

# Antisense to cyclin D1 reverses the transformed phenotype of human gastric cancer cells \*

CHEN Bing<sup>1</sup>, ZHANG Xue-Yong<sup>2</sup>, ZHANG Yu-Jing<sup>3</sup>, ZHOU Ping<sup>3</sup>, GU Yan<sup>4</sup> and FAN Dai-Ming<sup>2</sup>

**Subject headings** stomach neoplasms; cyclin D1; RNA, antisense; gene therapy

## Abstract

**AIM** To further investigate the effect of cyclin D1 on the biologic behavior of cancer cells and its potential role in gene therapy of tumor.

**METHODS** A cyclin D1 subcloning plasmid termed BKSD1 was constructed by subcloning the human cyclin D1 cDNA into Bluescript-KS, a plasmid vector with a pair of T7 and T3-promoters, with recombinant DNA technology of molecular biology. So, it is easy to generate digoxigenin (DIG)-labeled RNA probes of antisense and sense to cyclin D1 using RKSD1 as a template vector. PDORD1AS, an eukaryotic expression vector containing the full-length human cyclin D1 cDNA in its antisense orientation cloned into the retroviral vector pDOR-neo, was successfully constructed with BKSD1 to change restriction sites. A gastric cancer cell line, SGC7901/VCR, was transfected with pDORD1AS by Lipofect Amine-mediated introduction and a subline termed SGC7901/VCRD1AS, which had stable overexpression of antisense RNA to cyclin D1, was obtained by selection in G418. The subline, control subline transfected pDOR-neo and SGC7901/VCR were evaluated by methods of immunohistochemistry, flow cytometry, molecular hybridization, morphology and cell biology.

**RESULTS** Compared with control cell lines, SGC7901/VCRD1AS had a reduced expression of cyclin D1 (inhibition rate was about 36%), in-

creased cell size and cytoplasm to nucleus ratio, increased doubling time (42.2 h to 26.8 h and 26.4 h), decreased saturation density ( $18.9 \times 10^4$  to  $4.8 \times 10^5$  and  $4.8 \times 10^5$ ), increased percentage of cells in the G1/G0 phase (80.9%-64.6% and 63.8%), reacquired serum dependence, and a loss of tumorigenicity in nude mice (0/4 to 4/4 and 4/4).

**CONCLUSION** Stable overexpression of antisense RNA to cyclin D1 can reverse the transformed phenotype of human gastric cancer cells and may provide an approach of gene therapy for gastric cancer.

## INTRODUCTION

Studies on the functions of cellular proto-oncogenes and tumor suppressor genes indicate that most of these genes mediate signal transduction pathways that play a critical role in cell proliferation and differentiation as well as cell cycle control<sup>[1]</sup>. This has led to the realization that cycle regulatory proteins can also be directly involved in oncogenesis. Critical transitions in the eukaryotic cell cycle are regulated by the sequential activation of a series of cyclins and cyclin-dependent kinases (CDKs)<sup>[2]</sup>. Since the major regulatory events leading to mammalian cell proliferation and differentiation occur in the G1 phase of the cell cycle<sup>[3]</sup>, the deregulated expression of G1 phase cyclins or their related CDKs might cause loss of cell cycle control, thus enhancing oncogenesis. Cyclin D1 has been strongly implicated in controlling the G1 phase of the cell cycle. So, cyclin D1 gene is regarded as one of the best oncogene candidates<sup>[4]</sup>. Indeed, rearrangement, amplification, and overexpression of the cyclin D1 gene, which is located on the human chromosome 11q13 region, have been found in several types of human cancer<sup>[5]</sup>. Overexpression of cyclin D1 in rat fibroblasts enhanced their growth and tumorigenicity and cyclin D1 collaborated with an activated *ras* oncogene<sup>[6]</sup> or a defective adenovirus E1A oncogene<sup>[7]</sup> to increase the transformation of primary rodent fibroblast. The present study was undertaken to obtain more direct evidence that cyclin D1 plays a critical role in establishing and maintaining the transformed phenotype of these tumor cells. To this end,

<sup>1</sup>Department of Gastroenterology, Southwest Hospital, Third Military Medical University, Chongqing 400038, China

<sup>2</sup>Institute of Digestive Diseases, Xijing Hospital, Fourth Military Medical University, Xian 710033, China

<sup>3</sup>Columbia-Presbyterian Cancer Center, Columbia University, New York 10032, USA

<sup>4</sup>Department of Gastroenterology, Jiuxianqiao Hospital, Beijing 100016, China

CHEN Bing, male, born on 1960-11-27 in Nanjing City, China, associate professor of internal medicine, Ph.D. & MD in gastroenterology, post doctor of Third Military Medical University, China, having 24 papers published.

