African Journal of Biotechnology Vol. 10(65), pp. 14618-14624, 24 October, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.890
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Mutation analysis of the negative regulator cyclin G2 in gastric cancer

Jinlan Gao#, Qi Liu#, Xing Liu, Yang Zhang, Shusen Wang and Yang Luo*

The Research Center for Medical Genomics, MOH Key Laboratory of Cell Biology and Key Laboratory of Cell Biology, Ministry of Education, China Medical University, Shenyang 110001, China.

Accepted 5 August, 2011

Cyclin G2 is an unconventional cyclin which might have a potential negative role in carcinogenesis. In this study, the effect of cyclin G2 overexpression on gastric cell proliferation and expression levels of cyclin G2 in normal gastric cells and gastric cancer cells were investigated. Moreover, mutation analysis was performed in tissues from 57 gastric cancer patients. Our data demonstrate that ectopic expression of cyclin G2 inhibited the proliferation of gastric cells and the expression of cyclin G2 were significantly decreased in gastric cancer cells compared with normal cells. No mutations were found but four synonymous SNPs were detected in gastric cancer tissues, suggesting that mutations of cyclin G2 might not be the main reason for gastric carcinogenesis.

Key words: Cyclin G2, gastric cancer, negative regulator, mutation screen.

INTRODUCTION

Gastric cancer is one of the most common malignant tumors in the world. With a high death rate, it ranks as the second most common cause of cancer death worldwide. Hence, elucidating the mechanisms of gastric carcinogenesis is urgent for its prevention and treatment. Like other types of cancer, multiple oncogenes and tumor suppressor genes are involved in the development of gastric cancer as the results of genetic and/or epigenetic alterations (Wu et al., 2010).

Cyclins are critical regulators of the cell cycle. Highly conserved and characterized by a dramatic periodicity in protein abundance through the cell cycle, cyclins activate specific cyclin-dependent kinases (CDKs) to promote cell cycle progression (Liu et al., 2011; Hydbring and Larsson, 2010; Lang and Calegari, 2010). Based on their structural similarity and functional period during cell cycle, cyclins have been identified into different groups. Till now, more than 10 groups have been identified.

As a member of cyclin G family identified in 1996, cyclin G2 gene is located on 4q21.22 and encodes a 126-amino acid protein with the molecular weight of 39 kDa (Jensen et al., 1999). Cyclin G2 is highly expressed in cerebellum, thymus, spleen, prostate, kidney and the immune system (Horne et al., 1996, 1997). In contrast to the conventional cyclins, cyclin G2 is suggested to be a negative regulator of the cell cycle (Bennin et al., 2002; Horne et al., 1997). Overexpression of cyclin G2 is reported to promote cell cycle arrest and apoptosis (Arachchige et al., 2006). Moreover, its down-regulation has been reported in thyroid cancer, breast cancer, oral cancer and acute leukemia (Alevizos et al., 2001; Bogni et al., 2006; Choi et al., 2009; Jia et al., 2005; Kim et al., 2004). Our previous study showed that ectopic expression of cyclin G2 could inhibit colony forming efficiency in the gastric cancer cell line SGC-7901 (Tian et al., 2002). Additionally, the expression levels of cyclin G2 were demonstrated to correlate with gastric cancer progression (Choi et al., 2009). However, less is known about the underlying mechanisms of cyclin G2 on gastric carcinogenesis.

In this study, the regulation of cyclin G2 overexpression on cell viability and the expressions of cyclin G2 in gastric cells were investigated. Furthermore, mutation analysis was performed in tissues from 57 gastric cancer patients

*Corresponding author: E-mail: luoyang@mail.cmu.edu.cn. Tel: 86-24-23256666-5532.

Abbreviations: CDK, Cyclin-dependent kinase; **DHPLC,** denaturing high-performance liquid chromatography.

#These authors contributed equally to the work.

with PCR, denaturing high-performance liquid chromatography (DHPLC) and sequencing. Our results indicate that cyclin G2 is a putative negative proliferation regulator in gastric cells and cyclin G2 expressions are significantly down regulated in gastric cancer cell lines at mRNA level and protein level. In addition, no mutations are found but four synonymous SNPs are detected in mutation screen, suggesting that mutations of cyclin G2 might not be the main reason for gastric carcinogenesis.

MATERIALS AND METHODS

Clinical data

Matched normal and tumor tissues were obtained from 57 patients (44 men and 13 women) with primary gastric carcinoma from the Department of Oncology of the First Clinical College in China Medical University, from January 2003 to October 2005. According to tumor, node, metastasis (TNM) staging system (7th edition), there were 10 cases of I stage, 18 cases of II stage, 25 cases of III stage and 4 cases of IV stage. Normal tissues were 5 cm away from tumor tissues. All patients did not receive preoperative chemotherapy or radiotherapy. Approval for the study was received from the Ethics Committee of China Medical University.

