Overexpression of Cyclin L2 Inhibits Growth and Enhances Chemosensitivity in Human Gastric Cancer Cells

Hong-Li Li, Ding-Zhi Huang, Ting Deng, Li-Kun Zhou, Xia Wang, Ming Bai, Yi Ba*

Abstract

Cyclin L2 is a novel member of the cyclin family, recently implicated in the regulation of cell cycle progression and/or transcriptional regulation. The present study was undertaken to investigate the effects of overexpression on tumor cell growth and chemosensitivity in human gastric cells in vitro. Cyclin L2 was transfected into human gastric cancer cell line BCG823 and expressed with a mammalian expression vector pcDNA3.1. The effects and mechanisms of cyclin L2 on cell growth, cell cycling and apoptosis were studied. Compared to control vectors, overexpression of cyclin L2 inhibited the growth of BCG823 cells and enhance their chemosensitivity to fluorouracil, docetaxel and cisplatin. The anti-proliferative effects of cyclin L2 could be due to G0/G1 arrest and apoptosis. Cyclin L2 induced G0/G1 arrest and apoptosis involved upregulation of caspase-3 and down regulation Bcl-2 and survivin. The results indicated that overexpression of cyclin L2 protein may promote efficient growth inhibition and enhance chemosensitivity to chemotherapeutic agents in human gastric cancer cells by inducing G0/G1 cell cycle arrest and apoptosis.

Keywords: Cyclin L2 - gastric cancer - apoptosis - cell cycle - chemosensitivity
(human lung adenocarcinoma cell lines) were obtained from the American Type Culture Collection (ATCC, Baltimore, USA, except for HT-29 (human hepatocellular carcinoma cell lines). Cells were maintained in RPMI 1640 medium containing 100 U/ml penicillin, 100 mg/ml streptomycin and 10% fetal bovine serum (Gibco, UK) at 37 °C in a 5% CO₂ humidified atmosphere.

**Mammalian expression vector and cell transfection**

The full-length coding region of human cyclin L2 was cloned into the myc-6His-tagged expression vector pcDNA3.1/Myc-His (pCCNL2) and the antisense construct pCCNL2-AS or pCNA3.1 mock vector were all kindly provided by Prof. Cao Xue-Tao (Institute of Immunology, Second Military Medical University, Shanghai, China). The pCCNL2, pCCNL2-AS, or pcDNA3.1 mock vectors were transfected using liposomemediated LipofectAMINE reagent (Invitrogen, USA) into A549 cells according to the manufacturer’s instructions and screened under 500 μg/ml G418 (Calbiochem, USA) for 3-5 weeks. Cell clones of stably transfected cells were obtained by the limited dilution method.

**Cell proliferation assays**

Cell proliferation of transiently transfected BCG823 cells were assessed by cell viability using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) dye reduction assay. To initiate experiment, cells were seeded at 5×10⁴ cells/ml in 96-well plates and harvested at 0, 24, 48, 72 and 96 hour after transient transfection of pCCNL2, pCCNL2-AS, or pcDNA3.1 mock vector, respectively. One hundred μl of the culture medium containing MTT reagent (1 mg/ml) was added into the wells and incubated for additional 4 hours. Then the medium was removed and 100 ml of Me2SO (Sigma, USA) was added to each well. The plates were shaken at room temperature for 10 minutes. Cellular viability was determined by measuring the absorbance of the converted dye at a wavelength of 570 nm.

**Flow cytometry assay**

After transient transfection of pCCNL2, pCCNL2-AS, or pcDNA3.1 mock vectors for 24, 48, 72 hours, respectively, BCG823 cells were harvested and fixed overnight with 70% ethanol at 4 °C, followed by resuspension in 500 μl of phosphate buffered solution (PBS). After the addition of 10 μl RNase (10 mg/ml), cells were incubated for 30 minutes at 37 °C and stained with 10 ml propidium iodide (1 mg/ml). The cells suspension was analyzed on a Coulter Epics Elite flow cytometer (Beckman-Coulter, Miami, USA). Cell cycle analysis was performed with the Multicycle System (Phoenix Flow Systems, USA).