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**Correspondence to:** Dr CHEN Bing, Department of Gastroenterology, Southwest Hospital, Third Military Medical University, Chongqing 400038, China.

Tel. +86-23-68754259

E-mail.bingchen@cq.col.com.cn

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an antisense cyclin D1 cDNA was stably expressed in human gastric cancer cell line SGC7901/VCR that kept high expression of cyclin D1. This led to decreased levels of the endogenous cyclin D1 protein, marked inhibition of cell proliferation, and loss of tumorigenicity. These findings provide direct evidence that the cyclin D1 gene plays an essential role in the increased proliferation and oncogenesis of the gastric cells.

## MATERIALS AND METHODS

### Cell culture

The human cell lines used in the study, SGC7901/VCR gastric cancer cell line<sup>[8]</sup>, T24 bladder carcinoma cell line and K562 leukemia cell line, were obtained from Digestive Diseases Institute and Stomatology Biology Center of the Fourth Military Medical University, Xi'an, China. Cells were maintained in RPMI 1640 medium plus 100mL/L fetal calf serum (FCS). The medium for the cell lines containing the *neo* resistant gene was supplemented with G418.

### Construction of antisense cyclin D1 expression plasmids

The RKSD1 that contains the 1.1kb-human cyclin D1 cDNA in *Hind* III site is a present of Columbia-Presbyterian Cancer Center. A cyclin D1 subcloning plasmid termed BKSD1 was constructed by subcloning the human cyclin D1 cDNA into a subcloning vector Bluescript KS with recombinant DNA technology of molecular biology. pDORD1AS, an eukaryotic expression vector containing the 1.1 kb human cyclin D1 cDNA in its antisense orientation cloned into the retroviral vector pDOR-*neo*, was successfully constructed by the method as described before<sup>[9]</sup>.

### Lipofectamine mediated transduction

The pDORD1AS and vector control pDOR-*neo* plasmids were transfected into SGC7901/VCR gastric cancer cells using standard lipofectamine transfection procedure. A stable expression of cyclin D1 antisense RNA subline termed SGC7901/VCRD1AS was obtained by selection in G418.

### Immunohistochemistry

Exponentially proliferating cells grown on glass slides were subjected to immunohistochemical staining by using a monoclonal antibody DSC-6 against cyclin D1. T24 and K562 cell lines were used as positive and negative controls of cyclin D1 overexpression, respectively<sup>[6,10]</sup>.

### In situ hybridization

Using BKSD1 containing a pair of promoters for T7 and T3 RNA polymerase as a template vector, DIG-labeled, antisense and sense RNA probes of cyclin D1 was made by *in vitro* transcription of DNA. Ex-

ponentially proliferating cells grown on glass slides were subjected to *in situ* hybridization by a standard nonradioactive *in situ* hybridization procedure. Control cell lines included T24, K562, SGC7901/VCR and SGC7901/VCRneo which is a pDOR-*neo* transfected subline of SGC7901/VCR.

### Flow cytometric analysis

Cells were cultured in complete medium. When they were exponentially dividing, the cells were collected and analyzed for the cell cycle distribution (PI dyeing) and the expression level of cyclin D1 (immunofluorescence) by flow cytometry. All experiments were repeated three times.

### Doubling times and saturation density

Cells were plated in triplicate at a density of  $2.5 \times 10^4$  per well in 24-well plates in 1mL of RPMI 1640 plus-100mL/L-FCS. The number of cells per well was counted every day for 10 days. The doubling times and saturation densities of each cell line were calculated.

### Assessment of serum dependence

The growth rates of the two control cell lines and SGC7901/VCRD1AS cell line were measured at different concentrations of FCS. All cells were seeded initially of  $2.5 \times 10^4$  cells/well. Growth of each of the three cell lines was determined at three different serum concentrations (10%, 2.5% and 0.5%).

