Acta Oncologica, 2012; 51: 790–796

informa
healthcare

ORIGINAL ARTICLE

A role for *Cdkl1* in the development of gastric cancer

WEI SUN[1], LI YAO[2], BENCHUN JIANG[1], HUA SHAO[1], YING ZHAO[1] & QIANG WANG[1]

[1]*Department of General Surgery, Affiliated Shengjing Hospital, China Medical University, Shenyang 110004, Liaoning Province, China, and* [2]*Department of Nephrology, The First Affiliated Hospital, China Medical University, Shenyang 110001, Liaoning Province, China*

Abstract

Background. Cyclin-dependent kinase-like1 (*CDKL1*) is known as a new member of cyclin-dependent kinases. Whether genetic alterations of *CDKL1* gene are involved in the development and/or progression of gastric cancer is still unknown. *Material and methods*. Here, the expression of CDKL1 protein in paired specimens of gastric cancer tissues and corresponding normal gastric tissue ($n = 66$) was assessed by immunohistochemistry assay. We then used lentivirus-mediated knock down to specifically inhibit *CDKL1* expression in human gastric cancer cell lines. Cell proliferation potential in vitro was measured by MTT and clonogenic assays. Cell apoptosis was assessed by flow cytometry. *Results*. We show for the first time that high expression of *CDKL1* protein was observed in gastric cancer tissues compared with matched adjacent tissues. Loss of *CDKL1* function in both SGC7901 and MGC-803 gastric cancer cells significantly decreases cellular proliferation and increases apoptosis ($p < 0.01$). Furthermore, we show that the reduction of *CDKL1* with its siRNA stimulates the activation of *Bcl-2-interacting killer* (*Bik*) pro-apoptotic protein and attenuated the expression of proliferating cell nuclear antigen *PCNA*. *Conclusion.* In summary, our data suggest that *CDKL1* plays an important regulatory role in gastric cancer cell proliferation and survival, and therefore, may represent a new target for therapeutic intervention.

Gastric cancer is one of the most common malignances worldwide and accounts for the second most common cause of cancer related death [1]. With current therapy approaches, the prognosis of gastric cancer is still very poor. The pathogenesis of gastric cancer is complex and related to multiple factors. Dysregulation of well-established oncogenic pathways, such as those mediated by cell cycle regulators [2,3], nuclear factor-kappaB [4,5], cyclooxygenase-2 [6,7] and epidermal growth factor receptor [8,9] are implicated in gastric carcinogenesis. In this scenario, more complete understanding of the underlying physiological, cellular, and molecular mechanisms could have a major impact on developing new treatment strategies such as gene therapy, which promise to become the alternative choice of treatment in gastric cancer.

Disturbed regulation of the cell cycle is a hallmark of cancer [10]. Cell cycle progression is known to be modulated by concerted action of cyclins with cyclin-dependent kinases (CDKs) [11]. In human genome, 21 genes encode CDKs, while five additional genes encode a more distant group of proteins known as CDK like (CDKL) kinases. CDKL kinase family is not known to interact with cyclins, but considered as a separate branch of CDK family that is similar to the MAP kinases group of signal transducing enzymes [12].

CDKL1 gene, also known as *KKIALRE*, was the first member of CDKL kinase family that was cloned on the basis of its similarity to the *cyclin-dependent kinase 1* (*CDK*1) kinase domain [12]. This protein kinase (*CDKL1*) shares homology with *CDKL2* (also known as *KKIAMRE*) and they exhibit mutually exclusive expression in reproductive tissues: *CDKL1* in ovary and *CDKL2* in testis. They may therefore contribute to signal transduction within these highly differentiated tissues. Both CDKL1 and CDKL2 contain the conserved MAP kinase dual phosphorylation motif Thr-Xaa-Tyr (Thr-Asp-Tyr), and are

Correspondence: W. Sun, Department of General Surgery, Affiliated Shengjing Hospital, China Medical University, Sanhao Street 36, Shenyang 110004, Liaoning Province, China. Tel: +86 24 96615 31311. Fax: +86 024 96615 13631. E-mail: weisun11@126.com

(Received 18 October 2011; accepted 5 February 2012)

ISSN 0284-186X print/ISSN 1651-226X online
DOI: 10.3109/0284186X.2012.665611

activated by treatment of cells with epidermal growth factor (EGF) [13]. However, unlike other MAP kinases, the EGF-stimulated kinase activity does not require phosphorylation on Thr and Tyr within this motif. In addition, the CDKL1 protein is mainly a glial protein that is upregulated in gliosis and it probably plays no role in the hyperphosphorylation of tau in Alzheimer's disease brains [14]. We know that *CDK1* is a key cell cycle regulator and required in higher cells for entry into S-phase [15]. However, to our knowledge, whether *CDKL1* is involved in the mitosis in human cells as well as human pathology, especially in cancers is not clear.

