ARTICLE
Journal of Cellular Biochemistry 114:1343–1354 (2013)

Journal of Cellular Biochemistry

CtBP2 Contributes to Malignant Development of Human Esophageal Squamous Cell Carcinoma by Regulation of $p16^{INK4A}$

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ABSTRACT

C-terminal binding protein-2 (CtBP2), as a transcriptional co-repressor, has been shown to mediate the repression of $p16^{INK4A}$, a tumor suppressor gene product, in primary human cells. Here we aimed to investigate how the correlation between CtBP2 and $p16^{INK4A}$ influenced the development of esophageal squamous cell carcinoma (ESCC). Immunohistochemistry of ESCC tissue sections indicated that the CtBP2 and $p16^{INK4A}$ expressions were inversely correlated to each other with a linear regression coefficient of -0.747 ($P < 0.05$), and Western blot analysis revealed that CtBP2 was higher expressed in tumorous tissues than in adjacent non-tumorous tissues. Either CtBP2 or $p16^{INK4A}$ expression was significantly related to histological differentiation ($P = 0.016$ or 0.001) and to the expression of Ki-67, a proliferating marker ($P = 0.006$ or 0.02), and patients with higher CtBP2 and lower $p16^{INK4A}$ expressions had shorter overall survival. We also observed that CtBP2 modulated the cell proliferation and cell cycle in ECA109 cells, an ESCC cell line, by inhibiting $p16^{INK4A}$. Overexpression or knockdown of CtBP2 in ECA109 cells was found to inhibit or activate the mRNA or protein expression of $p16^{INK4A}$, which in turn altered the cell proliferation and cell cycle in ECA109 cells, as measured by flow cytometry and cell count assay. Additionally, after ECA109 cells silenced for CtBP2 were treated with cisplatin (an anti-ESCC agent), the $p16^{INK4A}$ expression was up-regulated, and the cell apoptosis was promoted, thus confirming the repression of $p16^{INK4A}$ by CtBP2. Collectively, all results suggested that CtBP2 might contribute to the progression of ESCC through a negative transcriptional regulation of $p16^{INK4A}$. J. Cell. Biochem. 114: 1343–1354, 2013.

KEY WORDS: C-TERMINAL BINDING PROTEIN 2 (CTBP2); ESOPHAGEAL SQUAMOUS CELL CARCINOMA (ESCC); MALIGNANT DEVELOPMENT; $P16^{INK4A}$

Esophageal squamous cell carcinoma (ESCC), the major histological form of esophageal cancer, is one of the most aggressive malignancies with poor prognosis in the world, especially in China and other Asian countries. Despite progress in surgical and chemotherapy treatment, the 5-year survival rate of ESCC patients is still as poor as 20–30% [Li, 2002; Enzinger and Mayer, 2003]. It is believed that abnormal intracellular signal transduction caused by oncogene and anti-oncogene dysfunction is one of the major pathogenic mechanisms of ESCC [Sherr, 2004]. Although many genetic changes in ESCC have been found, little is known about

Chengqi Guan and Hui Shi contributed equally to this work.

All the authors declare no conflict of interest.

Grant sponsor: National Basic Research Program of China; Grant numbers: 2011CB910604, 2012CB822104; Grant sponsor: National Natural Science Foundation of China; Grant numbers: 81272708 and 31070723; Grant sponsor: Natural Science Foundation of Jiangsu province; Grant number: BK2009157; Grant sponsor: Key Project Natural Science Foundation of Jiangsu University and College; Grant number: 11KJA310002; Grant sponsor: Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD); Grant sponsor: Nantong University Graduate Student Science and Technology Innovation Project; Grant number: YKC12013.

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Manuscript Received: 22 April 2012; Manuscript Accepted: 28 November 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 17 December 2012

DOI 10.1002/jcb.24475 • © 2012 Wiley Periodicals, Inc.

major oncogenes and tumor suppressor genes involved in the tumorigenesis of the disease.

$p16^{INK4A}$ is a key tumor-suppressor gene product that blocks the progression of the cell cycle by binding to either CDK4 or CDK6 and inhibiting the action of cyclin D [Serrano et al., 1993; Kamb et al., 1994; Nobori et al., 1994; Ortega et al., 2002]. Since an imbalance between $p16^{INK4A}$ and cyclin D leads to abnormal cell growth and tumor development, the p16 protein plays a negative regulatory role in the G1-S phase checkpoint and thus functions as a tumor suppressor protein [Sherr and Roberts, 1999]. The protein expression of $p16^{INK4A}$ has been found to be reduced in human primary tumors, including esophageal carcinomas, pancreatic carcinomas, gliomas, non-small cell lung carcinomas [Witkiewicz et al., 2011]. Inactivation of the $p16^{INK4A}$ gene in different human tumor types is initiated through multiple mechanisms, including homozygous deletion, mutation, and transcriptional inhibition by methylation [Hirama and Koeffler, 1995; Ruas and Peters, 1998]. The transcription regulation of $p16^{INK4A}$ is essential for cellular ageing and oncogenic stress response [Sandig et al., 1997; Kataoka et al., 2000; Katsuda et al., 2002], but the underlying mechanisms remain incompletely understood.

C-terminal binding protein (CtBP) was identified because of its interaction with the C-terminus of the adenovirus E1A oncoprotein [Boyd et al., 1993; Schaeper et al., 1995], and has since been thought of as a potential therapeutic target for the treatment of cancer [Bergman and Blaydes, 2006; Chinnadurai, 2009]. CtBP acts as a transcriptional corepressor to negatively modulate the transcriptional activation and oncogenesis of E1A [Subramanian et al., 1989; Sollerbrant et al., 1996]. In mammals, the CtBP2 family is encoded be two genes: CtBP1 and CtBp2. CtBP2, almost exclusively found in the nucleus [Zhao et al., 2006], functions as a transcriptional corepressor through regulation by nuclear NADH levels [Thio et al., 2004]. Evidence shows that CtBP2 modulates essential cellular processes, such as cell proliferation and cell apoptosis. In breast cancer-derived cell lines, loss of CtBP2 expression suppresses the cell proliferation and cell cycle progression [Bergman et al., 2009]. Disruption of CtBP2 in mice causes embryonic lethality in mice due to a variety of defects [Hildebrand and Soriano, 2002]. The suppressed expression of CtBP2 renders cancer cells hypersensitive to apoptosis [Grooteclaes et al., 2003; Zhang et al., 2005; Paliwal et al., 2006]. Moreover, CtBP2, as a transcriptional corepressor, mediates repression of tumor suppressor genes, such as p21, E-cadherin, and PTEN [Grooteclaes and Frisch, 2000; Hildebrand and Soriano, 2002; Paliwal et al., 2007]. The above results suggest that CtBP2 may play a prominent role in tumorigenesis and tumor progression. In general, however, an involvement of the CtBP2 expression in the development of cancers, especially of ESCC, has been not fully studied.

Very importantly, a recent study demonstrates that CtBP mediates the repression of $p16^{INK4A}$ in primary human fibroblasts and keratinocytes, and provides a common final target for regulating the balance among tumor suppression, regenerative capacity, and senescence [Mroz et al., 2008]. Driven by this finding, we hypothesized whether the $p16^{INK4A}$ gene expression is restrained by CtBP2 in ESCC. To test this hypothesis, here we examined CtBP2 and $p16^{INK4A}$ protein levels in ESCC tissue specimens by using immunohistochemistry and Western blotting, and determined the correlation between the expression of CtBP2 and $p16^{INK4A}$ with clinicopathological parameters. To further clarify the effects of CtBP2 on $p16^{INK4A}$ repression in ESCC, ECA109, an ESCC cell line, was transfected with CtBP2-enhanced green fluorescent protein (EGFP) or CtBP2-siRNA vectors to confirm the interplay between the expressions of CtBP2 and $p16^{INK4A}$ in ESCC.

MATERIALS AND METHODS

PATIENTS AND TISSUE SPECIMENS

A total of 90 cases with ESCC were retrieved from the archival files of the Department of Pathology, Affiliated Hospital of Nantong University from 2000 to 2004. The patients consisted of 63 males and 27 females with a mean age of 61 years (range, 31–80). None of the patients was treated with such preoperative therapies as radiation, chemotherapy, or immunotherapy. After obtaining informed consents from the patients, we collected their demographic characteristics and clinical data, which are listed in Table I. The tumor grade was classified as well (grade I, $n = 24$), moderately (grade II, $n = 53$), or poorly (grade III, $n = 13$) differentiated. In addition, invasion of lymphatic and blood vessels was assessed microscopically.

Tissue specimens were immediately processed after surgical removal. For histological examination, all tumorous and surrounding non-tumorous tissue portions were fixed in 10% buffered formalin and embedded in paraffin for sectioning. In addition, eight paired tumorous and adjacent non-tumorous tissue specimens were stored at $-80^{\circ}C$ for specific analysis.