Recombinant construction

The cyclin G2 encoding sequence was cloned into the p3xFLAG-CMV-7.1 eukaryotic expression vector using standard procedure and the recombinant plasmid p3xFLAG-CMV-G2 was verified by sequencing.

Cell culture and transfection

Human gastric epithelial immortalized cells GES-1 were maintained in Dulbecco's modified Eagle's medium; the human gastric cancer cell lines SGC-7901 and MGC-803 were maintained in RPMI-1640 medium. Cells were cultured with 10% fetal bovine serum in a humidified environment containing 5% CO_2 and held at a constant temperature of 37°C. Transient transfections were performed with Lipofectamine™ 2000 Reagent (Invitrogen) as described by the manufacturer.

Trypan blue exclusion test

GES-1 cells were seeded at 0.5×10^5 cells per well in 24-well plates. Transfections were performed as above and proliferation was determined using the trypan blue exclusion test. Cells were digested with 0.25% tryspin, collected and stained with 0.4% trypan blue (Sigma-Aldrich, USA) for 5 min at 24, 48 and 72 h post-transfection, respectively. The unstained cells were counted on a hemocytometer using an upright microscope (Olympus).

Methyl thiazolyl tetrazolium (MTT) assay

GES-1 cells were seeded and transfections were performed as described above. MTT (Sigma-Aldrich, USA) was added to each well at 24, 48 and 72 h post-transfection and left for 4 h, respectively (Zandi et al., 2010). The medium was removed after incubation and DMSO was added to dissolve the crystals by shaking the plate gently for 10 min. The absorbance at 570 nm was read with a microplate reader.

Reverse transcription polymerase chain reaction (RT-PCR)

Cellular RNA from GES-1, SGC-7901 and MGC-803 cells was extracted using TRIzol reagent and 1 µg of total RNA was reverse transcribed into cDNA. RT-PCR was performed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Primers were the following: 5′-TGT TGC CAT CAA TGA CCC CTT-3′ (sense) and 5′-CTC CAC GAC GTA CTC AGC G-3′ (antisense) for GAPDH; 5′-TGC CTA GCC GAG TAT TCT TCT-3′ (sense) and 5′-TGT TTG TGC CAC TTT GAA GTT G-3′ (antisense) for cyclin G2. The intensity of bands was quantified using LabWorks™ Image Acquisition and Analysis Software (Ultra-Violet Products Ltd., Cambridge, UK).

Preparation of cell lysates and western blot

Cells were washed twice with ice-cold PBS and rinsed in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 5 mM EDTA, 1% NP-40 with protease inhibitor cocktail (Roche). The lysates were sonicated and centrifuged at 12,000 rpm for 15 min at 4°C. Proteins were separated by SDS-PAGE, transferred to PVDF membrane and immunoblotting was performed using the standard protocol: Blocking with 5% nonfat milk in TBS, incubating primary antibody overnight and secondary antibody for 2 h, washing 3 times in TBST 0.05% and signals were revealed with super signal chemiluminescent substract (Thermo Scientific).

Genomic DNA extraction, primer design and PCR amplification

Genomic DNA was extracted from matched normal and cancer tissues from 57 gastric cancer patients using the established phenol/chloroform protocols as described previously (Chen et al., 2008). Primers to amplify each exon and flanking intronic sequences according to the genomic sequence of cyclin G2 were outlined in Table 1. PCR amplifications were performed according to the following conditions: initial denaturation at 94°C for 1 min, followed by 35 cycles with 30 s at 94°C, 30 s at 55°C, 1 min at 72°C, with a final extension step at 72°C for 5 min. Following PCR, reactions were denatured by heating to 95°C for 5 min and then cooled to 25°C over 30 min at 0.01°C/s to enhance heteroduplex formation. The products were examined on 1.5% agarose gels.

DHPLC analysis and sequencing

The fragments were directly injected into a DNASep column. Each fragment was analyzed under three partially denaturing temperatures using the Transgenomic WAVE DNA Fragment Analysis System and associated WAVE-Maker™ Software with the optimal temperature determined as previously reported (Chen et al., 2008). The chromatograms of each fragment were compared to those obtained from normal DNA. Fragments containing heteroduplexes with a shorter retention time were purified and sequenced for detecting variations.

Data analysis

Results were expressed as the mean ± S.D. of three independent experiments and statistical significance was determined by Student's *t* test for paired values. Differences at $P < 0.05$ were considered to be significant.

Table 1. Primer sequences and DHPLC conditions for mutation screen of cyclin G2.

