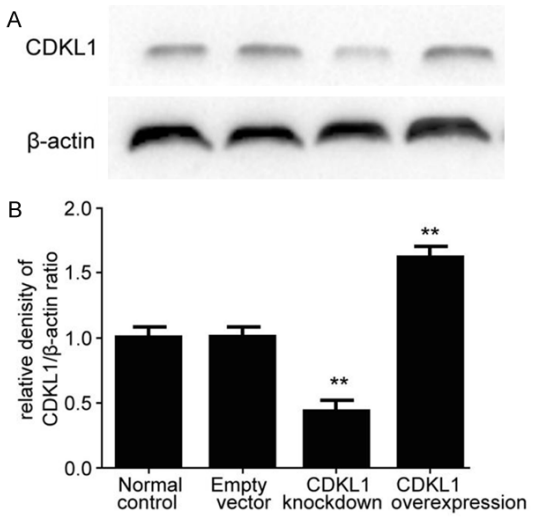

Figure 2. CDKL1 protein expression after CDKL1 gene knockdown and overexpression. A: Level of CDKL1 protein detected by western blotting. B: Relative expression fold of CDKL1 protein. The level of CDKL1 was decreased and increased in CDKL1 knockdown group and overexpression group respectively. **P < 0.01, compared with normal control.

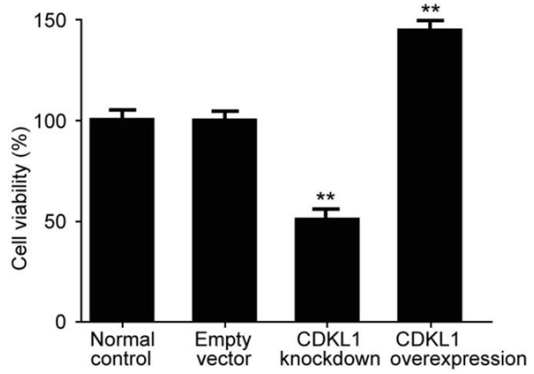

Figure 3. The impact of CDKL1 on cell proliferation. The cell proliferation in CDKL1 knockdown group was inhibited while that in overexpression group was induced. **P < 0.01, compared with normal control.

RNA extraction and PCR

The cells were washed by PBS for three times and treated by 1 ml Trizol on ice for 5 min. Next, the lysate was added with 200 μl chloroform at room temperature for 3 min. After centrifuged at 4°C and 12000 g for 15 min, the supernatant was mixed with 500 μl isopropanol at room temperature for 10 min. After centrifuged 4°C and 12000 g for 10 min, the sediment was washed by 1 ml ethanol for three times and dissolved in 20 μl DEPC water to obtain mRNA.

The primers for CDKL1 were designed and synthetized by Sigma [13, 14]. CDKL1, forward, 5'-CGAATGCTCAAGCAACTCAAGC-3', reverse, 5'-GCCAAGTTATGCTCTTCACGAG-3'. GAPDH was selected as internal control. GAPDH, forward, 5'-TGACTTCAACAGCGACACCCA-3', reverse, 5'-CACCCTGTTGCTGTAGCCAAA-3'. The reaction was composed of 50°C for 30 min, 95°C for 5 min, and followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 50 s. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

MTT assay

MGC-803 cells in logarithmic phase were digested by 0.25% trypsin and resuspended. Next, the cells were seeded in 96-well plate at 100 μl and transfected by 50 nm CDKL1 siRNA for 6 h. After changing the medium, the cells were further cultured for 18 h. Each group was repeated for eight times. After 24 h, the cells were added with 10 μl MTT solution for 4 h. At last, the crystal violet was dissolved by 150 μl DMSO and the plate was read at 490 nm to calculate cell survival.

Colony formation assay

The cells transfected by lentivirus were seeded in 6-well plate at 1000/well. After cultured for 7 days, the cells were washed by PBS and fixed by paraformaldehyde for 15 min. At last, the cells were stained by Giemsa for 20 min and the colony with more than 50 cells was counted under the fluorescence microscope.

Cell apoptosis

Cell apoptosis was determined by AV-PI staining. The cells were seeded in 6-well plate and transfected by lentivirus. After digested as single cell suspension, the cells were incubated in AV and PI on ice avoid of light for 10 min. At last, the cells were detected on BD FACS Calibur flow cytometry (BD Biosciences, San Diego, CA). Each experiment was repeated for three times.