IMMUNOHISTOCHEMICAL ANALYSES

The tissue sections were deparaffinized using a graded ethanol series, and endogenous peroxidase activity was blocked by soaking in 0.3% hydrogen peroxide. Thereafter, the sections were processed in 10 mmol/L citrate buffer (pH 6.0) and heated to $121^{\circ}C$ in an autoclave for 20 min to retrieve the antigen. After being rinsed in phosphate buffered saline (PBS, pH 7.2), 10% goat serum was applied for 1 h at room temperature to block non-specific reactions. The sections were then incubated overnight at $4^{\circ}C$ with anti-CtBP2 mouse polyclonal antibody (diluted 1:100; BD Biosciences Pharmingen, San Diego, CA), anti-$p16^{INK4A}$ rabbit antibody (diluted 1:150; Santa Cruz Biotechnology), or anti-Ki-67 mouse monoclonal antibody (diluted 1:100; Santa Cruz Biotechnology). Negative control slides were also processed in parallel using a non-specific immunoglobulin IgG (Sigma Chemical Co., St. Louis, MO) at the same concentration as the primary antibody. All slides were processed using the peroxidase antiperoxidase method (Dako, Hamburg, Germany). After being rinsed in PBS, the peroxidase reaction was visualized by incubating the sections with diaminobenzidine tetrahydrochloride in 0.05 mol/L Tris buffer (pH 7.6) containing 0.03% H_2O_2. After being rinsed in water, the sections were counterstained with hematoxylin, dehydrated, and coverslipped.

TABLE I. CtBP2 p16^{INK4A} Expression and Clinicopathological Parameters in 90 ESCC Specimens

		CtBP2			p16^{INK4A}		
Parameters	Total	Low (<0.81)	High (≥0.81)	*P*-value	Low (<0.52)	High (≥0.52)	*P*-value
Age (years)							
≤60	47	24	23	0.833	22	25	0.527
>60	43	21	22		23	20	
Gender							
Male	63	34	29	0.25	35	28	0.107
Female	27	11	16		10	17	
Histological differentiation							
Well	24	18	6	0.016*	5	19	0.001*
Mod	53	22	31		29	24	
Poor	13	5	8		11	2	
Distant metastasis							
Positive	23	12	11	0.809	14	9	0.227
Negative	67	33	34		31	36	
Tumor diameter (cm)							
≤5	80	39	41	0.502	42	38	0.18
>5	10	6	4		3	7	
Depth							
T1	10	5	5	0.15	2	8	0.141
T2	4	4	0		1	3	
T3	23	13	10		13	10	
T4	53	23	30		29	24	
Ki-67							
≤0.78	45	29	16	0.006*	17	28	0.02*
>0.78	45	16	29		28	17	

P-value statistical analyses were performed by the Pearson χ^2 test.
*$P < 0.05$ was considered significant.

IMMUNOHISTOCHEMICAL EVALUATION

All of the immunostained sections were evaluated in a blinded manner by independent experienced observers without knowledge of the clinical and pathological features of patients. For assessment of CtBP2, p16^{INK4A}, and Ki-67, five high-power fields in each specimen were selected randomly, and nuclear staining was examined. More than 500 cells were counted to determine the mean percentage of immunostained cell number to total cell number. In half of the samples, staining was repeated twice to avoid technical errors, but similar results were obtained in these samples.

CELL CULTURES AND CELL CYCLE ANALYSES

A human esophageal cancer cell line ECA109 was purchased from China Academy of Science cell library and maintained in RPMI 1640 (GibCo BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 100 U/ml penicillin–streptomycin mixture (GibCo BRL) at 37°C and 5% CO_2. For cell cycle analysis, the cells were fixed in 70% ethanol for 1 h at 4°C and then incubated with 1 mg/ml RNase A for 30 min at 37°C. Subsequently, the cells were stained with propidium iodide (50 mg/ml; Becton Dickinson, San Jose, CA) in PBS with 0.5% Tween-20, and analyzed using a Becton Dickinson flow cytometer BD FACScan (San Jose, CA) and Cell Quest acquisition and analysis programs. Gating was set to exclude cell debris, cell doublets, and cell clumps.

PROLIFERATION ASSAYS

Cell proliferation was measured using a commercial Cell Counting Kit (CCK)-8 (Dojindo, Kumamoto, Japan) following the manufacturer's instructions. Briefly, ECA109 cells were seeded onto 96-well cell culture cluster plates (Corning, Inc., Corning, NY) at a concentration of 2×10^4 cells/well in 100 μl culture medium, and grown overnight. Cell Counting Kit-8 reagents were added to a subset of wells for 2 h incubation at 37°C, and the absorbance was read in an automated plate reader.

WESTERN BLOT ANALYSIS

Tissues and cell samples were promptly homogenized in a homogenization buffer containing 1 M Tris–HCl pH 7.5, 1% Triton X-100, 1% NP-40 (Non-idet p-40), 10% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 0.5 M EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF, then centrifuged at 10,000*g* for 30 min to collect the supernatant. Protein concentrations were determined with a Bio-Rad protein assay (Bio-Rad, Hercules, CA). The supernatant was diluted in 2× SDS loading buffer and boiled. Proteins were separated with SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidine difluoride filter (PVDF) membranes (Millipore, Bedford, MA). The membranes were blocked with 5% dried skim milk in TBST (20 mM Tris, 150 mM NaCl, 0.05% Tween-20) for 2 h at room temperature. Then, the membranes were incubated overnight with polyclonal antibodies, which were: anti-CtBP2 (1:500, Becton, Dickinson and Company), anti-Cleaved Caspase3 (1:500, Cell Signaling), anti-p16^{INK4A} (1:500), anti-CDK4 (1:1,000), anti-CDK2 (1:1,000), anti-Cyclin D (1:500,), anti-Cyclin E (1:500), anti-EGFP (1:500), anti-β-actin (1:500), anti-PCNA (1:1,000, all the above antibodies from Santa Cruz Biotechnology). Then, horseradish peroxidase-linked IgG was used as the secondary antibody. Immunoreactive bands were visualized by chemiluminescence (NEN Life Science Products, Boston, MA). After the chemiluminescence was exposed to X-ray films, which were scanned using a Molecular Dynamics densitometer (Imaging Technology, Ontario, Canada). The band density was measured with a computer-assisted image-analysis system (Adobe Systems,

San Jose, CA) and normalized against β-actin level. The experiments were carried out on three separate occasions.

RNA ISOLATION AND REVERSE TRANSCRIPTASE-PCR (RT-PCR)

Total RNA of ECA109 cells were extracted using a Trizol extraction kit according to the manufacturer's procedure. Total RNA was reverse-transcribed using the Thermo Script RT-PCR system (Invitrogen). The primer pairs for $p16^{INK4A}$ were: sense, 5′-GGG-TAGAGGAGGTGCGG-3′ and antisense, 5′-CGGGGATGTCTGA-GGGA-3′. The primer pairs for p21 were: sense, 5′-ATGTCAG-AACCGGCTGGGGATGTC-3′, and antisense, 5′-GGGCTTCCTCTT-GGAGAAGATC-3′. The primer pairs for Bax were: sense, 5′-TT-CAGGGTTTCATCCAGGATCGAGCAGGGCGAATGGG-3′, and anti-sense, 5′-GATCAGTTCCGGCACCTTGGTGCACAGGGCCTTGAGC-3′. Cycling conditions were: 94°C for 45 s, 55°C for 45 s, 72°C for 30 s, and a total of 30 cycles. Glyceraldehyde-3-phosphate dehydroge-nase (GAPDH) was used as internal control and its primers were: sense, 5′-TGATGACATCAAGAAGGTGGTGAAG-3′ and antisense, 5′-TCCTTGGAGGCCATGTGGGCCAT-3′. Cycling conditions were: 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a total of 28 cycles. After amplification, the products were separated on an agarose (1.5%) gel (cast in the presence of ethidium bromide) and visualized under UV light.

PLASMID CONSTRUCTION

pcDNA3.1CtBP2 (1–445) was kindly donated by Dr. J. P. Blaydes (School of Medicine, University of Southampton). CtBP2 (1–445)-EGFP was generated by ligation of a XhoI/KpnI digested insert of pcDNA3.1CtBP2(1–445), into XhoI/KpnI-digested pEGFP-N3 (Clon-tech), followed by site directed mutagenesis (SDM; Quik Change, Stratagene), to place the EGFP in frame.

The human CtBP2siRNA expression vector, pSilencer 4.1-CMV, was successfully constructed. The sequence was submitted to a Blast search against the human genome sequence to ensure that only the CtBP2 gene was targeted. The sequences used were: sense: 5'-GATCCGCGCCTTGGTCAGTAATAGTTCAAGAGACTATTACTGA-CCAAGGCGCTTA-3', and antisense, 5'-AGCTTAAGCGCCTTGGT-CAGTAATAGTCTCTTGAACTATTACTGACCAAGGCGCG-3'.

ECA109 cells were seeded the day before transfection using 1,640 with 10% FBS but without antibiotics. Transient transfection of siRNA vectors and the non-slience vectors was carried out employing lipofectamine 2000 and plus reagent in Opti-MEM as suggested by the manufacturer. The cells were incubated with the pSilencer vectors and lipofectamine and plus reagent complexes for 4 h at 37°C. Transfected cells were used for subsequent experiments for 48 h after transfection. The experiments were repeated at least three times.