Fragment	Primer sequence (5' to 3')	PCR size (bp)	Tm (°C)	DHPLC temperatures (°C)
Exon1	5′-GATTACATTCTCTGTGTGGTGTC-3′ 5′-TTACCTCCTCCAAGCCCCATCA-3′	228	60.4	59.4 60.4
Exon2	5′-CTGTGTGGTGTCTTTACTGC-3′ 5′-CCCATCAGCTGCTTGAGCAA-3′	203	60.3	60.3 61.3
Exon3	5′-CCTTGGTTCTTGGTTTTTATTGC-3′ 5′-GTGCTAGGCACTGGGCATAGAA-3′	219	53.2	56.2 57.2
Exon4	5′-GACAGTTGTTAAAAGAAGCCTC-3′ 5'-CTGGGCAACAAGAGCGAAATT-3′	464	53.9	53.9 54.9
Exon5	5'-GGAAGTTTAATAAATCTTTGTTCTTTGC-3′ 5′-CAAGGCTAATACAGATGGCTG-3′	224	53.2	55.0 56.8
Exon6	5′-GCAACTGCCGACTCATCTTTTC-3′ 5′-CAAGTGTAAAAGTTAAGAGTCCC-3′	332	53.9	55.0 54.4 53.8
Exon7	5′-TTTGGGAGACATTGCCGTAACC-3′ 5′-GTAGGTGCTTGCTTTCTCTG-3′	356	55.5	55.5 57.4 58.5
Exon8	5′-CCTCACTAGAGATATAGCAGC-3′ 5′-GGCTAAACCAGGAAACTACC-3′	301	55.1	55.1 57.7

RESULTS

Ectopic expression of cyclin G2 inhibits proliferation of gastric cells

To investigate the regulation of cyclin G2 overexpression on the proliferation rate, GES-1 cell viability was examined by the trypan blue exclusion test and MTT assay, respectively.

For the trypan blue exclusion assay, cell numbers were monitored at the different time points. The cell number was suppressed to 85% at 24 h and it kept decreasing to 81% at 48 h and 79% at 72 h post-transfection ($P < 0.05$, Figure 1A).

In the MTT assay, the proliferative changes were presented as relative cell numbers based on the OD_{570} value. The actual absorbance value was obtained by the absorption value of each well after subtracting the blank absorbance value. Compared with the control, the number of cells transfected with cyclin G2 was reduced by 25, 22 and 26% at 24, 48 and 72 h post-transfection, respectively ($P < 0.05$, Figure 1B). Hence, over expression of cyclin G2 is demonstrated to inhibit gastric cell proliferation significantly.

Expressions of cyclin G2 are decreased in gastric cancer cells

RT-PCR was repeated 3 times to analyze the differences of cyclin G2 expression levels among gastric cancer SGC-7901, MGC-803 cells and the normal GES-1 cells. As shown in Figure 2A, the mRNA expression of cyclin G2 decreased to 40% in the poorly-differentiated MGC-803 cells while 85% in the well-differentiated SGC-7901 cells contract to that of GES-1. Furthermore, we also detected protein levels of cyclin G2 in gastric cancer cells. Consistent with RT-PCR data, Figure 2B demonstrated that protein expression of cyclin G2 decreased in gastric cancer cells. In addition, we observed that the protein expression of cyclin G2 was lower in MGC-803 cells than that of SGC-7901, suggesting that expression reductions of cyclin G2 at mRNA levels and protein levels might be involved in the progression of gastric carcino-