The present study aims to evaluate the expression of *CDKL1* in gastric cancer and elucidate a functional role of *CDKL1* in gastric cancer cell proliferation and apoptosis by using RNA interference (RNAi) to knock down the expression of *CDKL1*. Also, the apoptosis related molecules (*Bcl-2*, *Bik*, and $p21^{Waf1/cip1}$) and proliferation related marker (*PCNA*) were assessed in gastric cancer cells with loss of function of *CDKL1*. *PCNA* was chosen, primarily because it serves as a marker of cellular proliferation. *Bcl-2* anti-apoptotic protein and *Bcl-2-interacting killer* (*Bik*) pro-apoptotic protein were also chosen. $p21^{Waf1/cip1}$ was chosen given its role as an inhibitor of apoptosis. Importantly, we found that *CDKL1* is highly expressed in cancer areas compared to adjacent non-carcinoma tissues and suppression of *CDKL1* inhibits cell proliferation and induces apoptosis of gastric cancer cells. Taken together, our findings suggest that *CDKL1* plays an important regulatory role in gastric cancer cell growth, and therefore, may represent a new target for therapeutic intervention.

Material and methods

Immunohistochemical staining

The investigated specimens were obtained from 66 patients in the Department of Gastrointestinal Surgery, the Affiliated Shengjing Hospital of China Medical University. The paraffin-embedded blocks of those were cut 3-μm thick and used for further immunostaining as previously described [16]. The samples were incubated with mouse monoclonal antibody to CDKL1 (diluted 1: 200; Abcam) and horseradish peroxidase (HRP)-conjugated rabbit polyclonal secondary antibody to mouse IgG-H&L (1: 200 dilution; Abcam). The color was developed with 0.02% diaminobenzidine (DAB) solution. Negative controls were performed by replacing the primary antibody with phosphate-buffered saline (PBS). The stained sections were evaluated by two blinded pathologists independently and results were strongly consistent between the two readings. Expression of CDKL1 proteins was evaluated as positive if the stained cells were distributed in $>10\%$ of area. Each slice was scored according to the staining intensity of the positive cells and the percentage of positive cells to total cancer cells. Briefly, light yellow, brownish-yellow and dark-brown were scored as 0, 1, 2, and 3; and the positivity of 0, $<1/3$, 1/3 to 2/3, and $>2/3$ were scored as 0, 1, 2, and 3. The two combined score of 0 was defined as "−", 2 to 3 as "+", 4 as "++", 5 to 6 as "+++".

Cell culture

The gastric cancer lines, GES, SGC7901, MKN45, MGC-803, were obtained from Shanghai institutes for biological science (SIBS) cell bank. Cells were maintained in BPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) plus 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) in a 5% CO_2 incubator at 37°C.

Lentiviral CDKL1-siRNA vector construction and packaging

Small interfering RNA (siRNA) target sequence (CCAGCAAGTGTTTAGCACGAA) for *CDKL1* (NM_004196) was designed and short harpin RNA (shRNA) constructs was synthesized. Non-sense sequence (TTCTCCGAACGTGTCACGT) was designed and used as RNAi control. shRNA targeting *CDKL1* and control shRNA were respectively cloned into pGCSIL-GFP plasmid vector (GeneChem, Shanghai, China) which contains the green fluorescent protein (GFP) gene as a reporter with an internal CMV promoter. *CDKL1*-siRNA and scramble siRNA (scr-siRNA) plasmids were prepared and confirmed by DNA sequencing.

The *CDKL1*-siRNA (or Scr-siRNA) plasmid, pHelper 1.0 and pHelper 2.0 plasmids (GeneChem) were transfected 293T cells based on the instruction of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) when the cell density reached 70–80%. Lentivirus particles expressing *CDKL1*-siRNA or scr-siRNA (Lv-*CDKL1*-siRNA or Lv-scr-siRNA) were harvested from cell culture medium 48 hours after transfaction. Lentivirus was concentrated using Plus-20 centrifugal ultrafiltration device (Millipore, USA), and were kept at −80°C.

Infection of gastric cancer cells by lentivirus

When SGC7901 and MGC-803 cells were grown to 30% confluence in BPMI 1640 medium, appropriate amount of lentivirus was added according to the multiplicity of infection (MOI). The number of

GFP-positive cells was determined using fluorescence microscopy (Olympus, Tokyo, Japan) three days after infection.