STATISTICAL ANALYSIS

The Stat View 5.0 software package was used for statistical analysis. The associations among the CtBP2 expression, $p16^{INK4A}$ expression, and clinicopathological features were analyzed using the χ^2 test. For analysis of survival data, Kaplan–Meier curves were constructed, and the log-rank test was performed. Multivariate analysis was performed using Cox's proportional hazards model, and the risk ratio and its 95% confidence interval were recorded for each marker. Probability values of <0.05 were considered statistically significant in all of the analyses.

RESULTS

CtBP2 EXPRESSION CORRELATES INVERSELY WITH $p16^{INK4A}$ EXPRESSION IN ESCC

Immunohistochemical staining was performed to examine the physiological or pathological correlation among the expression of CtBP2, $p16^{INK4A}$, and Ki-67, a proliferation index, in ESCC tissue sections. In most ESCC specimens, the percentage of CtBP2-positive tumor cells was positively related to the percentage of Ki-67-positive tumor cells, but was inversely related to the percentage of $p16^{INK4A}$-positive tumor cells (Fig. 1). In brief, the low expression of CtBP2 or Ki-67 was accompanied by the high expression of $p16^{INK4A}$ (Fig. 1A–C), and the high expression of CtBP2 or Ki67 by the low expression of $p16^{INK4A}$ in the same ESCC specimen (Fig. 1D–F). The statistics on the expressions of CtBP2 and $p16^{INK4A}$ in 90 ESCC tissue specimens indicated that the median percentage of CtBP2- and $p16^{INK4A}$-positive cells was 81% and 52%, respectively (Table I).

Western blot analysis was performed to detect the expressions of CtBP2 and $p16^{INK4A}$ in eight paired tumorous and adjacent non-tumorous tissue specimens. There was an inverse correlation between CtBP2 and $p16^{INK4A}$ expressions. The CtBP2 expression was obviously higher in tumor tissues than in adjacent non-tumorous tissues, in which little or null expression of CtBP2 was observed. In contrast, the $p16^{INK4A}$ expression was lower in tumor tissues than in adjacent non-tumorous tissues (Fig. 2).

CORRELATION OF CtBP2 AND $p16^{INK4A}$ EXPRESSION WITH CLINICOPATHOLOGIC VARIABLES

For each clinicopathological variable, patients (or tumor tissue specimens) were divided into high and low CtBP expresser groups or high and low $p16^{INK4A}$ expresser groups depending on whether the percentage of CtBP- or $p16^{INK4A}$-positive cells was $\geq$ and $<$ the median percentage (cutoff value) of 81% or 52%, respectively (Table I). Statistics indicated that the CtBP2 expression was significantly related to histological differentiation ($P=0.016$) and to the Ki-67 expression ($P=0.006$), but not related to other clinicopathologic variables, such as distant metastasis ($P=0.809$), tumor diameter ($P=0.502$), and depth ($P=0.15$); and likewise the $p16^{INK4A}$ expression was significantly related to histological differentiation ($P=0.001$) and to the Ki-67 expression, but not related to other clinicopathologic variables ($P=0.227$, 0.18, and 0.141). Concretely, 75% or 79% of patients with grade I (well differentiated) were low CtBP2 expressers or high $p16^{INK4A}$ expressers, respectively; in contrast, 58% or 55% of patients with grade II (moderate differentiated) were high CtBP2 expressers or low $p16^{INK4A}$ expressers, respectively; and 62% or 85% of patients with grade III (poor differentiated) were high CtBP2 expressers or low $p16^{INK4A}$ expressers. In other words, the patients with grade I tended to have low CtBP2 expression or high $p16^{INK4A}$ expression, but the patients with grade II or III tended to have high CtBP2 expression or low $p16^{INK4A}$ expression (Table I). In addition, there was an inverse correlation between $p16^{INK4A}$ and CtBP2 expressions in ESCC tissue specimens with a correlation coefficient of -0.747 ($P<0.05$, Fig. 3).

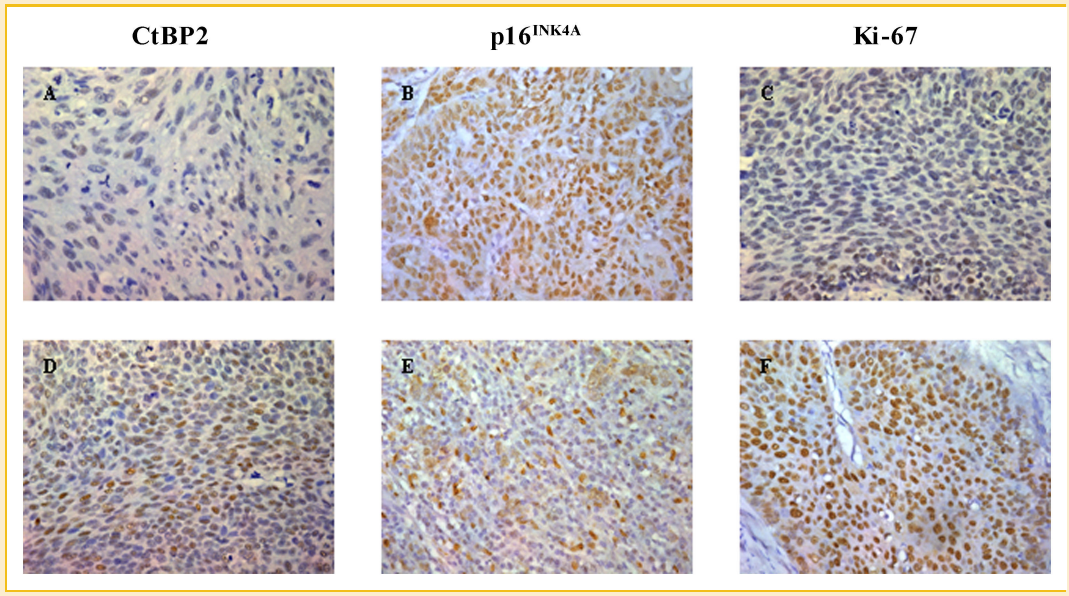

Fig. 1. Immunohistochemical analysis of CtBP2 and $p16^{INK4A}$ expressions. Paraffin-embedded ESCC tumorous tissue sections were stained with antibodies against CtBP2, $p16^{INK4A}$, and Ki-67 and counterstained with hematoxylin. A–C: Low CtBP2 and low Ki-67 but high $p16^{INK4A}$ expressions were observed in the same tissue specimen. D–F: high CtBP2 and high Ki-67 but low $p16^{INK4A}$ expressions were observed in the same tissue specimen. The experiment details were described in Materials and Methods Section. Magnification 400×.

PROGNOSTIC SIGNIFICANCE OF CtBP2 EXPRESSION AND $p16^{INK4A}$ EXPRESSION

We carried out Kaplan–Meier analysis to study the correlation between CtBP2 or $p16^{INK4A}$ expression with patient survival. Survival analysis was restricted to 90 patients with the follow-up data and immunohistochemistry result. The Kaplan–Meier survival curves of low versus high expressers of CtBP2 (Fig. 4A) or $p16^{INK4A}$ (Fig. 4B) showed a significant separation. Moreover, patients with the phenotype of CtBP2 $\geq$81% and $p16^{INK4A}$ <52% had the shorter cumulative survival (Fig. 4C). Multivariate analysis using the Cox proportional hazards model demonstrated that CtBP2 ($P = 0.011$), $p16^{INK4A}$ ($P = 0.004$), Ki67 ($P = 0.006$), Histological differentiation ($P = 0.046$) were independent prognostic indicators of overall survival (Table II).

THE EXPRESSION OF CtBP2 AND CELL CYCLE INHIBITOR $p16^{INK4A}$ IN PROLIFERATING ECA109 CELL LINE

To investigate the changes of CtBP2, $p16^{INK4A}$, and CDK4 during cell cycle progression of ESCC, ECA109 cells were subjected to serum starvation and serum refeeding. We observed that the cells were arrested in the G1 phase by serum deprivation for 72 h, after which the percentage of cells in the G1 phase increased from 39.08% to 73.35% (Fig. 5A). Upon serum re-addition, the cells were released from the G1 phase and re-entered the S phase. As expected, the CtBP2 expression was increased in the cells as early as 6 h after serum re-addition (Fig. 5B and C). The CtBP2 expression was lower in the G0 and early G1 phases and higher in the S phase, namely, the CtBP2 expression was gradually increased with the cell cycle progression. In contrast, the $p16^{INK4A}$ expression showed an opposite change trend, namely, the $p16^{INK4A}$ expression was decreased with the cell cycle progression. Meanwhile, the expression of CDK4, which is negatively regulated by $p16^{INK4A}$ [Xiong et al., 1993], was up-regulated. These results further confirmed that CtBP2 and $p16^{INK4A}$ were inversely related to each other, playing a central role in the regulation of cell proliferation.