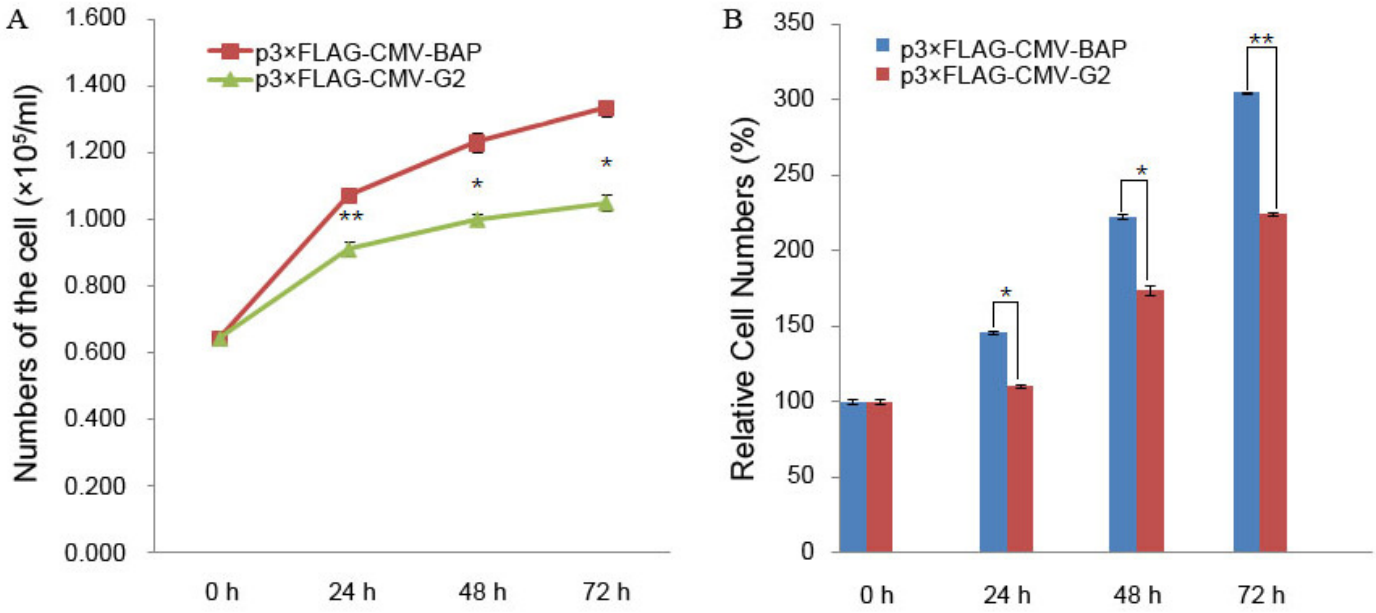

Figure 1. Overexpression of cyclin G2 significantly inhibits cell proliferation of gastric cells**.** Trypan blue exclusion tests (A) and MTT assays (B) were performed on GES-1 cells at 24, 48 and 72 h post-transfection, respectively. The data presented were mean ± S.D. of triplicate wells. Advanced $^{*}P < 0.05$ and $^{**}P < 0.01$.