Real time RT-PCR

A total of 8×10^3 cells transduced with control lentivirus or cells transduced with Lv- *CDKL1*-siRNA were seeded into a 12-well plate, respectively. Cells of each group were harvested after culture for five days. Total cellular RNA was extracted using Trizol reagent (Invitrogen) and reversely transcripted to cDNA by M-MLV reverse transcriptase (Promega Corp.) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qPCR) products were detected with SYBR Green I dye by using Thermal Cycler Dice Real Time System TP800 (TAKARA, Japan). *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene was amplified as internal control [17]. The primer sequences used were as follows: *GAPDH*-forward, TGACTTCAACAGCGACACCCA; *GAPDH*-reverse, CACCCTGTTGCTGTAGCCAAA; *CDKL1*-forward, CGAATGCTCAAGCAACTCAAGC, *CDKL1*-reverse, GCCAAGTTATGCTCTTCACGAG. Relative quantitation was done by taking the difference [Delta C(T)] between the C(T) of *GAPDH* and C(T) of each gene and computing 2[-Delta Delta C(T)] [18].

Western blot

Lentivirus infected cells for five days were collected and total protein was isolated and quantitated by the bicinchoninic acid (BCA) method. Protein (20 μg) was loaded onto a 10% SDS-PAGE and transferred to PVDF membrane. Then, CDKL1, Bik, $p21^{Waf1/Cip1}$, Bcl-2 and PCNA proteins were detected by respective antibodies using enhanced chemiluminescence (ECL) kit (Amersham) and visualized by exposure to x-ray film. GAPDH was used as loading control. The antibodies used were as follows: anti-CDKL1 (1: 500 dilution; Abcam), anti-Bik (1: 800 dilution; Santa-Cruz Biotechnology), anti-$p21^{Waf1/Cip1}$ (1: 800 dilution; Cell Signaling), anti-Bcl-2 (1: 800 dilution; Abcam), HRP-conjugated rabbit polyclonal anti-mouse IgG (H&L) antibody and anti-rabbit IgG (H&L) antibody (1: 5000 dilution; Santa-Cruz Biotechnology).

MTT cell proliferation assay

Lentivirus infected cells at the logarithmic phase were seeded into 96-well plates at the density of 2000 cells/well, and were incubated in 5% CO_2 incubator at 37°C. After one, two, three, four and five days of culture, the cell viability was assessed by MTT assay. Briefly, 20 μl MTT (5 mg/ml) was added and incubated for four hours. The reaction was terminated by removal of the supernatant and addition of 100 μl DMSO. The optical density (OD) of each well was measured at 490 nm using ELISA reader.

Assay of cell colony formation

Cells at the logarithmic phase after infection were inoculated in six-well plates at the density of 800 cells/well. Culture medium was changed every three days. When the cell number in most single colony was greater than 50, cells were fixed for 30 minutes in paraformaldehyde (Sangon Biotech Shanghai Co. Ltd), washed with PBS and stained with Giemsa dye (Chemicon). After washing with ddH_2O, the number of colonies was counted under the fluorescence microscope micropunlisher 3.3 RTV (Olympus).

Detection of apoptosis in gastric cancer cell line SGC7901

The protocol provided in Annexin V Apoptosis Detection Kit APC (eBioscience Inc., San Diego, CA, USA) was strictly followed. Briefly, cell culture supernatants and adherent cells in six-well plate were collected after seven days of Lv-*CDKL1*-siRNA transduction. Cells were washed with PBS by centrifugation at 1500 rpm. Then cells were washed once in 1X Binding Buffer and double stained with Annexin V APC/PI. After incubating for 10 minutes at room temperature in the dark, cells were analyzed by flow cytometry (FACS Calibur, BD Biosciences Innovation, USA).

Statistical analysis

All data were expressed as mean ± SD of three independent experiments, in which each assay was performed in at least triplicate. The Student's t-test was used to evaluate the differences between the control cells and *CDKL1* knockdown cells using SPSS 13.0 software. Statistic analysis concerning normal-tumor samples was performed using χ^2-test. $p < 0.05$ was considered as statistically significant.

Results

Expression of CDKL1 in gastric cancer

Sixty-six gastric cancer tissue specimens were analyzed for expression of CDKL1 protein using the immunohistochemistry assay. As shown in Table I and Figure 1, gastric cancer samples had a higher rate of positivity for CDKL1 expression in comparison to adjacent normal stomach tissue ($p < 0.0001$).