Cell cycle

The cells were seeded in 6-well plate and transfected by lentivirus. After digested as single cell suspension, the cells were fixed by 70% ethanol for 1 h. Then the cells were resuspended and stained by propidium iodide on ice. At last, the

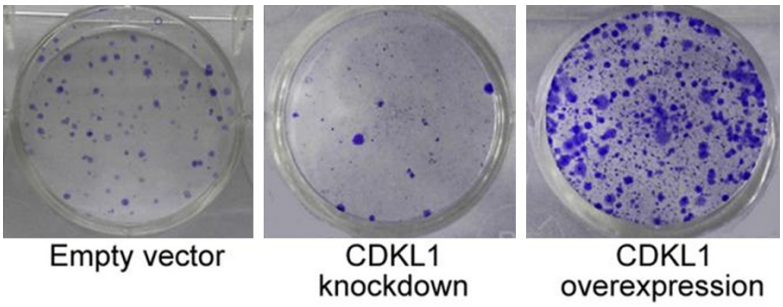

Figure 4. The impact of CDKL1 on cell colony formation. The cell colony formation in CDKL1 knockdown group was suppressed while that in overexpression group was increased, compared to that in empty vector group.

cells were filtered by 50 μm nylon net and detected on BD FACS Calibur flow cytometry (BD Biosciences, San Diego, CA). Each experiment was repeated for three times.

Statistical analysis

Each experiment was repeated for three times. The data were depicted as mean ± standard deviation and compared by t test, one-way ANOVA, or SNK method. $P < 0.05$ was considered as statistical significance.

Results

CDKL1 expression in human gastric cancer and adjacent tissues

Western blot and real-time PCR were applied to test CDKL1 protein and mRNA expressions in human gastric cancer and adjacent tissues. As shown in **Figure 1**, CDKL1 protein and mRNA levels significantly increased in gastric cancer tissue compared with adjacent tissue. It suggested that CDKL1 overexpressed at transcription level.

CDKL1 protein expression after CDKL1 gene knockdown and overexpression

After lentivirus transfection, CDKL1 protein expression was detected by Western blot. As shown in **Figure 2**, CDKL1 protein obviously declined and upregulated in knockdown group and overexpression group compared with empty vector group and control, respectively.

The impact of CDKL1 on cell proliferation

Cell proliferation was detected after lentivirus transfection. As shown in **Figure 3**, cell proliferation obviously attenuated in CDKL1 knockdown group compared with control. It significantly enhanced in CDKL1 overexpression group. It suggested that CDKL1 showed correlation with cell proliferation.

The impact of CDKL1 on cell colony formation

Giemsa staining was used to evaluate colony formation. As shown in **Figure 4**, cell colony formation markedly reduced after CDKL1 knockdown, while it obviously increased after CDKL1 overexpression. It revealed that CDKL1 promoted gastric cancer cell colony formation.

The impact of CDKL1 on cell cycle and apoptosis

Flow cytometry was selected to determine the impact of CDKL1 on cell cycle and apoptosis. As shown in **Figure 5A**, gastric cancer cell proportion increased in G1 phase and declined in G2/M phase after CDKL1 knockdown, indicating downregulation of CDKL1 blocked cells in G1 phase. On the contrary, CDKL1 overexpression accelerated cell cycle by decreasing cell number in G1 phase and increasing cell proportion in G2/M phase. As shown in **Figure 5B**, CDKL1 knockdown apparently elevated cell apoptosis, whereas CDKL1 overexpression exhibited the opposite effect, suggesting that CDKL1 knockdown may block cell cycle and enhance cell apoptosis.

Discussion

Gastric cancer is the most common gastrointestinal malignancy. Its incidence shows a younger trend following the improvement of living conditions. It was demonstrated that its occurrence and development is closely related to unhealthy living habit and environment pollution [4, 15]. Thus, it is urgent to discuss the potential therapeutic target of gastric cancer.

CDKL1 is the newcomer of CDK. CDKs can bind with cyclin to regulate cell cycle, differentiation, and apoptosis [16, 17]. Loss of cell cycle control leads to uncontrolled cell proliferation,