**Chemotherapy-induced cytotoxicity**

BCG823 cells were seeded in a 96-well plate at a concentration of 5×10⁴ cells/well for 16 h and then transfected with pCCNL2, pCCNL2-AS, or pcDNA3.1 mock vectors for 24 hours respectively. Cells were incubated with medium contain chemotherapeutic agents in different concentrations (fluorouracil at the concentration of 0.625, 2.5, 10, 40, 120 mg/L, docetaxel at the concentration of 10, 50, 100, 500, 1000 nmol/L, or cisplatin at the concentration of 0.125, 0.5, 2, 8, 32 mg/L, respectively) for 48 h. Cell viability was examined by MTT assay at 570 nm (OD readings). The suppression rate was calculated using the formula: Suppression rate = (1-ODtreatment/ODcontrol) × 100%.

**Protein extraction**

BCG823 cells were plated in 6-well plates and transient transfection of pCCNL2, pCCNL2-AS, or pcDNA3.1 mock vector for 24 hours, respectively. Floating and adherent cells were harvested and combined. Cells were lysed with RIPALysis buffer containing protease inhibitor Cocktail II (Upstate, Lake Placid, NY, USA). The samples were reduced at 70 °C for 10 minutes, and cooled on ice before being stored at -80 °C.

**Western blot**

Western blot was performed as described previously (Barton et al., 2005). Briefly, proteins (20 μg/lane) were separated by 12% SDS-PAGE. The SeeBlue prestained standard (Invitrogen, USA) was used to determine protein size. After electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (GE Health, USA) using the XCell II Blot Module (Invitrogen, USA) for 1.5 hours at 30 V. The membrane was blocked with 5% nonfat milk in PBST (PBS, 0.1% Tween 20) for 1 hour. After being washed three times with PBST, the membrane was then incubated with primary antibody diluted in PBST, 5% nonfat dry milk powder at room temperature with agitation for 1 hour. Blots were then probed with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, USA), and protein bands were detected using the enhanced chemiluminescence (ECL) system (Cell Signaling, USA) and quantified by densitometry using Bio-Rad Quantity One software (Bio-Rad, Hercules, USA).

**Antibodies**

All of the antibodies, excluding BCI-2 (Cell signaling), were commercially obtained from Santa Cruz (Berkeley, USA). The sequence of cyclin L2 cDNA was sub-coned inframe into pGEX-4T3 (Amersham Biosciences, USA) for glutathione S-transferase (GST) fusion protein expression. GST-CCNL2 fusion protein expressed in Escherichia coli BL21 (Stratagene, CAN) was affinity-purified with glutathione-sepharose 4B (Amersham Biosciences) and eluted in 10 mmol/L reduced glutathione. An antiserum specific for the long form of cyclin L2 was produced by immunization of rabbits with a peptide corresponding to amino acids 437-448 within the C-terminal serine/arginine-rich (RS) domain.

**Statistical analysis**

Values were expressed as mean ± standard deviation (SD). Results were evaluated by analysis of variance (ANOVA) using SPSS 11.0 software (SPSS, Chicago, USA), unless otherwise specified. A P value < 0.05 was considered to represent statistical significance.
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Results

Transfection of pCCNL2 into BCG823 cells

Human cyclin L2 is expressed widely in normal human tissues and tumors cells. We have detected the expression of human cyclin L2 in three human solid tumor cell lines. As shown in Figure 1A, cyclin L2 is expressed a relatively higher level in human gastric cancer cell lines BCG823 and human hepatocellular carcinoma cell lines HT-29, but lower in human lung adenocarcinoma cell lines A549. (A) Human cyclin L2 was higher expressed in human gastric cancer cell lines BCG823 and human hepatocellular carcinoma cell lines HT-29, but lower in human lung adenocarcinoma cell lines A549. (B) The protein of cyclin L2 expressed by BCG823 cells which transfected pCCNL2 was further verified using western blot. (C) Cell proliferation of transiently transfected BCG823 cells was determined by MTT. The results represent means of three independent experiments. Asterisk indicates P < 0.05 compared with the control.

Figure 1. Cyclin L2 Expression in Vitro and Effect on the Growth of BCG823 Cells. (A) Human cyclin L2 was higher expressed in human gastric cancer cell lines BCG823 and human hepatocellular carcinoma cell lines HT-29, but lower in human lung adenocarcinoma cell lines A549. (B) The protein of cyclin L2 expressed by BCG823 cells which transfected pCCNL2 was further verified using western blot. (C) Cell proliferation of transiently transfected BCG823 cells was determined by MTT. The results represent means of three independent experiments. Asterisk indicates P < 0.05 compared with the control.