Table I. Expression pattern of CDKL1 in gastric cancer tissues and adjacent tissues. Immunoblot results were scored in four categories: negative expression, low-level expression, middle-level expression and high-level expression.

		CDKL1 expression				
	Number of cases	Negative (−)	Low (+)	Middle (++)	High (+++)	p-value
Cancer tissues	66	31	21	11	3	
Carcinoma						<0.0001
Normal tissues	66	63	3	0	0	

These results indicated that CDKL1 might be upregulated in tumorigenesis process of gastric cancer.

Knock down of CDKL1 gene expression by CDKL1-siRNA

CDKL1 protein was initially found to be expressed in a serial of gastric cancer cell lines (GES, SGC7901, MKN45 and MGC-803) as confirmed by Western blot (Figure 2A). In order to study the role of *CDKL1* in gastric cancer proliferation and apoptosis, we designed *CDKL1*-targeting shRNA which have been widely used in studying gene functions of the mammals [19–21]. Next, we constructed shRNA-expressing lentivirus targeting *CDKL1*. Successfully lentivirus infection on two common gastric cancer cell lines, SGC7901 and MGC-803 cells, was evidenced by the fluorescence microscopy three days after infection (Figure 2B). Clearly, more than 80% of cells showed GFP expression and kept normal cell morphology (Figure 2B).

qPCR and Western blot were used to detect the expression levels of *CDKL1* after lentivirus infection for five days when Lv-*CDKL1*-siRNA efficiently transduced both gastric cancer cell lines, reaching up to 100% of cells expressing GFP. It was clearly showed in Figure 2C and D that the mRNA levels of *CDKL1* was decreased by 76.4% and 82.2% in SGC7901 and MGC-803 cells compared with the control ($p<0.05$, $p<0.001$). Moreover, expression of CDKL1 protein was also significantly inhibited in CDKL1 knock-down gastric cancer cells (Figure 2E and F).

Effect of CDKL1-siRNA on cell growth and colony formation of gastric cancer cells

After infection by *CDKL1*-siRNA-expressing lentivirus for five days, we investigated the cell viability for five consecutive days by MTT assay. The number of viable cells in *CDKL1*-siRNA group was decreased compared to Scr-siRNA group. Statistical analysis results showed that the proliferation of SGC7901 and MGC-803 cells was significantly inhibited after knock down of *CDKL1* gene by CDKL1-siRNA ($p<0.001$, Figure 3A and B). In addition, colony-formation assay was performed to determine tumorigenesis in vitro. Cells in the control group were in cluster growth and formed

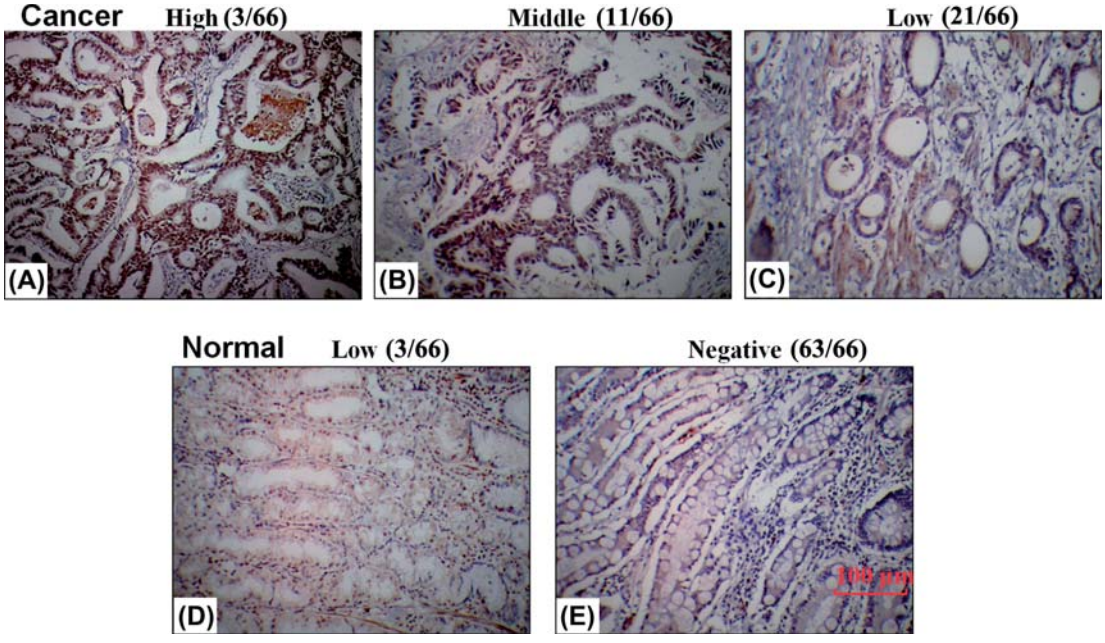

Figure 1. Images of immunohistochemical expression of CDKL1 in human gastric cancer compared with matched adjacent tissues. The specimens were viewed with an Olympus CKX41 microscope at magnification of 20×. (A, B, C) Representative photos of high-level, middle-level and low-level expression of CDKL1 protein in human gastric cancer. (D, E) Representative photos of low-level and negative expression of CDKL1 protein in adjacent tissues.