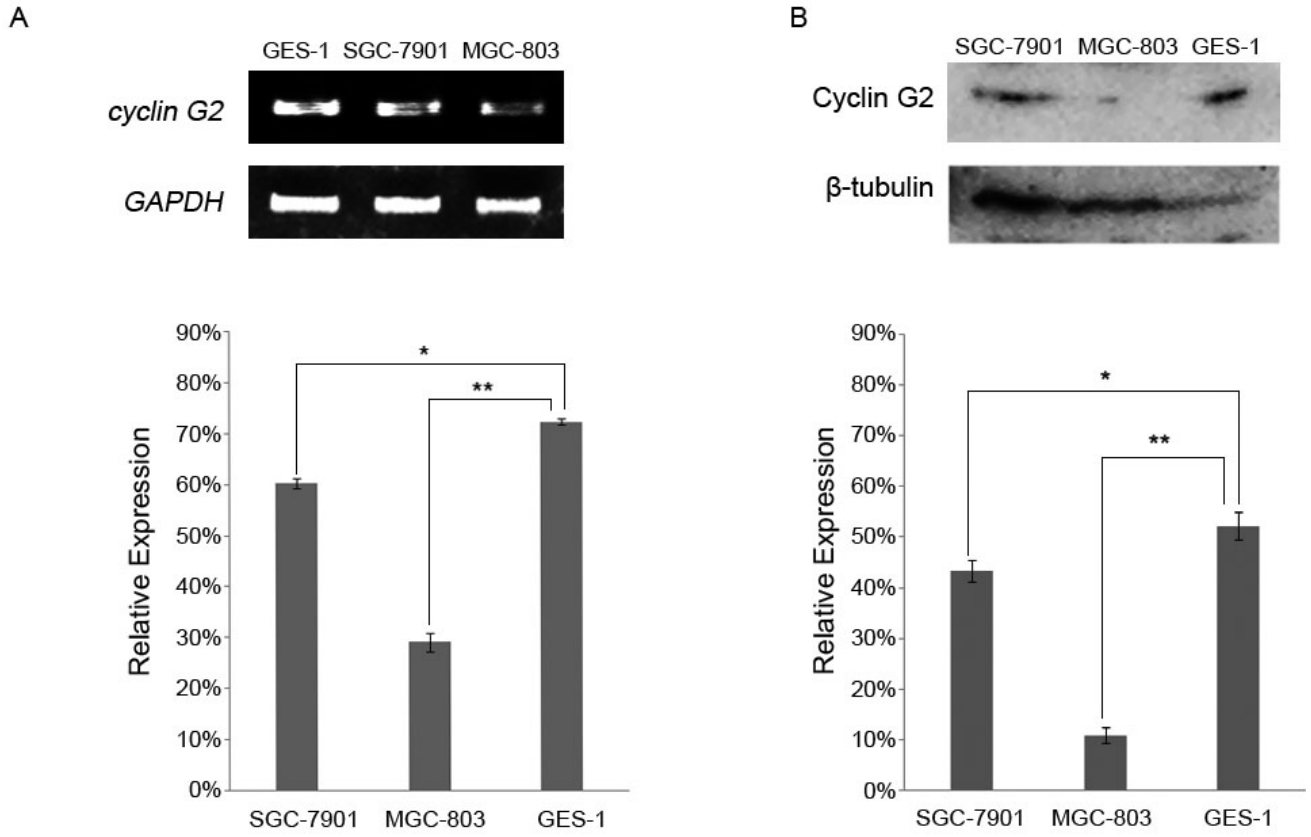

Figure 2. Expressions of cyclin G2 are decreased in gastric cancer cells. The expressions of cyclin G2 in the SGC-7901 and BGM-803 gastric cancer cell lines and normal gastric GES-1 cells were analyzed by semi-quantitative RT-PCR (A) and western blot (B), with GAPDH and β-tubulin as internal control, respectively. The intensity of bands was quantified using LabWorks™ Image Acquisition and Analysis Software. $^{*}P < 0.01$ and $^{**}P < 0.001$.

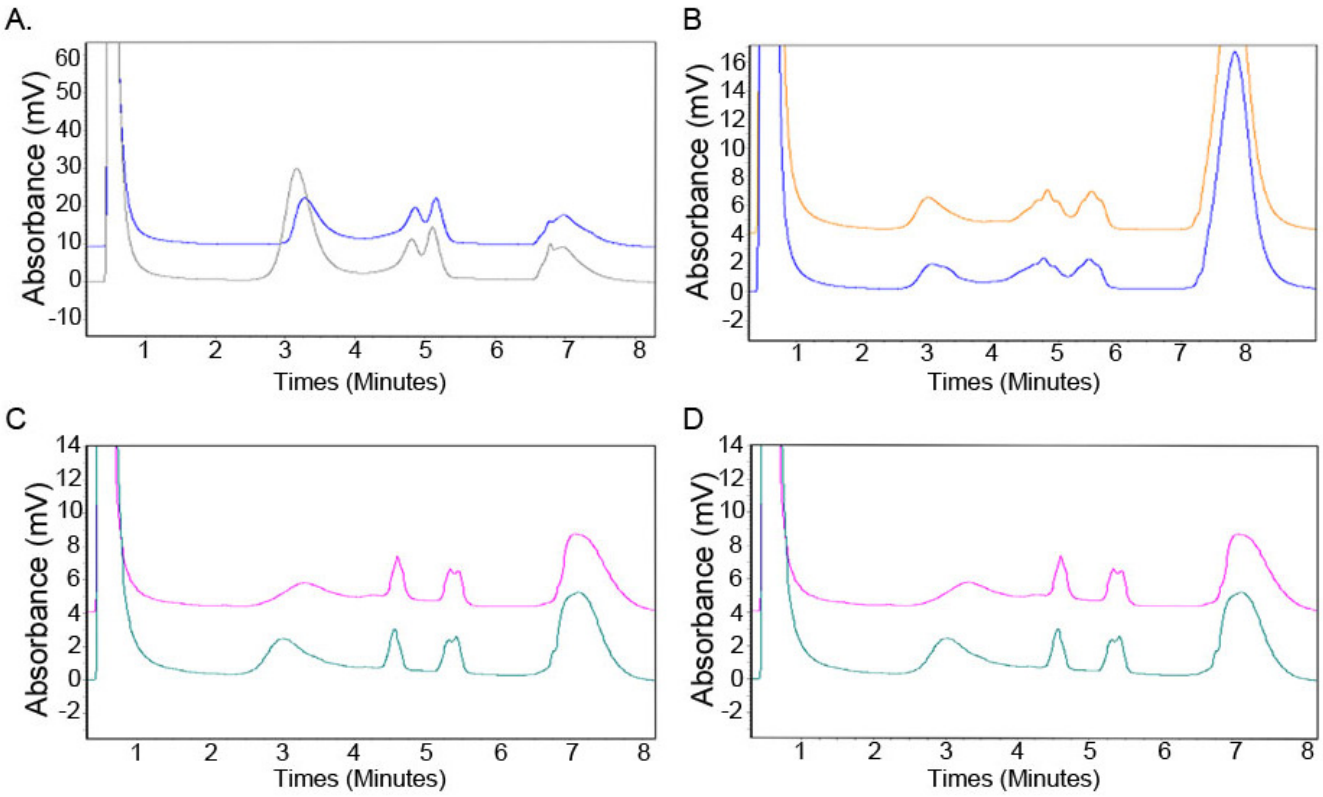

Figure 3. DHPLC detection of cyclin G2 mutation. The chromatogram profile of tumor tissues compared with that of the matched normal tissues. Heteroduplexes were detected in exon2 (A), exon7(B,C) and exon8 (D).

genesis ($P < 0.01$).