### Soft agar assay

Growth in 3g/L-Noble agar was assayed. In brief, cells were suspended in RPMI1640 plus 200mL/L-FCS containing 3g/L-agar and plated in triplicate in 24-well plates. After 2 weeks of growth, the cells were counted by microscopy. All experiments were repeated two times and similar results were obtained.

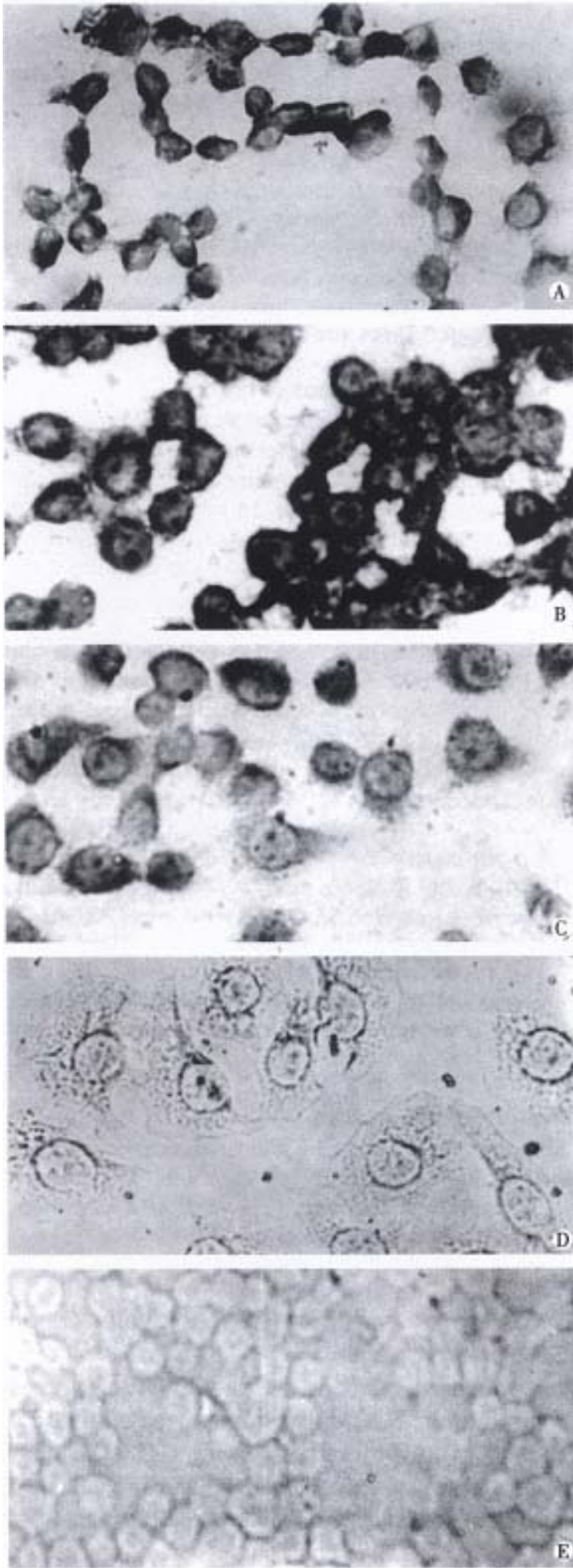
### Tumorigenicity assays

Cells of  $5 \times 10^5$  were injected subcutaneously into multiple sites in athymic (nude) mice. The animals were monitored for tumor formation every week and sacrificed one month later.