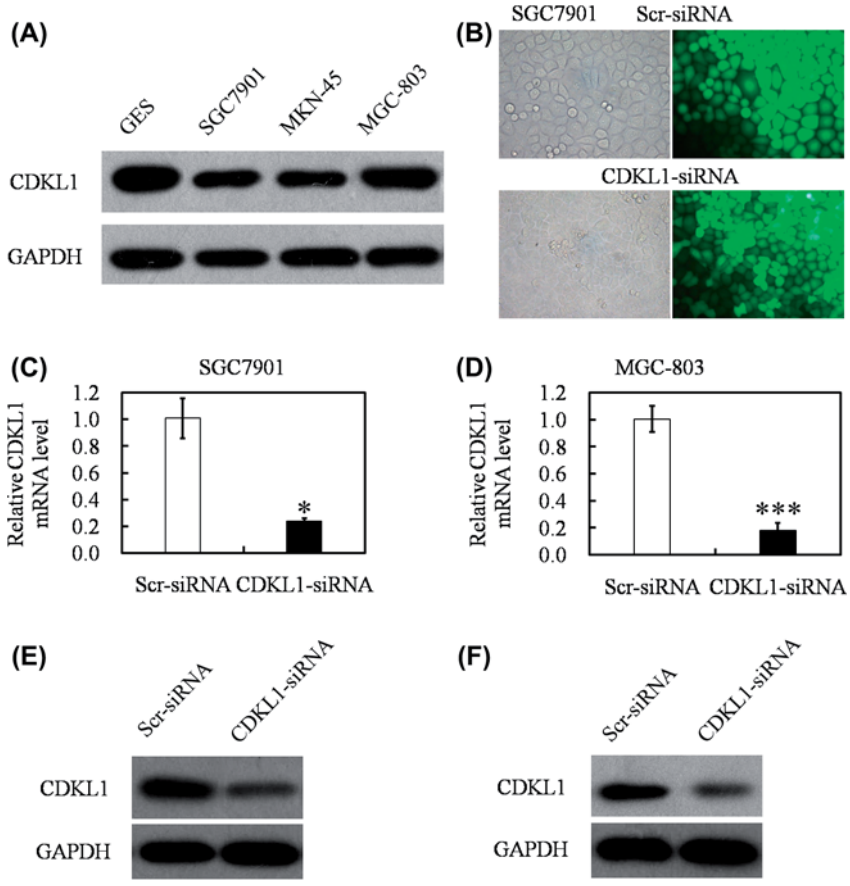

Figure 2. Infection of gastric cancer cells by *CDKL1*-siRNA-expressing lentivirus. (A) Analysis of CDKL1 protein in gastric cancer cell lines (GES, SGC7901, MKN45, MGC803) by Western blot assay. (B) The lentiviral vector containing *CDKL1* siRNA or a scrambled sequence was constructed and respective lentivirus was packaged. SGC-7901 cells were infected with *CDKL1* siRNA lentivirus or control lentivirus for three days and observed under fluorescence microscopy in bright field (left panel) and fluorescent field (right panel) with 400 × magnification. (C, D) Expression levels of mRNA were measured by qPCR. In comparison with control, *CDKL1* siRNA lentivirus infection resulted in 76.4% and 82.2% decrease in the expression level of CDKL1 mRNA in SGC7901 (C) and MGC-803 (D) cells, respectively. (E, F) Expression levels of protein were measured by Western blot. CDKL1 protein was significantly downregulated in CDKL1 siRNA lentivirus infected SGC7901 (E) and MGC-803 (F) cells. *, $p < 0.05$ and ***, $p < 0.001$.

colonies 14 days after infection. Consistently, the colony formation was completely inhibited in both *CDKL1* knock-down cell lines. In comparison with the control, the number of colonies in SGC7901 and MGC-803 cells was decreased by 56.5% and 86.2%, respectively ($p < 0.001$, Figure 3C and D).