Mutation analysis of cyclin G2 with DHPLC and sequencing

We further investigated if mutations of cyclin G2 contributed to its expression reductions. Genomic DNA was extracted from 57 matched normal and tumor tissues and PCR was performed as described above. With DHPLC analysis, 9 cases of heteroduplexes were identified in PCR products amplified exon2 (2 cases), exon7 (1 case) and exon8 (6 cases) as shown in Figure 3.

PCR products showing aberrant chromatograms on DHPLC analysis were subjected to DNA sequencing. Four SNPs in cyclin G2 were detected in the gastric cancer tissues, all of which were synonymous demonstrated as following: E29E (222G>A) in cyclin G2 exon2; IVS7-57A>G in cyclin G2 intron7; IVS7-55T>C in cyclin G2 intron7; IVS8+31A>G in cyclin G2 intron8 (Figure 4). No mutation was detected.

DISCUSSION

G-type cyclins have well conserved amino acid sequences among mammals. Cyclin G1 and G2 share 53% amino acid sequence identity, 60% nucleotide sequence identity and they are both downstream regulated by homeobox A-10 (Yue et al., 2005). However, they show several differences. Cyclin G1 is identified as one of the transcriptional targets of the tumor suppressor gene p53 while cyclin G2 expression is known to be independent of p53 (Bates et al., 1996). In contrast to the nuclear localization of cyclin G1, cyclin G2 shows nucleocytoplasmic shuttling (Arachchige et al., 2006). The level of cyclin G1 is stable in cell cycle while cyclin G2 expression oscillates tightly with the cell cycle, peaking at late S/G_2 phase (Bennin et al., 2002).

Cyclin G2 modulates cellular division by associating with phosphatase 2A (PP2A), ubiquitin Skp1 and Skp2 and regulating their activities (Bennin et al., 2002; Xu et al., 2008). Moreover, mRNA expression of cyclin G2 was reported to elevate at G_0 phase and considerably reduce when cells enter cell cycle, suggesting its potential role in G_0 phase (Martínez-Gac et al., 2004). In addition, cyclin G2 was reported to be involved in the differentiation of the uterus and adipocytes (Aguilar et al., 2010; Yue et al., 2005). No known active CDK partner of cyclin G2 has yet been described.

In our study, ectopic expression of cyclin G2 was detected to inhibit gastric cell proliferation. Furthermore, the expression reduction of cyclin G2 were detected in gastric cancer cells compared with normal cells. Our