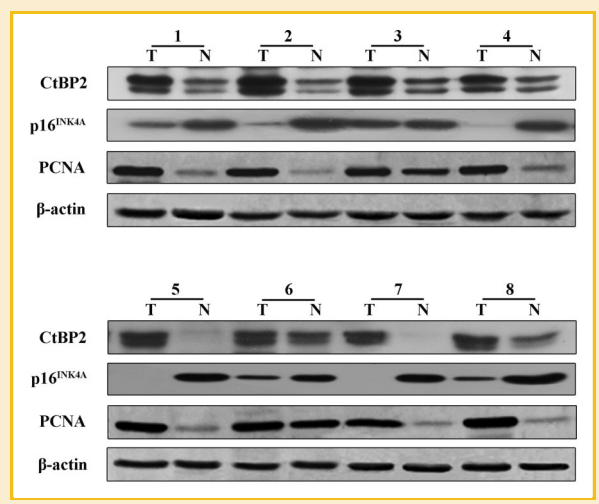

Fig. 2. Expressions of CtBP2 and $p16^{INK4A}$ in eight paired ESCC tumorous (T) and adjacent non-tumorous (N) tissue specimens. A representative Western blot image showed that the CtBP2 expression was significantly higher in tumorous tissues than in paired adjacent non-tumorous tissues. PCNA was used as a tumor proliferative marker. β-actin was used as a control for protein load and integrity.

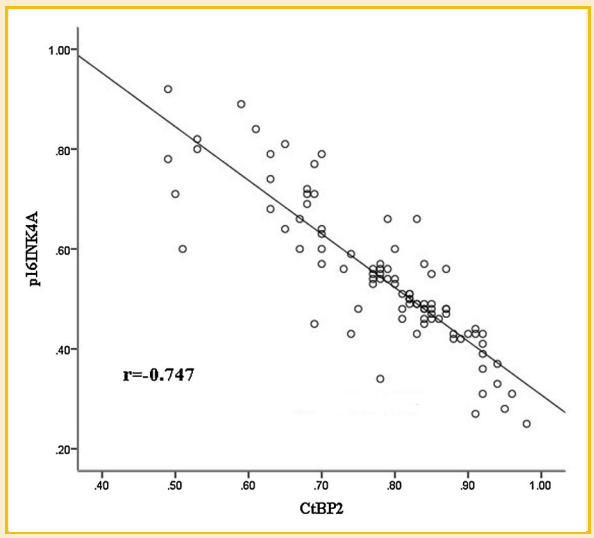

Fig. 3. Relationship between CtBP2 and $p16^{INK4A}$ expressions in ESCC. The scatterplot of the CtBP2 expression against the $p16^{INK4A}$ expression exhibited an approximately linear relationship between two variables with a Spearman's regression coefficient of -0.747 ($P < 0.05$).

INHIBITORY EFFECT OF CtBP2 OVEREXPRESSION ON $p16^{INK4A}$ EXPRESSION IN ECA109 CELLS

CtBP2-EGFP was transiently tansfected into ECA109 cells, and the CtBP2 overexpression was confirmed by Western blot analysis (Fig. 6A). We subsequently detected the expression $p16^{INK4A}$ and other CtBP2 targets: p21 and Bax by RT-PCR and Western blot analysis, and found that the expression of $p16^{INK4A}$ as well as p21 and Bax was all decreased in the cells at the mRNA and protein level (Fig. 6B). Cell growth assay also revealed that the cell growth rate was significantly higher in CtBP2-EGFP cells than in control-EGFP cells (Fig. 6C). To explore the mechanism underlying the growth promotion by CtBP2 overexpression, the cell cycle distributions of CtBP2-GFP and control-GFP cells were determined by flow cytometry. The percentage of CtBP2-EGFP cells in the S phase of CtBP2-GFP cells was obviously increased as compared with that of control-EGFP cells (Fig. 6D), suggesting that CtBP2 was able to promote the G1/S transition. The expression of several key cell cycle regulators, including cyclin D, cyclin E, CDK2, and CDK4, was determined in CtBP2-EGFP and control-EGFP cells. The comparison indicated that the expression of all the regulators in CtBP2-EGFP cells was increased as compared to that in control-EGFP cells (Fig. 6E).

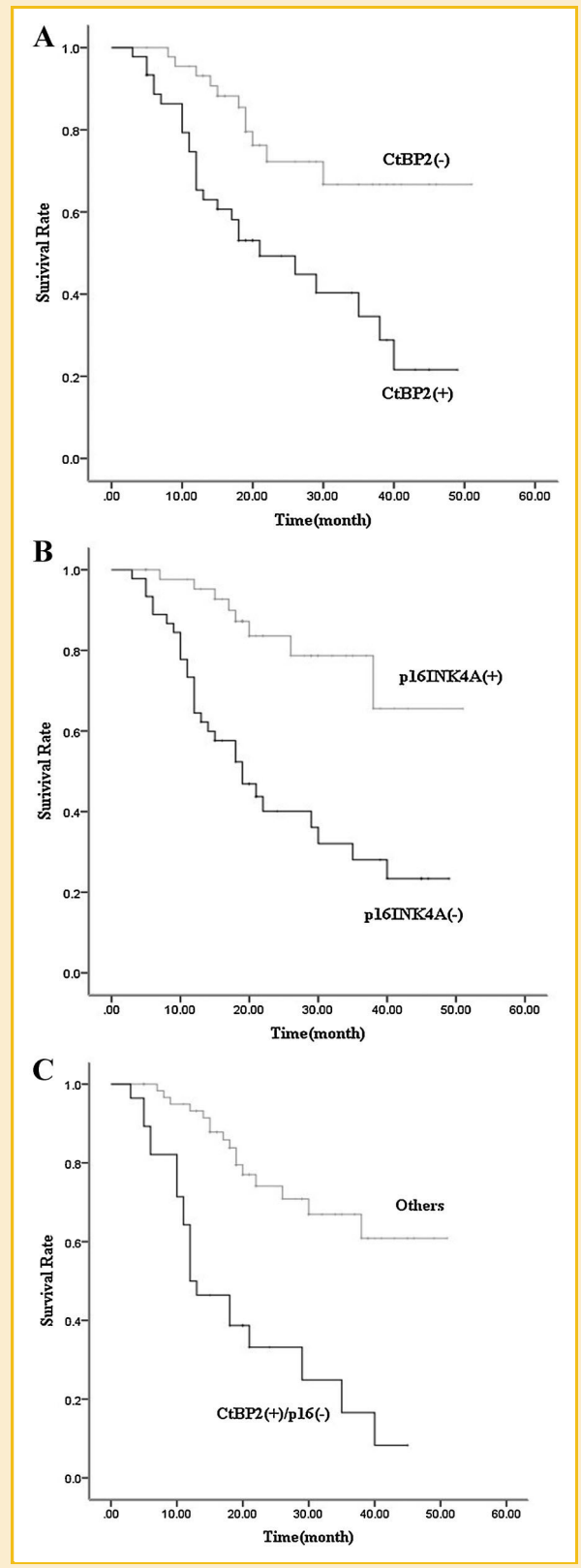

Fig. 4. Correlation between cumulative survival with the expression of CtBP2, $p16^{INK4A}$, and CtBP2/$p16^{INK4A}$. Based on the mean percentage of CtBP2- or $p16^{INK4A}$-positive cells, patients were divided into high CtBP2 expressers ($\geq$81%) and low CtBP2 expressers ($<$81%) or divided into high $p16^{INK4A}$ expressers ($\geq$52%) and low $p16^{INK4A}$ expressers ($<$52%). A: Patients in the high CtBP2 expresser group had significantly shorter overall survival. B: Patients in the low $p16^{INK4A}$ expresser group had significantly shorter overall survival. C: Patients having the mean percentage of CtBP2-positive cells $\geq$81% and the mean percentage of $p16^{INK4A}$-positive cells $<$52% showed the shortest overall survival.

TABLE II. Contribution of Various Potential Prognostic Factors to Survival by Cox Regression Analysis in 90 ESCC Specimens

	Hazard ratio	95.0% Confidence interval	*P*-value
Age	0.914	0.443–1.889	0.809
Gender	0.509	0.228–1.134	0.099
Histological differentiation	0.502	0.255–0.987	0.046*
Tumor diameter	0.797	0.209–3.043	0.740
Depth	1.189	0.728–1.942	0.490
Distant metastasis	0.658	0.296–1.461	0.303
CtBP2	3.074	1.290–7.325	0.011*
$p16^{INK4A}$	0.234	0.086–0.637	0.004*
Ki67	0.301	0.127–0.715	0.006*

Statistical analyses were performed by the log-rank test.
*$P < 0.05$ was considered significant.

ACTIVATION EFFECT OF CtBP2 SILENCING ON $p16^{INK4A}$ EXPRESSION IN ECA109 CELLS

To construct a siRNA targeted CtBP2, the ECA109 cells were transiently tansfected with CtBP2-siRNA, which significantly down-regulated the CtBP2 expression in cells (Fig. 7A) and concomittantly up-regulated the expression of $p16^{INK4A}$ and other CtBP2 targets: p21 and Bax in cells at the mRNA levels and protein level (Fig. 7B). Silencing of CtBP2 resulted in a significant inhibition of the cell growth rate (Fig. 7C). Flow cytometry showed that CtBP2-siRNA was able to inhibit the cell cycle at the G1/S transition (Fig. 7D). And the decreased expression of cyclin D, cyclin E, CDK2, and CDK4 in CtBP2-silenced cells was detected as compared to that in control-silenced cells (Fig. 7E). These results supported the above finding that that the promotion of tumor cell proliferation by CtBP2 was through its inactivation of $p16^{INK4A}$ expression.