Effect of CDKL1-siRNA on cell apoptosis of gastric cancer cells

In order to study the mechanism of *CDKL1* in modulating cell growth, cells apoptosis was measured using Annexin V-APC staining and flow cytometry after *CDKL1*-siRNA-expressing lentivirus infection. We found that knock down of *CDKL1* gene by CDKL1-siRNA caused a significant increase in the population of apoptotic cells, compared to the control group ($p < 0.01$, $p < 0.001$, Figure 4A and B).

Expression of apoptosis related genes

To elucidate to the mechanisms by which *CDKL1* promotes gastric cancer growth and survival, we then determined proliferation and apoptosis-related gene using qPCR and Western blot. As shown in Figure 5A, knock down of CDKL1 significantly promotes the expression of *Bik* and inhibits the expression of *Bcl-2* gene ($p < 0.05$, $p < 0.01$). Furthermore, the reduction of *CDKL1* led to increased expression of Bik and $p21^{Waf1/Cip1}$ protein and decreased expression of Bcl-2 and PCNA proteins (Figure 5B).

Discussion

Like almost all other cancers, gastric cancer has a molecular genetic basis which relies on the disorders in normal cellular regulatory mechanisms that governs cell growth, apoptosis and cell division [22]. In present

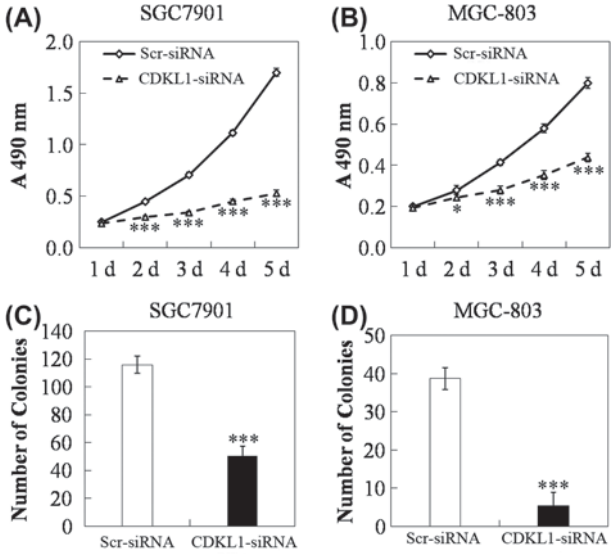

Figure 3. The effect of *CDKL1* knock down on the proliferation and colony formation of gastric cancer cells. (A, B) Analysis of cell viability of CDKL1 siRNA lentivirus or control lentivirus infected cells for one to five days. The proliferation of both SGC7901 (A) and MGC-803 (B) cells was significantly inhibited after *CDKL1* knock down. (C, D) Colony formation in lentivirus infected cells was assayed 14 days after culture. Statistical results of colony number showed that the colony-forming ability was impaired in *CDKL1*-siRNA lentivirus infected SGC7901 (C) and MGC-803 (D) cells. *, $p < 0.05$ and ***, $p < 0.001$.

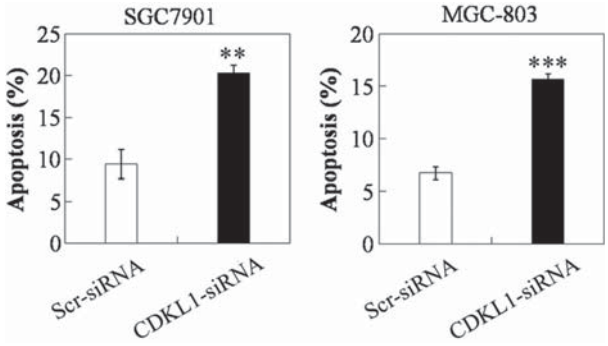

Figure 4. Detection of apoptotic cells by flow cytometry. After seven days of lentivirus infection, SGC7901 (A) and MGC-803 (B) cells were cultured in six-well plates and subjected to Annexin V staining/flow cytometric analysis. Results showed that *CDKL1* knock down resulted in inducing the apoptosis of both gastric cancer cells. **, $p < 0.01$ and ***, $p < 0.001$.

research, we observed a significant upregulation of CDKL1 protein in human gastric cancer tissues (Figure 1). We then employed lentivirus-mediated knock down to specifically inhibit *CDKL1* gene expression and demonstrated that loss of *CDKL1* function significantly impaired the proliferation and induced apoptosis of SGC7901 and MGC-803 cells. The results of the present study demonstrated that *CDKL1* may be a tumor promoter for gastric cancer.