Figure 2. Effect of Combination of Cyclin L2 and Chemotherapeutic Agents on BCG823 Cells Proliferation by MTT in Vitro. Overexpression of cyclin L2 enhanced the chemosensitivity of human gastric cancer cell lines BCG 823 cells to fluorouracil (A), docetaxel (B) and cisplatin (C). The results represent means of three independent experiments. Asterisk indicates P < 0.05 compared with the control.

Figure 3. Analysis of Cell-cycle arrest and Apoptosis in Combination of Cyclin L2 and Chemotherapeutic Agents on BCG 823 Cells. (A) After transfecting with pCCNL2, pCCNL2-AS or pcDNA3.1, cells were treated with Fluorouracil, Docetaxel or Cisplatin in different concentrations, respectively. (B) Effect of CCNL2 overexpression on caspase-3, Bcl-2 and survivin. BCG 823 cells were transfected with pcDNA3.1 (mock), pCCNL2, or pCCNL2-AS, respectively, with parental cells as control. Whole cells lysates were analyzed for caspase-3, Bcl-2 and survivin by Western blot. b-actin was used as a loading control. Representative blots from three independent experiments are shown.

Anti-proliferative effects of cyclin L2 on BCG 823 cells

Addressing whether overexpression of human cyclin L2 would affect the growth of BCG823 cells may help understanding the molecular mechanisms of the cellular malignant phenotype, and provide insights of invasion and metastasis of tumor cells. Proliferation of BCG823 cells transiently transfected with pCCNL2 were inhibited with that of mock vector-transfected cell or parental BCG823 cells (P < 0.05), especially in cells transfected with pCCNL2-AS (P < 0.01). The results suggested that overexpression of human cyclin L2 has inhibited growth of human gastric cancer cells in vitro.

Effect of cyclin L2 or chemotherapeutic agents on BCG823 cell proliferation

Next we assessed whether overexpression of cyclin L2 affect chemosensitivity of Fluorouracil, Docetaxel or Cisplatin to BCG823 cells. After transfecting with pCCNL2, pCCNL2-AS or pcDNA3.1, cells were treated.
with pCCNL2 for 24 hours, cell cycle analysis revealed an increase of G0/G1 phase and an evident apoptosis by comparison to the control cells (Table 1).

Next we assessed whether overexpression of cyclin L2 affects chemotherapeutic agents mediated apoptosis. After transfecting with pCCNL2, pCCNL2-AS or pDNA3.1, cells were treated with Fluorouracil, Docetaxel or Cisplatin in different concentrations respectively. We found that BCG823 cells accumulated in the G0/G1 phase and after transfected with pCCNL2 for 24 hours, in contrast to cells transfected with mock vector or parental cells (Figure 3A, Table 2). In addition, compared with control group cells, its significant increases in the proportion of apoptosis cells which transfected pCCNL2 and then exposed to chemotherapeutic agents, respectively (Figure 3A, Table 2).

Overexpression of cyclin L2 up-regulates the expression of caspase-3 and down-regulates the expression of Bcl-2 and survivin

It is well known that cell cycle arrest and apoptosis are associated with activation of caspases and anti-apoptosis proteins. To examine the molecular mechanisms underlying the G0/G1 phase arrest and apoptosis in BCG823 cells induced by overexpressed cyclin L2, we examined the levels of the apoptosis protein Bcl-2 and survivin by Western blot analysis. As shown in Figure 3B, transient expression of cyclin L2 induced an increase in caspase-3 (2- and 3-fold, respectively) in BCG823 cells within 24 hours post-transfection compared with pCCNL2-AS and mock vector-transfected, or parental BCG823 cells. Conversely, Bcl-2 and survivin expression were down-regulated in BCG823 cells overexpression of human cyclin L2. These results demonstrate that overexpression of human cyclin L2 modulates the expression of critical apoptotic factors, leading to cell cycle arrest and apoptosis.

**Discussion**

Cyclins have been shown to play an important role in the regulation of the cell cycle. Certain cyclins and their pair proteins, CDKs, are recognized to have critical roles in the regulation of the transcription (Graaf et al., 2004; Egloff et al., 2006). Cyclin L2 and the closely related isof orm, cyclin L1 (originally named cyclin L) differ from all other members of the cyclin family by the presence of a C-terminal RS domain. The RS domain is a hallmark of many proteins involved in pre-mRNA progression of the cell cycle and signal transduction of apoptosis and oncogenesis (Graaf et al., 2004; Yang et al., 2004; Loyer et al., 2008). In the present study, we observed that overexpression of human cyclin L2 have direct anti-proliferative effects on BCG823 cells, and also able to induce cellular apoptosis in vitro. Elucidating the mechanism in specific cell type is important for understanding the growth regulation in particular tumor type. Therefore, cell cycle progression was further analyzed by flow cytometry in this study. Both G0/G1 phase arrest and apoptosis were found to contribute to cyclin L2 mediated growth suppression in BCG823 cells, which is consistent with the cell cycle arrest and apoptosis