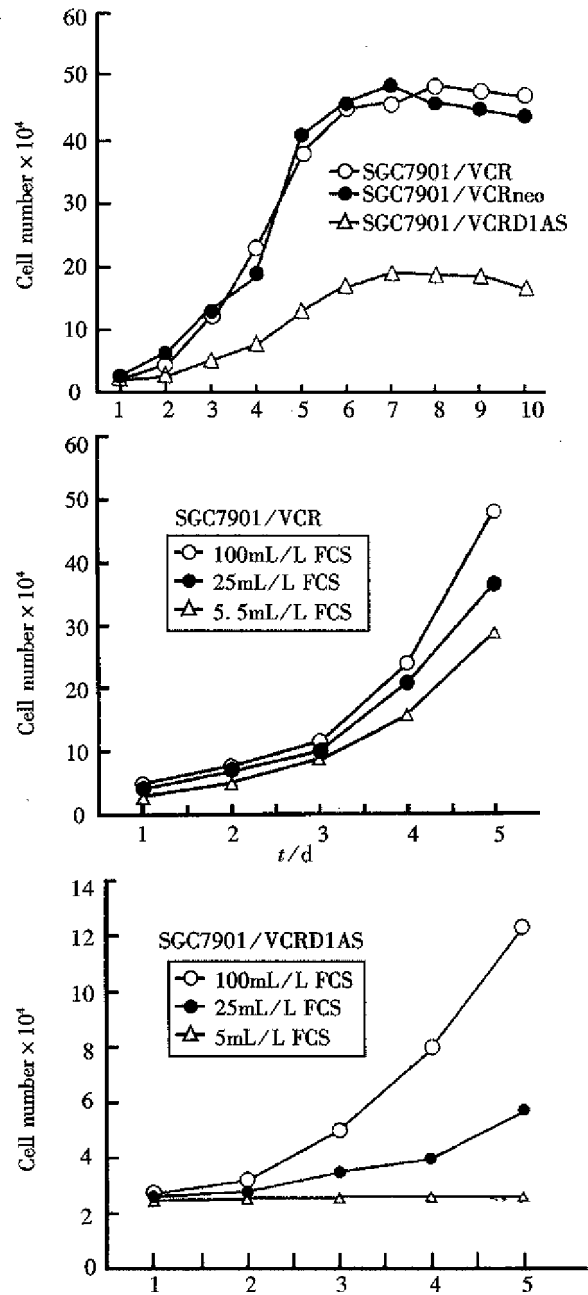
## RESULTS

### Expression of antisense RNA of cyclin D1 in SGC7901/VCR cells

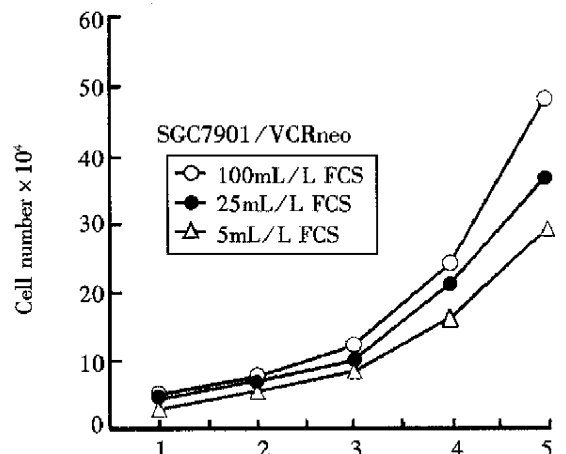
We introduced an antisense cyclin D1 cDNA sequence into the SGC7901 cell line, whose cyclin D1 gene was overexpressed. Following G418 selection, the drug resistant (*neo*+) cell SGC7901/VCRD1AS was randomly collected from the cultures infected with the pDORD1AS construct. As controls, *neo*+ clone SGC7901/VCRneo was selected from SGC7901/VCR culture infected with pDOR-*neo* vector lacking the antisense cyclin D1 cDNA sequence.



**Figure1** The result of *in situ* hybridization of cyclin D1 mRNA. A T24; B. SGC7901/ VCR; C. SGC7901/ VCRneo; D. SGC7901/VCRD1AS; E. K562



**Figure2** Growth curve of SGC7901/VCRD1AS and control cell lines.



**Figure3** Serum dependence of SGC7901/VCRD1AS and control cell lines.

It turned out by immunohistochemistry, flow cytometry and *in situ* hybridization that the expression levels of cyclin D1 protein and mRNA were much lower ( $P < 0.01$ , inhibition rate of cyclin D1 protein expression level by flow cytometry assay was 36%) in SGC7901/VCRD1AS than in SGC7901/VCR and SGC7901/VCR neo control cells.

### Characterization of morphology, growth properties and cell cycle progression of antisense cyclin D1 expressing cells

The observation results of morphology. The SGC7901/VCR and SGC7901/VCR neo control cells displayed a loss of contact inhibition and formed dense foci when they grew to a high saturation density. The SGC7901/VCRD1AS cells grew at monolayer and low cell density, manifested an increased cytoplasm to nucleus ratio, and were much flatter and larger in cell size than the control cells (Figure 1).