Sequential activation and inactivation of CDK serine-threonine kinases family modulate the progression of cells through the cell cycle. In particular, *CDK1* controls progression from the S phase through G2 and into the M phase. Loss of cell cycle control, leading to uncontrolled proliferation, is common in human cancers. *CDK1* has been viewed to play the most crucial role of the G2/M modulators in cell cycle progression and cell proliferation of hepatocellular carcinomas (HCC) and significantly predicts the recurrence of this carcinoma [23]. Furthermore, a correlation was found between the immunoexpression of CDK1 and G2 cyclins and they play an important role in the progression of thyroid malignant lymphoma [24].

CDKL protein kinases, comprising five members, *CDKL1* to *CDKL5*, were identified based on their structural relation to CDKs. Although CDKL have a similar structure to CDKL in mammals, their function in cancer is not so clear. In our study, we found that knock down of *CDKL1* effectively inhibits cell proliferation and colony formation in gastric cancer cells (Figure 3), indicating that *CDKL1* is a positive

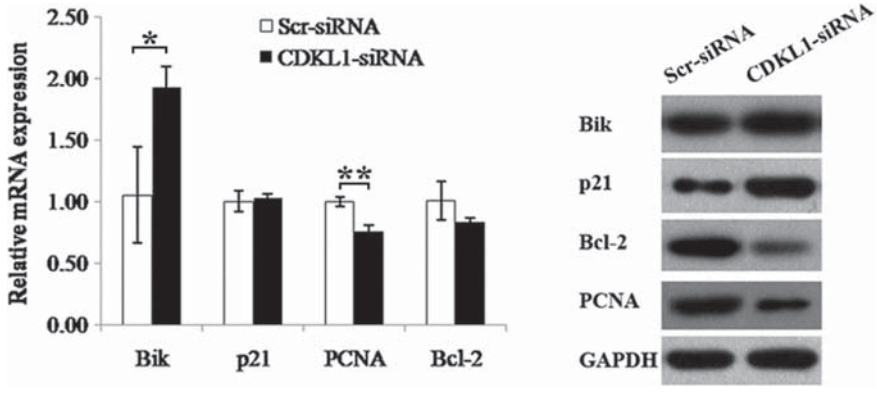

Figure 5. Analysis of *CDKL1* downstream target genes by Western blot. Expression of proliferation and apoptotic markers was observed in *CDKL1*-siRNA lentivirus infected SGC7901 cells by qPCR (A) and Western blot (B). The reduction of *CDKL1* with its siRNA stimulates the activation of apoptotic molecules, such as *Bik* and *p21* $^{Waf1/Cip1}$ and attenuated the expression of *Bcl-2* and *PCNA*. *, $p < 0.05$ and **, $p < 0.01$.

regulator of cell proliferation. In addition, we found that knock down of *CDKL1* can significantly increase apoptosis of gastric cancer cells (Figure 4). These results indicated that *CDKL1* is an inhibitor of apoptosis in gastric cancer. Furthermore, differential expression of proliferation and apoptosis-related genes was observed in *CDKL1* knock-down cells (Figure 5). Among them, Bcl-2 and Bik proteins were two members of Bcl-2 family. *Bcl-2* is anti-apoptotic gene, while *Bik* is an apoptosis-inducing gene. In addition, $p21^{Waf1/cip1}$ is cyclin-dependant kinase inhibitor well known to prevent cyclin D/CDK 4, 6 and cyclin E/CDK 2 mediated phosphorylation of the retinoblastoma protein (Rb) and to block progression of the cell cycle in the G1 phase. Moreover, overexpression of $p21^{Waf1/cip1}$ also triggered apoptosis. *PCNA*, an amplifier of cell proliferation, has been widely used as a tumor marker for cancer cell progression and patient prognosis. Collectively, the increase expression of *Bik* and $p21^{Waf1/cip1}$, as well as the decrease of *Bcl-2* and *PCNA* expression in *CDKL1* knock-down cells will be account for the enhancement of cell apoptosis and the inhibition of proliferation.

In summary, these findings might extend our knowledge of the biological progression of gastric cancer and may provide a new therapeutic target for treatment of this disease. However, subsequent study of the signaling pathway which modulates *CDKL1* function in gastric cancer still needs to be elucidated.

Acknowledgements

This study was supported by grants-in-aid from the project of Department of Education, No. L2010628, Liaoning Province, China.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74–108.

[2] Begnami MD, Fregnani JH, Nonogaki S, Soares FA. Evaluation of cell cycle protein expression in gastric cancer: Cyclin B1 expression and its prognostic implication. Hum Pathol 2010;41:1120–7.