INCREASED $p16^{INK4A}$ EXPRESSION IN CISPLATIN-TREATED ECA109 CELLS BY CtBP2 TRANSCRIPTION REPRESSION

It has been reported that cisplatin, a popular anticancer drug, up-regulates the $p16^{INK4A}$ expression in different cancer cells, including osteosarcoma cells, human non-small cell lung cancer cells, head, and neck cancer cells [Al-Mohanna et al., 2004; Yip et al., 2006; Fang et al., 2007]. Herein, we examined whether cisplatin also promoted up-regulation of the $p16^{INK4A}$ expression in ESCC cells. Exposure of ECA109 cells to cisplatin for 48 h induced a dose-dependent decrease in the CtBP2 protein level in ECA109 cells, as measured by Western blot Analysis. Concomitantly with down-regulation of the CtBP2 expression, cisplatin also resulted in a dose-dependent up-regulation of the expression of $p16^{INK4A}$, as well as cleaved-caspase 3, an apoptosis marker (Fig. 8A). Moreover, we noted that cisplatin-induced upregulation of the $p16^{INK4A}$ expression was enhanced in CtBP2-silenced ECA109 cells (Fig. 8B). We also found that after cisplatin treatment, the cell death in CtBP2-silenced cells was elevated as compared to that in control-silenced

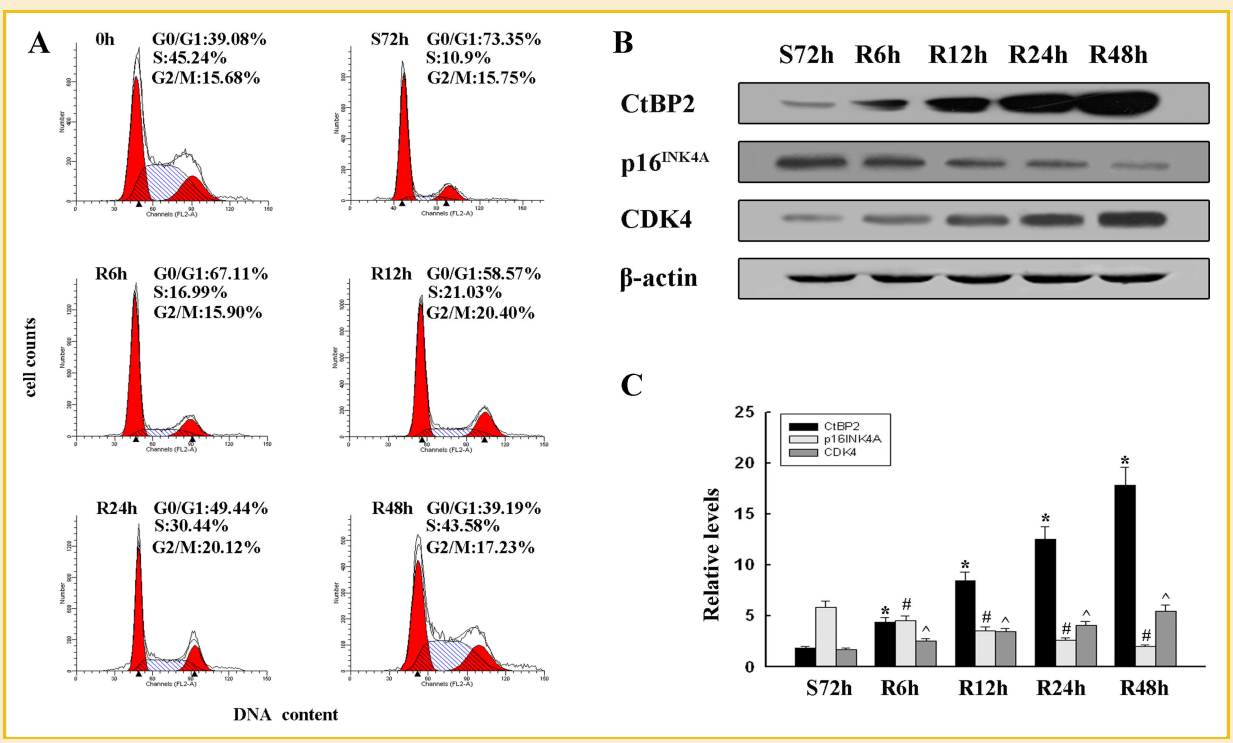

Fig. 5. The expression of CtBP2, $p16^{INK4A}$, and CDK4 in proliferating ECA109 cells. A: Flow cytometry was used to analyze the cell cycle progression of ECA109 cells that were subjected to serum starvation (S) for 72 h and refeeding (R) for 0, 6, 12, 24, and 48 h. B: A representative Western blot image showed that the expression of CtBP2, $p16^{INK4A}$, and CDK4 in ECA109 cells that were subjected to serum starvation (S) for 72 h and refeeding (R) for 0, 6, 12, 24, and 48 h. β-actin was used as a control for protein load and integrity. C: A bar chart demonstrated the relative protein expression of CtBP2, p16INK4A, and CDK4 in ECA109 cells at different time points, as measured by Western blot analysis. Data are presented as means ± SEM of three independent measurements (*, #, ^$P < 0.01$ vs. control at S72h).

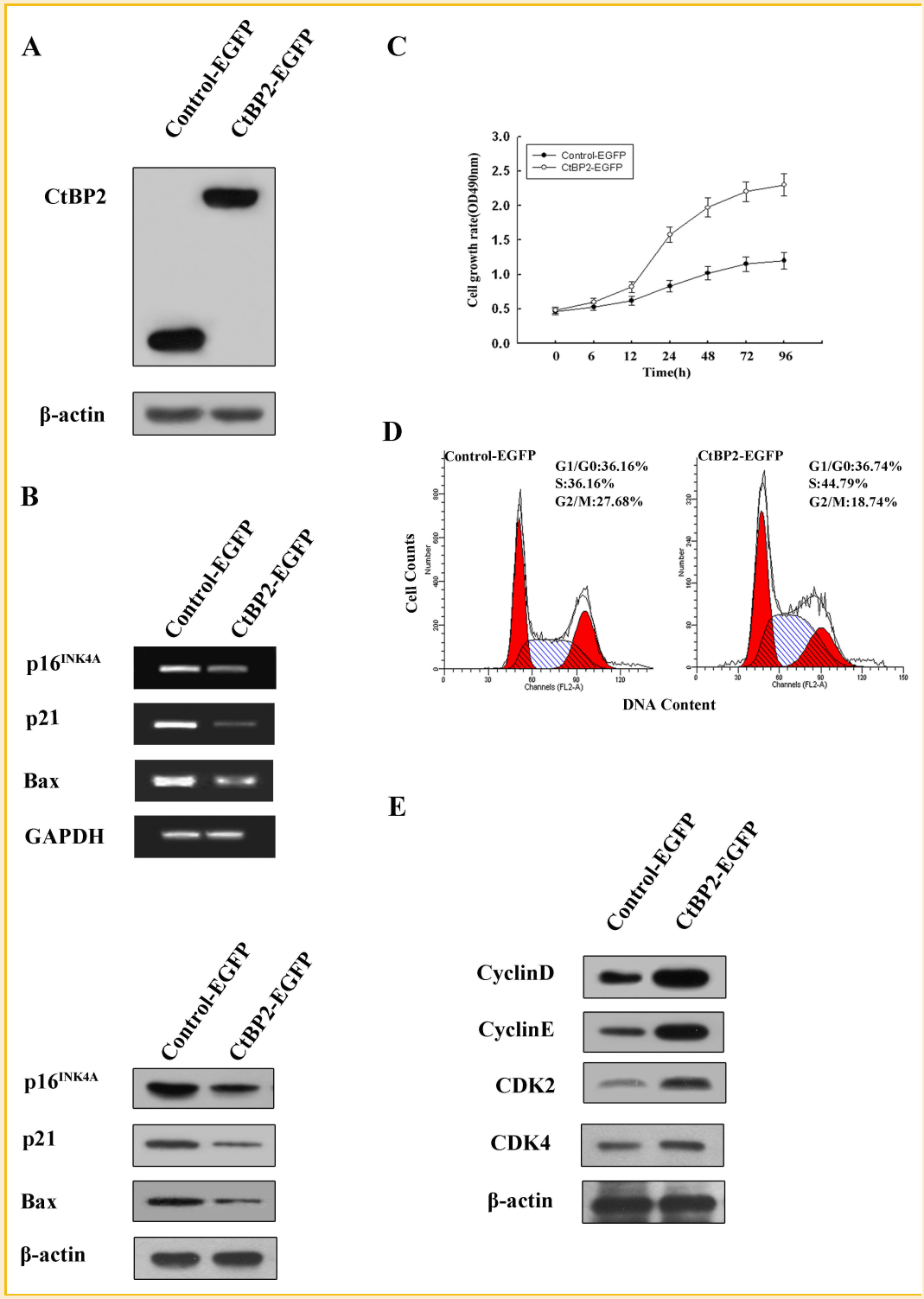

Fig. 6. Overexpression of CtBP2 promoted the cell proliferation and the expression of cell cycle-related molecules in ECA109 cells. A: A representative Western blot image showed that the CtBP2 protein expression in the cells was increased by treatment of CtBP2-EGFP. B: Representative PCR and Western blot images showed that the mRNA or protein expression of $p16^{INK4A}$, p21, or Bax in the cells was decreased by treatment of CtBP2-EGFP. C: The comparison in growth curves between the cells treated with CtBP2-EGFP and with control-EGFP, as measured by CCK-8 assay ($P < 0.05$). D: DNA content between control-EGFP and CtBP2-EGFP treated cells were compared by flow-cytometry. E: A representative Western blot image showed the expression of cyclin D, cyclin E, CDK2, and CDK4 in control-EGFP and CtBP2-EGFP treated cells, respectively. GAPDH or β-actin was used as internal control for RT-PCR or Western blot analysis, respectively.