The analysis of cell biology. As Figures 2, 3 and Table 1 show, compared with control cells, the SGC7901/VCRD1AS cells displayed a much longer doubling time ( $P < 0.01$ ), an increased percentage of cells in the G1/G0 phase, required serum dependence to some extent, a much lower saturation density ( $P < 0.01$ ), and a marked inhibition on tumorigenicity in nude mice and soft agar cloning efficiency ( $P < 0.01$ ).

**Table 1 Growth properties and tumorigenicity of SGC7901/VCRD1AS and control cell lines**

Cell line	SGC7901/VCR	SGC7901/VCRneo	SGC7901/VCRD1AS
Doubling time (h)	26.8	26.4	42.2
Saturation density ( $\times 10^5$ )	4.8	4.8	1.9
Cell cycle distribution (%)			
G1/G0	64.6	63.8	80.9
S	25.9	27.8	13.0
G2-M	9.4	8.3	6.1
Colony forming efficiency (%)	5.47	5.50	0.03
Tumor formation in nude mice	4/4	4/4	0/4

### DISCUSSION

In previous studies, the anti-cyclin D1 antibodies or cyclin D1 antisense plasmids were microinjected into fibroblast<sup>[10]</sup> or B-cell lymphoma cell lines<sup>[11]</sup> during

the G1 interval. The cells were prevented from entering the S phase. These results indicated that cyclin D1 is required for cells to undergo the G1-S transition. However, because of the transient nature of these microinjection studies, they did not address the role of cyclin D1 overexpression in continuously dividing cells, in growth control and tumorigenesis. Overexpression of cyclin D1 in fibroblasts shortened the G1 phase<sup>[6]</sup>. In our study, stable overexpression of antisense RNA to cyclin D1 can inhibit the cell growth and reverse the transformed phenotype of gastric cancer cells besides a marked decrease in the mRNA and protein level of cyclin D1. For example, a much longer doubling time and increased percentage of G1/G0 cells reveal G1 phase arrest, a much lower saturation density and reacquired serum dependence announce restored adjustability by exogenous signals, a monolayer growth feature in low density and increased cytoplasm to nucleus ratio manifest reacquired anchorage dependent and somewhat epithelial feature. Our present studies provide the evidence that inhibition of the expression of cyclin D1 in tumor cells that overexpress this gene can reverse their transformed phenotype. It is of clinical significance because large numbers of human tumors display overexpression of cyclin D1. Our findings may provide a potential approach of gene therapy for gastric cancer<sup>[12]</sup>.

### REFERENCES

- 1 Oliff A, Gills JB, Cormick FM. New molecular targets for cancer therapy. *Sci Am*, 1996;275:110-112
- 2 Sherr CJ. G1 phase progression: cycling on cue. *Cell*, 1994;79:551-555
- 3 Weinberg RA. How cancer arises. *Sci Am*, 1996;275:32-34
- 4 Resnitzky D, Reed SI. Different roles for cyclin D1 and E in regulation of the G1-S transition. *Mol Cell Biol*, 1995;15:3463-3469
- 5 Sherr CJ. Cancer cell cycle. *Science*, 1996;274:1672-1677
- 6 Jiang W, Kahn SM, Zhou P, Zhang YJ, Cacace A, Infante SD. Overexpression of cyclin D1 in rat fibroblasts causes abnormalities in growth control, cell cycle progression and gene expression. *Oncogene*, 1993;8:3447-3457
- 7 Hunter T. Cyclins and cancer. *Cell*, 1991;66:1071-1074
- 8 Cai XI, Fan DM, Zhang XY. Establishment and phenotype of MDR human gastric cancer cell lines. *Chin Clin Tumor*, 1995;21(Suppl):67-72
- 9 Chen B, Zhang XY, Wang CJ, Hui HX, Jing M, Fan DM. Construction and appraisal of eukaryotic expression vector antisense to cyclin D1. *J Cell Mol Immunol*, 1997;13:45-46
- 10 Kurzrock R, Ku S, Talpa Z. Abnormalities in the PRAD1 (CYCLIN D1/BCL-1) oncogene are frequent in cervical and vulva squamous cell carcinoma cell lines. *Cancer*, 1995;75:584-590
- 11 Lukas J, Pngano Mi, Staskova Z, Draetta G, Bartek J. Cyclin D1 protein oscillates and is essential for cell cycle progression in human tumor cell lines. *Oncogene*, 1994;9:707-718
- 12 Xu CT, Pan BR. Current status of gene therapy in gastroenterology. *WJG*, 1998;4:85-88