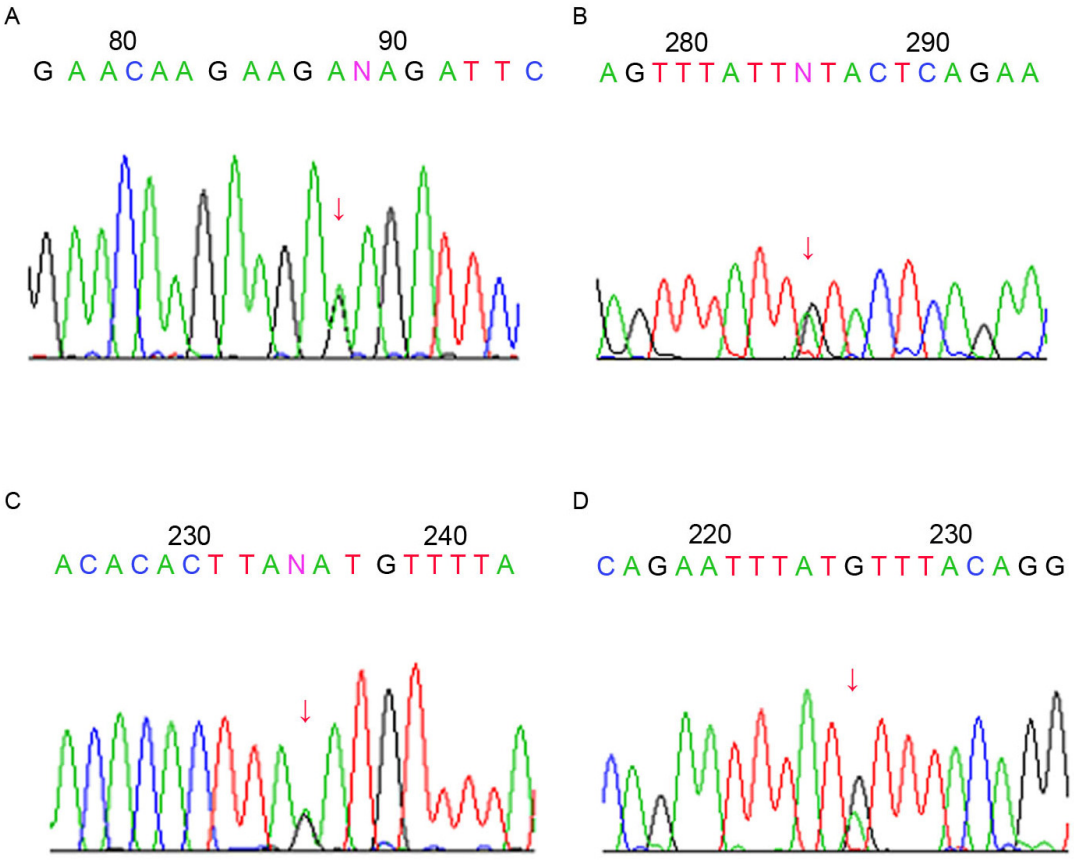

Figure 4. Four SNPs were detected with DNA sequencing in the 57 gastric cancer tissues. A: E29E (222G>A) in cyclin G2 exon2; B: IVS7-57A>G in cyclin G2 intron7; C: IVS7-55T>C in cyclin G2 intron7; D: IVS8+31A>G in cyclin G2 intron8.

results demonstrate the negative modulation of cyclin G2 in gastric carcinogenesis and suggest that the expression of cyclin G2 might be regulated in gastric cancer. To unambiguously evaluate the role of cyclin G2, we performed mutation analysis by screening each of its exons and some introns in 57 gastric cancer tissues. No mutations were detected in our screens, suggesting that mutation of cyclin G2 might not be the main cause for gastric carcinogenesis and other reasons such as loss of heterozygosity (LOH) and/or epigenetic modifications of cyclin G2 might contribute to the gastric carcinogenesis. Moreover, the expression of cyclin G2 was reported to be regulated by phosphoinositide 3-kinase (PI3K), Fox0 genes, c-jun NH (2)-terminal kinase, mTOR signaling and estrogen-occupied estrogen receptor. In gastric cancer, the role of these factors in modulating cyclin G2 expression awaits further investigation (Ito et al., 2003; Le et al., 2007; Stossi et al., 2006; Kasukabe et al., 2008).

In summary, this study provides the evidence that cyclin G2 is a negative regulator in gastric cell proliferation and its expressions are regulated in gastric cancer cells. In addition, no mutation of cyclin G2 was detected in tissues from 57 gastric cancer patients, suggesting that mutations of cyclin G2 might not be the main reason for leading to gastric carcinogenesis.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (No: 81170543) and the Program of the Education Commission of Liaoning Province (2008807).

REFERENCES

Aguilar V, Annicotte JS, Escote X, Uendrell J, Langin D, Fajas L (2010). Cyclin G2 regulates adipogenesis through PPAR gamma coactivation. Endocrinology, 151(11): 5247-5254.

Alevizos I, Mahadevappa M, Zhang X, Ohyama H, Kohno Y, Posner M, Gallagher GT, Varvares M, Cohen D, Kim D, Kent R, Donoff RB, Todd R, Yung CM, Warrington JA, Wong DT (2001). Oral cancer *in vivo* gene expression profiling assisted by laser capture microdissection and microarray analysis. Oncogene, 20: 6196-6204.

Arachchige Don AS, Dallapiazza RF, Bennin DA, Brake T, Cowan CE, Horne MC (2006). Cyclin G2 is a centrosome-associated