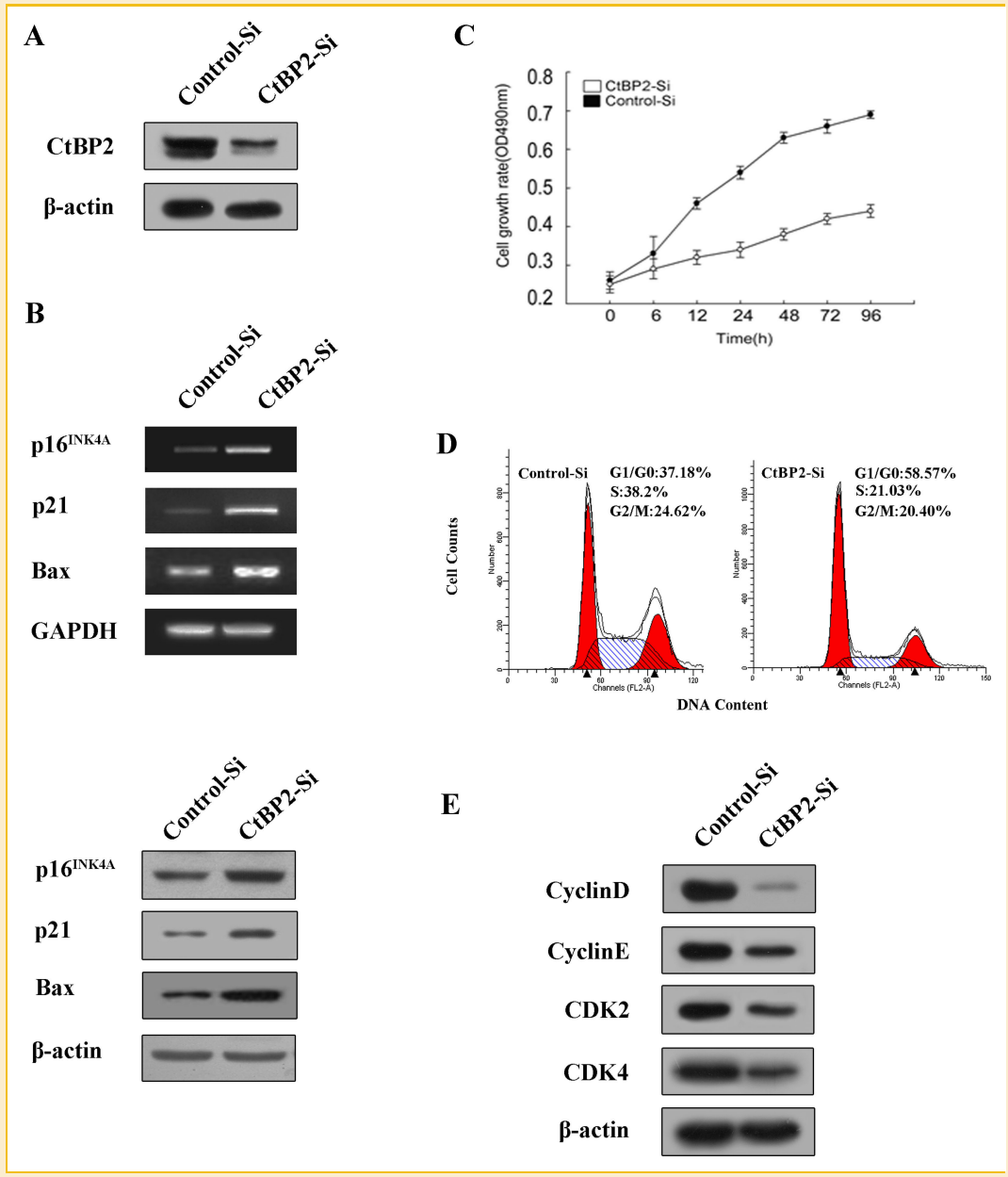

Fig. 7. Silencing of CtBP2 expression suppressed the cell proliferation and the expression of cell cycle-related molecules in ECA109 cells. A: A representative Western blot image showed that the CtBP2 expression in the cells was decreased by treatment of CtBP2-siRNA. B: Representative PCR and Western blot images showed that the mRNA or protein expression of $p16^{INK4A}$, p21, or Bax in the cells was decreased by treatment of CtBP2- siRNA. C: The comparison in growth curves between the cells treated with CtBP2-siRNA and with control-siRNA, as measured by cell counting assay ($P < 0.05$). D: DNA content between control-siRNA and CtBP2-siRNA treated cells were compared by flow-cytometry. E: A representative Western blot image showed the expression of cyclin D, cyclin E, CDK2, and CDK4 in control-siRNA and CtBP2-siRNA treated cells, respectively. GAPDH or β-actin was used as internal control for RT-PCR or Western blot analysis, respectively.

cells due to accumulation of $p16^{INK4A}$ in CtBP2-silenced cells (Fig. 8C). These results suggested that CtBP2 acted as a negative regulator of $p16^{INK4A}$ even in cisplatin treated ESCC cells.

DISCUSSION

The occurrence and development of ESCC are a comprehensive pathologic process involving complex alterations in oncogenes and tumor suppressor genes, which play roles in cell proliferation, cell cycle control and cell apoptosis through regulation of multiple signal transduction pathways. In this study, we tried to investigate the influences of CtBP2, a transcriptional corepressor, on $p16^{INK4A}$, a tumor suppressor, and to examine the role of this influences on the development of ESCC.

Immunostaining of CtBP2 was mainly localized in the nuclei of ESCC cells, and the CtBP2 expression was inversely associated with the $p16^{INK4A}$ expression, but positively associated with cell