**Table 1. Cyclin L2 Delays G0/G1 Progression and Induces Apoptosis**

<table>
<thead>
<tr>
<th>Groups</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
<th>Apoptosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental</td>
<td>58.1±5.12</td>
<td>32.5±5.52</td>
<td>9.40±0.74</td>
<td>0.62±0.21</td>
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<tr>
<td>Mock</td>
<td>60.6±2.50</td>
<td>32.5±1.06</td>
<td>6.99±1.78</td>
<td>2.01±0.63</td>
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<tr>
<td>CCNL2</td>
<td>72.0±5.75 *</td>
<td>17.5±5.45</td>
<td>10.8±3.25</td>
<td>7.34±0.51 *</td>
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<tr>
<td>CCNL-AS</td>
<td>18.0±0.48</td>
<td>80.5±3.04</td>
<td>0.46±0.41</td>
<td>0.19±0.06</td>
</tr>
</tbody>
</table>

Cell cycle and apoptosis rates of BCG 823 cells were evaluated by flow cytometry as described in “Materials and Methods”; Data were represented as mean±SD of three independent experiments; *P<0.05, §P<0.01.

**Table 2. Cyclin L2 Enhance Chemotherapeutic Sensitivity**

<table>
<thead>
<tr>
<th>Groups</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
<th>Apoptosis (%)</th>
</tr>
</thead>
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<tr>
<td>Cisplatin</td>
<td>56.3±2.86</td>
<td>23.2±1.53</td>
<td>11.6±2.84</td>
<td>11.5±1.60</td>
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<tr>
<td>Mock</td>
<td>56.0±2.05</td>
<td>33.7±2.01</td>
<td>10.4±2.00</td>
<td>13.5±2.34</td>
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<tr>
<td>CCNL2</td>
<td>67.6±2.65 *</td>
<td>21.4±1.69</td>
<td>11.1±1.68</td>
<td>44.2±5.09 *</td>
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<tr>
<td>CCNL-AS</td>
<td>56.2±1.73</td>
<td>38.4±1.75</td>
<td>5.46±1.06</td>
<td>8.48±2.57</td>
</tr>
<tr>
<td>Fluorouracil</td>
<td>56.6±1.34</td>
<td>34.3±2.63</td>
<td>9.13±0.71</td>
<td>13.1±1.06</td>
</tr>
<tr>
<td>Mock</td>
<td>57.3±1.41</td>
<td>35.2±1.71</td>
<td>7.54±0.71</td>
<td>14.4±2.85</td>
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<tr>
<td>CCNL2</td>
<td>68.1±1.23 *</td>
<td>0.72±0.21</td>
<td>17.7±2.32</td>
<td>35.3±6.24 *</td>
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<tr>
<td>CCNL-AS</td>
<td>46.1±1.34</td>
<td>38.0±2.83</td>
<td>15.9±1.48</td>
<td>7.69±1.29</td>
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<tr>
<td>Docetaxel</td>
<td>4.12±0.78</td>
<td>36.9±1.92</td>
<td>59.0±2.14</td>
<td>8.03±1.89</td>
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<tr>
<td>Mock</td>
<td>2.18±1.27</td>
<td>34.8±2.14</td>
<td>63.0±3.13</td>
<td>9.28±2.29</td>
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<tr>
<td>CCNL2</td>
<td>44.8±1.41 *</td>
<td>17.3±2.71</td>
<td>37.9±1.71</td>
<td>29.7±9.71 *</td>
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<tr>
<td>CCNL-AS</td>
<td>5.33±1.64</td>
<td>22.1±2.01</td>
<td>72.6±3.64</td>
<td>4.52±1.21</td>
</tr>
</tbody>
</table>

Cell cycle and apoptosis rates of BCG 823 cells to cyclin L2 combination with therapeutic agents were evaluated by flow cytometry as described in “Materials and Methods”; Data were represented as mean±SD of three independent experiments; *P<0.05, §P<0.01.