[3] Kim DH. Prognostic implications of cyclin B1, p34cdc2, p27(Kip1) and p53 expression in gastric cancer. Yonsei Med J 2007;48:694–700.

[4] Camp ER, Li J, Minnich DJ, Brank A, Moldawer LL, MacKay SL, et al. Inducible nuclear factor-kappaB activation contributes to chemotherapy resistance in gastric cancer. J Am Coll Surg 2004;199:249–58.

[5] Lee BL, Lee HS, Jung J, Cho SJ, Chung HY, Kim WH, et al. Nuclear factor-kappaB activation correlates with better prognosis and Akt activation in human gastric cancer. Clin Cancer Res 2005;11:2518–25.

[6] Park ES, Do IG, Park CK, Kang WK, Noh JH, Sohn TS, et al. Cyclooxygenase-2 is an independent prognostic factor in gastric carcinoma patients receiving adjuvant chemotherapy and is not associated with EBV infection. Clin Cancer Res 2009;15:291–8.

[7] de Maat MF, van de Velde CJ, Umetani N, de Heer P, Putter H, van Hoesel AQ, et al. Epigenetic silencing of cyclooxygenase-2 affects clinical outcome in gastric cancer. J Clin Oncol 2007;25:4887–94.

[8] Xia L, Yuan YZ, Xu CD, Zhang YP, Qiao MM, Xu JX. Effects of epidermal growth factor on the growth of human gastric cancer cell and the implanted tumor of nude mice. World J Gastroenterol 2002;8:455–8.

[9] Moon WS, Tarnawski AS, Chai J, Yang JT, Majumdar AP. Reduced expression of epidermal growth factor receptor related protein in gastric cancer. Gut 2005;54:201–6.

[10] Wu WK, Cho CH, Lee CW, Fan D, Wu K, Yu J, et al. Dysregulation of cellular signaling in gastric cancer. Cancer Lett 2010;295:144–53.

[11] Malumbres M, Harlow E, Hunt T, Hunter T, Lahti JM, Manning G, et al. Cyclin-dependent kinases: A family portrait. Nat Cell Biol 2009;11:1275–6.

[12] Meyerson M, Enders GH, Wu CL, Su LK, Gorka C, Nelson C, et al. A family of human cdc2-related protein kinases. EMBO J 1992;11:2909–17.

[13] Taglienti CA, Wysk M, Davis RJ. Molecular cloning of the epidermal growth factor-stimulated protein kinase p56 KKIAMRE. Oncogene 1996;13:2563–74.

[14] Yen SH, Kenessey A, Lee SC, Dickson DW. The distribution and biochemical properties of a Cdc2-related kinase, KKIALRE, in normal and Alzheimer brains. J Neurochem 1995;65:2577–84.

[15] Pacek M, Prokhorova TA, Walter JC. Cdk1: Unsung hero of S phase? Cell Cycle 2004;3:401–3.

[16] Wegiel B, Bjartell A, Ekberg J, Gadaleanu V, Brunhoff C, Persson JL. A role for cyclin A1 in mediating the autocrine expression of vascular endothelial growth factor in prostate cancer. Oncogene 2005;24:6385–93.

[17] Zhang S, Yang JH, Guo CK, Cai PC. Gene silencing of TKTL1 by RNAi inhibits cell proliferation in human hepatoma cells. Cancer Lett 2007;253:108–14.

[18] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402–8.

[19] Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, Kopinja J, et al. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat Genet 2003; 33:401–6.

[20] Raoul C, Abbas-Terki T, Bensadoun JC, Guillot S, Haase G, Szulc J, et al. Lentiviral-mediated silencing of SOD1 through RNA interference retards disease onset and progression in a mouse model of ALS. Nat Med 2005;11:423–8.

[21] Ebert BL, Pretz J, Bosco J, Chang CY, Tamayo P, Galili N, et al. Identification of RPS14 as a 5q-syndrome gene by RNA interference screen. Nature 2008;451:335–9.

[22] Janjigian YY, Shah MA. Molecularly targeted therapies in advanced gastric cancer. Minerva Gastroenterol Dietol 2011; 57:75–88.

[23] Ito Y, Takeda T, Sakon M, Monden M, Tsujimoto M, Matsuura N. Expression and prognostic role of cyclin-dependent kinase 1 (cdc2) in hepatocellular carcinoma. Oncology 2000;59:68–74.

[24] Ito Y, Yoshida H, Matsuzuka F, Matsuura N, Nakamura Y, Nakamine H, et al. Cdc2 expression in primary thyroid lymphoma: Its relationship with biological aggressiveness and G2 cyclins. Pathol Res Pract 2003;199:533–8.