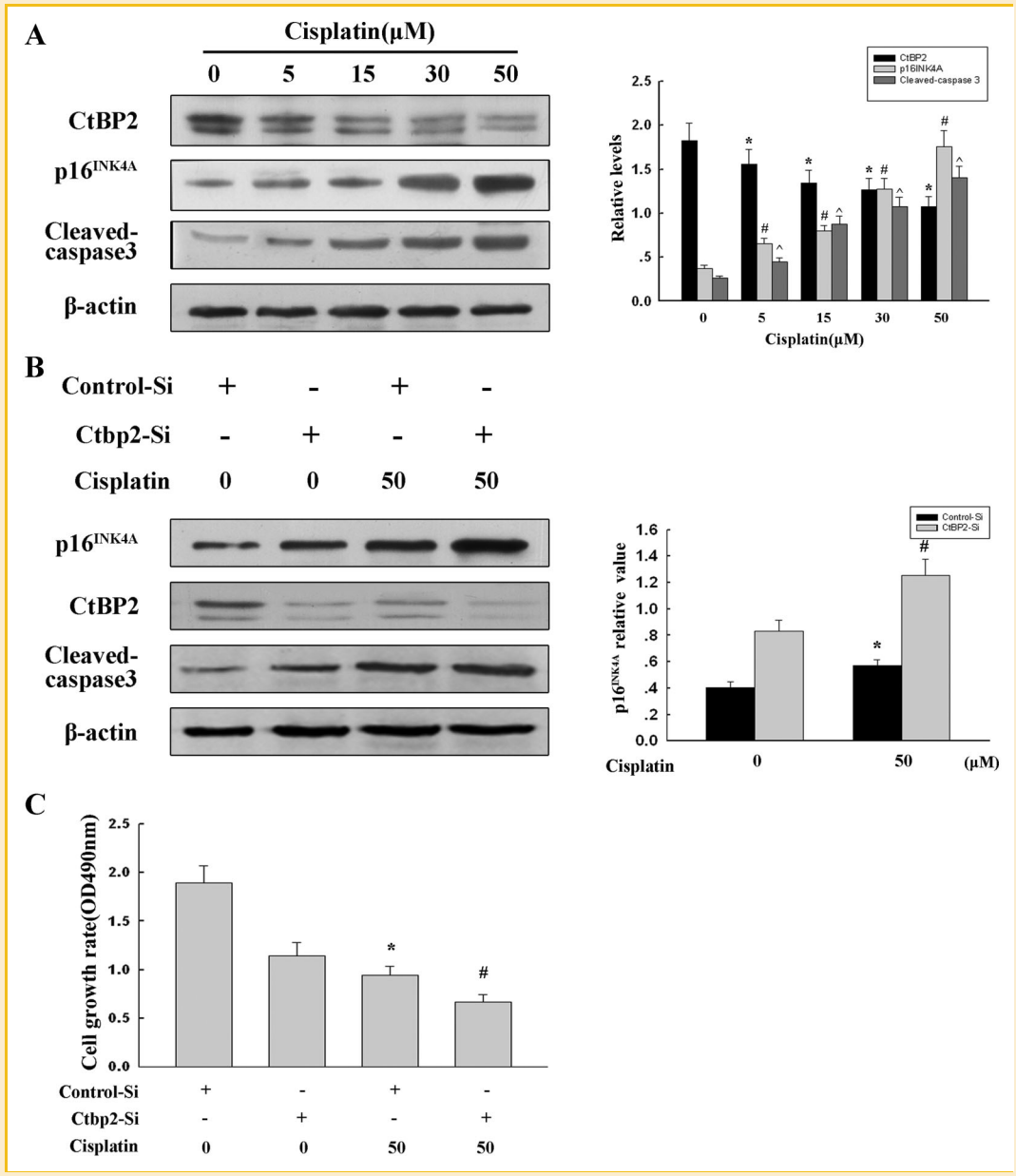

Fig. 8. The p16^{INK4A} expression in cisplatin-treated ECA109 cells was increased by CtBP2 transcription repression. A: Western blot analysis (a representative image and a bar chart) indicated the dose-dependent effect of cisplatin on the CtBP2 and p16^{INK4A} expressions in ECA109 cells, which were treated by cisplatin at different concentrations for 48 h. β-actin was used as a control for protein load and integrity. B: Western blot analysis (a representative image and a bar chart) indicated the effects of cisplatin on the CtBP2 and p16^{INK4A} expressions in ECA109 cells, which were transfected with control-siRNA or CtBP2-siRNA for 24 h, respectively, and then subjected to treatment with or without (i.e., with vehicle) cisplatin for 48 h, respectively. β-actin was used as a control for protein load and integrity. Data were presented as means ± SEM. $^{*}P < 0.05$ and $^{\#}P < 0.05$ versus controls (non-treated with cisplatin), respectively. C: A bar chart show that the comparison in the cell growth rate in ECA109 cells, which were transfected with control-siRNA or CtBP2-siRNA for 24 h, respectively, and then subjected to treatment with or without (i.e., with vehicle) cisplatin for 48 h, respectively, as measured by CCK-8 assay. Data were presented as means ± SEM. $^{*}P < 0.05$, $^{\#}P < 0.05$ versus controls (non-treated with cisplatin), respectively.

proliferation identified by the expression of Ki-67 (a proliferating cell marker) that was expressed specifically in the cell nucleus during the period from the late G1 to S phase. Western blot analysis revealed the different CtBP2 expressions between tumors and adjacent non-tumorous tissues. The correlation analysis for CtBP2 and p16^{INK4A} with clinicopathological variables demonstrated that the CtBP2 or p16^{INK4A} expression was significantly associated with histological differentiation or the Ki-67 expression. From these analysis results, we recognized that the higher CtBP2 or lower p16^{INK4A} expression seemed to be linked with the more malignant grade of histological differentiation in ESCC. Loss of p16^{INK4A} and its relationship to hypermethylation, clinicopathological parameters, and prognosis in ESCC patients have been studied [Fujiwara et al., 2008], but the possible role of CtBP2 on ESCC patients' prognosis is still unclear. In this study, survival analysis confirmed a significant correlation between overall survival with the CtBP2 and

$p16^{INK4A}$ expression. This result, together with the above finding for an inverse correlation between the expressions of CtBP2 and $p16^{INK4A}$, suggested that both the higher CtBP2 and lower $p16^{INK4A}$ expressions were associated with the poorer prognosis. The survival rate of patients with the higher CtBP2 expression was lower than that of patients with the lower CtBP2 expression. Therefore, the CtBP2 expression in ESCC might serve as a predicator for the clinical outcome.

We also clarified that CtBP2 expression modulates the malignant phenotype of ESCC possibly via the inhibition of the $p16^{INK4A}$ expression. The CtBP2 expression was increased during the period from late G1/S phase, whereas the $p16^{INK4A}$ expression was decreased concomitantly with the accumulation of CtBP2. We found that the CtBP2 expression was up-regulated during the cell cycle in ESCC. Further study revealed that CtBP2 overexpression in E109 cells promoted not only the cell growth by mediating down-regulation of the $p16^{INK4A}$, but also the G1/S phase transition. As is known, the G1/S phase transition is a major checkpoint for cell cycle progression, and two complexes, cyclin D1-CDK4 and cyclin E-CDK2, are critical positive regulators during this transition [Gutierrez et al., 2002; Vermeulen et al., 2003]. Consistent with the previous knowledge, our results showed that the expression of cyclin D1-CDK4 and cyclin E-CDK2 was increased in CtBP2-overexpressing ECA109 cells. On the other hand, we found that the silencing of CtBP2 in ECA109 cells inhibited tumorigenicity through the cell cycle arrest at the G1/S checkpoint and that the $p16^{INK4A}$ expression was significantly increased after ECA109 cells were transferred with CtBP2-siRNA vector. These results suggest that increasing the function and activity of $p16^{INK4A}$ by inhibiting the CtBP2 activity might represent an attractive strategy for cancer therapy.

We noticed that the CtBP2 expression was increased either in tumor tissues from ESCC patients or in an ESCC cell line. The increased expression of CtBP2 might be ascribed to an important fact that CtBP2 is a target for inhibition by the alternative reading frame (ARF). Arf (p14Arf in human and p19Arf in mouse) is a multifunctional tumor suppressor. Recent evidence suggests that the expression of p14Arf is downregulated in ESCC cells [Cheng et al., 2009], and so the CtBP2 expression is increased in ESCC cells.

Researchers have studied the biochemical process that is involved in CtBP2-regulated inhibition of p16 in primary human cells. First, CtBP2 knockdown increases the mRNA expression of p16. Second, exogenous CtBP2 represses the p16-promoted transcription of a luciferase reporter gene. Third, CtBP knockdown may affect epigenetic marks at the p16 promoter in a way consistent with transcriptional activation [Mroz et al., 2008]. In this study, we identified CtBP2 as an upstream regulator of the $p16^{INK4A}$ expression in ESCC, and considered that the CtBP-mediated repression in ESCC might be governed by the wide variety of mechanisms, which have to be systematically studied in future work.

To assess the possibility of proteolysis of $p16^{INK4A}$, CtBP2-EGFP-transfected ECA109 cells were exposed to 25 μM of cycloheximide, a protein synthesis inhibitor, for different times, followed by Western blot analysis. The time course in the $p16^{INK4A}$ expression suggested that $p16^{INK4A}$ was not rapidly proteolyzed in CtBP2-overexpressing ECA109 cells due to a quite long half-life of the protein (data not shown). It seemed that CtBP2 regulated $p16^{INK4A}$ primarily at the transcriptional rather than posttranslational level. Additionally, the role of CtBP2 in tumorigenesis was not limited to CtBP-mediated alterations in the expression of $p16^{INK4A}$ and even other targets, for example, p21 and Bax (as measured by this study). As is reported, CtBP-regulated cellular phenotypes related to initiation and development of tumors include epithelial–mesenchymal transition, cell cycle progression, cell apoptosis, senescence and mitotic fidelity [Chinnadurai, 2002, 2009; Bergman et al., 2006, 2009; Paliwal et al., 2007; Chen et al., 2008; Mroz et al., 2008]. Therefore, CtBP might directly affect the development of ESCC.

In most cancers, defective apoptosis is associated with the unscheduled cellular growth, which enables neoplastic cells to survive beyond their normal lifespan, to evade several cellular controls, and to promote angiogenesis [Hanahan and Weinberg, 2000]. Therefore, it became clear that cellular resistance to the apoptotic signals constitutes a major step in carcinogenesis. Moreover, defects in apoptosis affect the therapeutic outcome, since most of the agents used in cancer treatment stimulate apoptotic pathways to cause cancer cells death [Johnstone et al., 2002]. Cisplatin is a potent chemotherapeutic agent used to treat a wide range of human malignancies, including ESCC. The cytotoxicity of this anticancer drug is mediated principally through the apoptotic pathways. Caspase-3 has a key role in apoptosis, and cleaved caspase-3 is up-regulated in response to cisplatin treatment. The results in this study showed that CtBP2-siRNA significantly promoted the cell death inECA109 cells due to the combined regulation of $p16^{INK4A}$ by CtBP2 and cisplatin.

In summary, our observations supported the concept that CtBP2, as a negative transcriptional regulator of $p16^{INK4A}$, might contribute to the progression of ESCC. Hence the regulation of CtBP2/$p16^{INK4}$ is likely to become a useful tool for both diagnostic and prognostic applications in ESCC. Further studies will be needed to delineate the molecular mechanism by which CtBP2 participates in the transcription repression of $p16^{INK4A}$ in ESCC.