**Cyclin L2 treatment with chemotherapeutic agents induces cell cycle arrest and apoptosis**

According to the results of MTT assay, we further analyzed the effect of overexpression of CCNL2 on cell cycle and apoptosis by flow cytometry. BCG 823 cells were transiently transfected with pCCNL2 and compared with that of mock vector or parental BCG 823 cells. BCG823 cells transfected with pCCNL2-AS were used to eliminate endogenous CCNL2. After transfected
analysis in human hepatocellular carcinoma SMMC7721 cell lines and human lung carcinoma A549 cell lines (Yang et al., 2004; Li et al., 2007).

Systemic chemotherapy is widely accepted as palliative treatment for patients with unresectable or metastatic gastric cancer (Wagner et al., 2006; Ajani et al., 2007; Bang et al., 2010). Despite some advances in treatment of advanced gastric cancer, there is still no a satisfactory regimen which could be accepted as standard regimen for gastric cancer (Wagner et al., 2006; Ohtsu et al., 2011). Chemotherapeutic drug resistance is a fundamental problem in cancer management, responsible for most cases of treatment failure in patients with metastatic cancer (Swanton et al., 2007). An emerging understanding of the molecular pathways that characterize cell growth, cell cycle, apoptosis, angiogenesis and invasion has provided novel targets in cancer therapy (Shapiro et al., 2006; Ohtsu et al., 2011). Accordingly, combining various chemotherapeutic agents with nontoxicoty agents is of great importance for improving the efficacy of chemotherapeutics and overcoming resistance to cytotoxic drugs (Tabernero et al., 2005). Some previous studies reported that combined treatment with cyclin-dependent kinase pathway inhibitors and chemotherapeutic agents for cancer treatment resulted in additive therapeutic effects (Shapiro., 2006; Lazzarini et al., 2008). In the present study, we firstly demonstrated that combination of cyclin L2 and chemotherapeutic agents such as fluorouracil, docetaxel or cisplatin resulted in enhanced cell growth inhibition in BCG823 cells in vitro. Cell cycle analysis revealed that combination cyclin L2 with conventional chemotherapeutic agents enhanced the arrest of BCG823 cells in G0/G1 phase and an evident apoptosis. The current studies directly support the incorporation of cyclin L2 into chemotherapeutic drugs treatment regimens as a means to improve the chemosensitivity of each drug. Despite there is still an urgent need to develop novel drugs that can target different sites and pathway of the cell cycle while avoiding drug induced cytotoxicity in the clinical applications, the data should be further validated in animal model in future.

As we known, fluorouracil interferes with DNA synthesis by blocking thymidylates, an enzyme involved in the conversion of deoxyuridylic acid to thymidylic acid (Li et al., 2008). Docetaxel can impair the dynamic of microtubules, promote their polymerization, and arrest the cells in mitosis by binding to the β subunit of tubulin (Hernandez-Vargas et al., 2007). Cisplatin can covalently bond to proteins, RNA, and especially DNA, forming DNA cross-linking and intranstrand N-7 adducts (Reedijk et al., 1985). To investigate the mechanisms of cyclin L2-induced cell cycle arrest, we evaluated the expression of apoptosis-related protein in BCG823 cells. Caspases-3 is one of key effector molecules that are required in most apoptotic pathway (Alenzi, et al., 2010). Overexpression of cyclin L2 induced an increase in caspase-3 in BCG823 cells 24 hours post-transfection compared with mock vector-transfected, or parental BCG823 cells. Conversely, Bcl-2 and survivin expression were down-regulated in BCG823 cells which overexpressed human cyclin L2. These data suggested that cyclin L2 might activate caspase-3 cascade by suppressing antiapoptotic protein levels. These different targeted sites and pathways of the cell cycle from the chemotherapeutic agents such as fluorouracil, docetaxel or cisplatin might be partly causes of cyclin L2 enhanced chemosensitivity in human gastric cancer cells.

In summary, this study demonstrates that overexpression of cyclin L2 could inhibit the growth of human gastric adenocarcinoma BCG823 cells and enhance the chemosensitivity to fluorouracil, docetaxel and cisplatin in vitro. These effects could be due to cyclin L2 induced G0/G1 arrest and apoptosis involved upregulation of caspase-3 and down regulation Bcl-2 and survivin. It seems to be reasonable to perform further studies to identify the therapeutic benefit of cyclin L2 in animal model with solid tumors, and also to evaluate whether the combination of cyclin L2 with cytotoxic agents enhances antitumor effect in vivo.

Acknowledgements

The author(s) declare that they have no competing interests.

References