nucleocytoplasmic shuttling protein that influences microtubule stability and induces a p53-dependent cell cycle arrest. Exp. Cell Res. 312: 4181-4204.
Bates S, Rowan S, Vousden KH (1996). Characterisation of human cyclin G1 and G2: DNA damage inducible genes. Oncogene, 13: 1103-1109.
Bennin DA, Arachchige AS, Brake T, Mckenzie JL, Rosenbaum H, Ortiz L, DePaoli-Roach AA, Horne MC (2002). Cyclin G2 associates with protein phosphatase 2A catalytic and regulatory B' subunits in active complexes and induces nuclear aberrations and a G_1/S phase cell cycle arrest. J. Biol. Chem. 277: 27449-27467.
Bogni A, Cheng C, Liu W, Yang W, Pfeffer J, Mukatira S, French D, Downing JR, Pui CH, Relling MV (2006). Genome-wide approach to identify risk factors for therapy-related myeloid leukemia. Leukemia, 20: 239-246.
Chen YN, Chen H, Xu Y, Zhang X, Luo Y (2008). Expression of pituitary homeobox 1 gene in human gastric carcinogenesis and its clinicopathological significance. World J. Gastroenterol. 14: 292-297.
Choi MG, Noh JH, An JY, Hong SK, Park SB, Baik YH, Kim KM, Sohn TS, Kim S (2009). Expression levels of cyclin G2, but not cyclin E, correlate with gastric cancer progression. J. Surg. Res. 157(2): 168-174.
Horne MC, Donaldson KL, Goolsby GL, Tran D, Mulheisen M, Hell JW, Wahl AF (1997). Cyclin G2 is up-regulated during growth inhibition and B cell antigen receptor-mediated cell cycle arrest. J. Biol. Chem. 272: 12650–12661.
Horne MC, Donaldson KL, Goolsby GL, Tran D, Neubauer M, Wah AF (1996). Cyclin G1 and cyclin G2 comprise a new family of cyclins with contrasting tissue-specific and cell cycle-regulated expression. J. Biol. Chem. 271: 6050-6061.
Hydbring P, Larsson LG (2010). Tipping the balance: Cdk2 enables Myc to suppress senescence. Cancer Res. 70(17): 6687-6691.
Ito Y, Yoshida H, Uruno T, Nakano K, Takamura Y, Miya A, Kobayashi K, Yokozawa T, Matsuzuka F, Kuma K, Miyauchi A (2003). Decreased expression of cyclin G2 is significantly linked to the malignant transformation of papillary carcinoma of the thyroid. Anticancer. Res. 23: 2335-2338.
Jensen MR, Audolfsson T, Keck CL, Zimonjic DB, Thorgeirsson SS (1999). Gene structure and chromosomal localization of mouse cyclin G2 (CCNG2). Gene, 230: 171-180.
Jia JS, Xu SR, Ma J, Ha S, Guo XN, Wang Y (2005). Expression of cyclin g2 mRNA in patients with acute leukemia and its clinical significance. Zhongguo Shi Yan Xue Ye Xue Za Zhi, 13: 254-259.
Kasukabe T, Okabe-Kado J, Honma Y (2008). Cotylenin A, a new differentiation inducer, and rapamycin cooperatively inhibit growth of cancer cells through induction of cyclin G2. Cancer Sci. 99: 1693-1698.
Kim Y, Shintani S, Kohno Y, Zhang R, Wong DT (2004). Cyclin G2 dysregulation in human oral cancer. Cancer Res. 64: 8980-8986.
Lang C, Calegari F (2010). Cdks and cyclins link G(1) length and differentiation of embryonic, neural and hamatopoietic stem cells. Cell cycle 9(10): 1893-1900.
Liu Q, Gao JL, Liu X, Wang SS, Luo Y (2011). Monitoring cyclin-dependent kinase 2 (CDK2) activity with Kinase-Glo, a luminescence-based ATP quantitative reagent. Afr. J. Biotechnol. 10(7): 1217-1220.
Martínez-Gac L, Marqués M, García Z, Campanero MR, Carrera AC (2004). Control of cyclin G2 mRNA expression by forkhead transcription factors: novel mechanism for cell cycle control by phosphoinositide 3-kinase and forkhead. Mol. Cell Biol. 24: 2181-2189.
Stossi F, Likhite VS, Katzenellenbogen JA, Katzenellenbogen BS (2006). Estrogen-occupied estrogen receptor represses cyclin G2 gene expression and recruits a repressor complex at the cyclin G2 promoter. J. Biol. Chem. 281: 16272-16278.
Tian YL, Liu FR, Liu J, Jiang L, Luo Y, Zhang X (2002). Ectopic expression of cyclin G2 inhibits cell proliferation in HeLa cancer cell line. Chin. J. Cancer, 21: 577-581.
Wu WK, Cho CH, Lee CW, Fan D, Wu K, Yu J, Sung JJ (2010). Dyregulation of cellular signaling in gastric cancer. Cancer Lett. 295(2): 144-153.
Xu GX, Bernaudo S, Fu GD, Lee DY, Yang BB, Peng C (2008). Cyclin G2 is degraded through the ubiquitin-proteasome pathway and mediates the antiproliferative effect of activin receptor-like 7. Mol. Biol. Cell 19: 4968-4979.
Yue LM, Daikoku T, Hou XN, Li ML, Wang HB, Nojima H, Sudhansu KD, Sanjoy KD (2005). Cyclin G1 and cyclin G2 are expressed in the periimplantation mouse uterus in a cell-specific and progesterone-dependent manner: evidence for aberrant regulation with Hoxa-10 deficiency. Endocrinology, 146: 2424–2433.
Zandi K, Tajbakshs S, Nabipour I, Rastian Z, Yousefi F, Sharafian S, Sartari K (2010). *In Vitro* antitumor activity of *Gracilaria corticata*(a red alga) against Jurkat and molt-4 human cancer cell lines. Afr. J. Biotechnol. 9(40): 6787-6790.