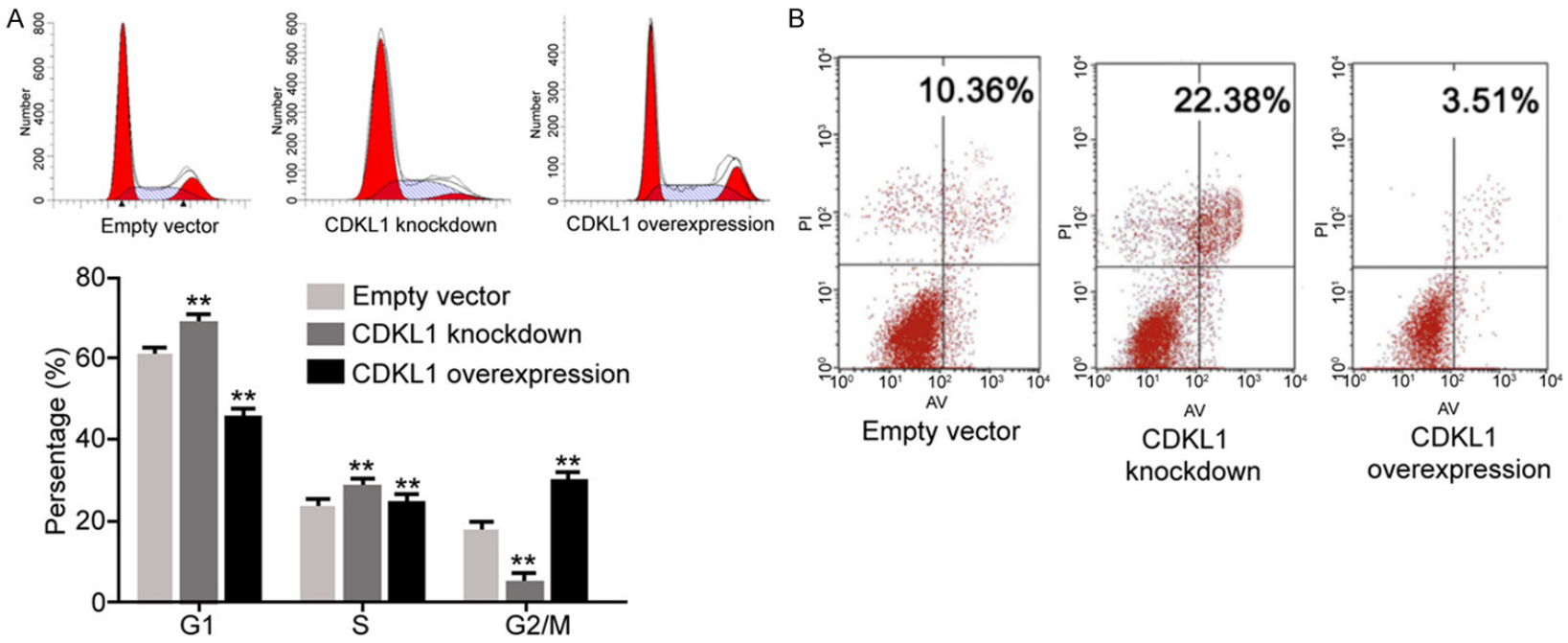

Figure 5. The impact of CDKL1 on cell cycle and apoptosis. A: Cell cycle analysis. CDKL1 knockdown decreased the cell arrest at G2/M phage while CDKL1 overexpression increased the cell arrest at G2/M phage. **P < 0.05, compared with empty vector group. B: Cell apoptosis. CDKL1 knockdown facilitated the cell apoptosis and its overexpression inhibited the apoptosis.

which is the main cause of tumor volume increase [18]. It was proposed that CDKL1 dysfunction inhibits cell proliferation and increases cell apoptosis in breast cancer and melanoma [12, 19]. Furthermore, CDKL1 was found upregulated in gastric cancer and breast cancer [12, 13]. However, the specific mechanism of CDKL1 in the occurrence and development of gastric cancer remains to be unclear.

Our results showed that CDKL1 overexpressed in human gastric cancer tissue. It also expresses in MGC-803 cell line. CDKL1 knockdown significantly suppressed cell proliferation and colony formation, arrested cell cycle, and promoted cell apoptosis. CDKL1 upregulation exhibited the opposite effect, suggesting that CDKL1 may be associated with the occurrence and development of gastric cancer.

Molecular targeted therapy has become the key in the investigation of gastric cancer [20]. Targeted therapy based on RNA interference showed great potential because of its effectiveness and specificity [21]. The candidate target for therapy includes the genes focusing on cell proliferation, migration, angiogenesis, and drug resistance. A good target should have high specificity and less cytotoxicity to normal cells [22]. They may show no genotoxicity related to chemotherapy [23]. In conclusion, specific downregulation of CDKL1 could be treated as a target for gastric cancer therapy.

In this study, we adopted single shRNA to knockdown CDKL1, which may be affected by off-target effect. Therefore, we applied CDKL1 overexpression by lentivirus transfection to observe the potential opposite effect. CRISPR/Cas9 could be used in the future to knockout CDKL1 gene. It may provide more strict result to select CDKL1 as therapeutic target combining with animal experiment.

Conclusion

CDKL1 was associated with gastric cancer cell growth, colony formation, apoptosis, and cell cycle. It could be considered as a valuable target for gastric cancer therapy.

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Disclosure of conflict of interest

None.

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