ACKNOWLEDGMENTS

This work was supported by the National Basic Research Program of China (973 Program, Nos. 2011CB910604 and 2012CB822104); the National Natural Science Foundation of China (Nos. 81272708 and 31070723); Natural Science Foundation of Jiangsu province (No. BK2009157); Key Project Natural Science Foundation of Jiangsu University and College (No. 11KJA310002); A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD); Nantong University graduate student science and technology innovation project (No. YKC12013).

REFERENCES

Al-Mohanna MA, Manogaran PS, Al-Mukhalafi Z, Al-Hussein KA, Abou-sekhra A. 2004. The tumor suppressor p16(INK4a) gene is a regulator of apoptosis induced by ultraviolet light and cisplatin. Oncogene 23:201–212.

Bergman LM, Blaydes JP. 2006. C-terminal binding proteins: Emerging roles in cell survival and tumorigenesis. Apoptosis 11:879–888.

Bergman LM, Morris L, Darley M, Mirnezami AH, Gunatilake SC, Blaydes JP. 2006. Role of the unique N-terminal domain of CtBP2 in determining the subcellular localisation of CtBP family proteins. BMC Cell Biol 7:35.

Bergman LM, Birts CN, Darley M, Gabrielli B, Blaydes JP. 2009. CtBPs promote cell survival through the maintenance of mitotic fidelity. Mol Cell Biol 29:4539–4551.

Boyd JM, Subramanian T, Schaeper U, La Regina M, Bayley S, Chinnadurai G. 1993. A region in the C-terminus of adenovirus 2/5 E1a protein is required for association with a cellular phosphoprotein and important for the negative modulation of T24-ras mediated transformation, tumorigenesis and metastasis. EMBO J 12:469–478.

Chen YW, Paliwal S, Draheim K, Grossman SR, Lewis BC. 2008. p19Arf inhibits the invasion of hepatocellular carcinoma cells by binding to C-terminal binding protein. Cancer Res 68:476–482.

Cheng TH, Hsu PK, Li AF, Hung IC, Huang MH, Hsu HS. 2009. Correlation of p53, MDM2 and p14(ARF) protein expression in human esophageal squamous cell carcinoma. J Cancer Res Clin Oncol 135:1577–1582.

Chinnadurai G. 2002. CtBP, an unconventional transcriptional corepressor in development and oncogenesis. Mol Cell 9:213–224.

Chinnadurai G. 2009. The transcriptional corepressor CtBP: A foe of multiple tumor suppressors. Cancer Res 69:731–734.

Enzinger PC, Mayer RJ. 2003. Esophageal cancer. N Engl J Med 349:2241–2252.

Fang K, Chiu CC, Li CH, Chang YT, Hwang HT. 2007. Cisplatin-induced senescence and growth inhibition in human non-small cell lung cancer cells with ectopic transfer of p16INK4a. Oncol Res 16:479–488.

Fujiwara S, Noguchi T, Takeno S, Kimura Y, Fumoto S, Kawahara K. 2008. Hypermethylation of p16 gene promoter correlates with loss of p16 expression that results in poorer prognosis in esophageal squamous cell carcinomas. Dis Esophagus 21:125–131.

Grooteclaes ML, Frisch SM. 2000. Evidence for a function of CtBP in epithelial gene regulation and anoikis. Oncogene 19:3823–3828.

Grooteclaes M, Deveraux Q, Hildebrand J, Zhang Q, Goodman RH, Frisch SM. 2003. C-terminal-binding protein corepresses epithelial and proapoptotic gene expression programs. Proc Natl Acad Sci U S A 100:4568–4573.

Gutierrez C, Ramirez-Parra E, Castellano MM, del Pozo JC. 2002. G(1) to S transition: more than a cell cycle engine switch. Curr Opin Plant Biol 5(6):480–486.

Hanahan D, Weinberg RA. 2000. The hallmarks of cancer. Cell 100:57–70.

Hildebrand JD, Soriano P. 2002. Overlapping and unique roles for C-terminal binding protein 1 (CtBP1) and CtBP2 during mouse development. Mol Cell Biol 22:5296–5307.

Hirama T, Koeffler HP. 1995. Role of the cyclin-dependent kinase inhibitors in the development of cancer. Blood 86:841–854.

Johnstone RW, Ruefli AA, Lowe SW. 2002. Apoptosis: A link between cancer genetics and chemotherapy. Cell 108(2):153–164.

Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, Stockert E, Day RS III, Johnson BE, Skolnick MH. 1994. A cell cycle regulator potentially involved in genesis of many tumor types. Science 264:436–440.

Kataoka M, Wiehle S, Spitz F, Schumacher G, Roth JA, Cristiano RJ. 2000. Down-regulation of bcl-2 is associated with p16INK4-mediated apoptosis in non-small cell lung cancer cells. Oncogene 19:1589–1595.

Katsuda K, Kataoka M, Uno F, Murakami T, Kondo T, Roth JA, Tanaka N, Fujiwara T. 2002. Activation of caspase-3 and cleavage of Rb are associated with p16-mediated apoptosis in human non-small cell lung cancer cells. Oncogene 21:2108–2113.

Li K. 2002. Mortality and incidence trends from esophagus cancer in selected geographic areas of China circa 1970–90. Int J Cancer 102(3):271–274.

Mroz EA, Baird AH, Michaud WA, Rocco JW. 2008. COOH-terminal binding protein regulates expression of the p16INK4A tumor suppressor and senescence in primary human cells. Cancer Res 68:6049–6053.

Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA. 1994. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature 368:753–756.

Ortega S, Malumbres M, Barbacid M. 2002. Cyclin D-dependent kinases, INK4 inhibitors and cancer. Biochim Biophys Acta 1602:73–87.

Paliwal S, Pande S, Kovi RC, Sharpless NE, Bardeesy N, Grossman SR. 2006. Targeting of C-terminal binding protein (CtBP) by ARF results in p53-independent apoptosis. Mol Cell Biol 26:2360–2372.

Paliwal S, Kovi RC, Nath B, Chen YW, Lewis BC, Grossman SR. 2007. The alternative reading frame tumor suppressor antagonizes hypoxia-induced cancer cell migration via interaction with the COOH-terminal binding protein corepressor. Cancer Res 67:9322–9329.

Ruas M, Peters G. 1998. The p16INK4a/CDKN2A tumor suppressor and its relatives. Biochim Biophys Acta 1378:F115–F177.

Sandig V, Brand K, Herwig S, Lukas J, Bartek J, Strauss M. 1997. Adenovirally transferred p16INK4/CDKN2 and p53 genes cooperate to induce apoptotic tumor cell death. Nat Med 3:313–319.

Schaeper U, Boyd JM, Verma S, Uhlmann E, Subramanian T, Chinnadurai G. 1995. Molecular cloning and characterization of a cellular phosphoprotein that interacts with a conserved C-terminal domain of adenovirus E1A involved in negative modulation of oncogenic transformation. Proc Natl Acad Sci U S A. 92:10467–10471.

Serrano M, Hannon GJ, Beach D. 1993. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature 366:704–707.

Sherr CJ. 2004. Principles of tumor suppression. Cell 116:235–246.

Sherr CJ, Roberts JM. 1999. CDK inhibitors: Positive and negative regulators of G1-phase progression. Genes Dev 13:1501–1512.

Sollerbrant K, Chinnadurai G, Svensson C. 1996. The CtBP binding domain in the adenovirus E1A protein controls CR1-dependent transactivation. Nucleic Acids Res 24:2578–2584.

Subramanian T, La Regina M, Chinnadurai G. 1989. Enhanced ras oncogene mediated cell transformation and tumorigenesis by adenovirus 2 mutants lacking the C-terminal region of E1a protein. Oncogene 4:415–420.

Thio SSC, Bonventre JV, Stephen I, Hsu H. 2004. The CtBP2 co-repressor is regulated by NADH-dependent dimerization and possesses a novel N-terminal repression domain. Nucleic Acids Res 32:1836–1847.

Vermeulen K, Van Bockstaele DR, Berneman ZN. 2003. The cell cycle: A review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif 36:131–149.

Witkiewicz AK, Knudsen KE, Dicker AP, Knudsen ES. 2011. The meaning of p16ink4a expression in tumors: Functional significance, clinical associations and future developments. Cell Cycle 10:2497–2503.

Xiong Y, Zhang H, Beach D. 1993. Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation. Genes Dev 7(8):1572–1583.

Yip HT, Chopra R, Chakrabarti R, Veena MS, Ramamurthy B, Srivatsan ES, Wang MB. 2006. Cisplatin-induced growth arrest of head and neck cancer cells correlates with increased expression of p16 and p53. Arch Otolaryngol Head Neck Surg 132:317–326.

Zhang Q, Nottke A, Goodman RH. 2005. Homeodomain-interacting protein kinase-2 mediates CtBP phosphorylation and degradation in UV-triggered apoptosis. Proc Natl Acad Sci U S A 102:2802–2807.

Zhao LJ, Subramanian T, Zhou Y, Chinnadurai G. 2006. Acetylation by p300 regulates nuclear localization and function of the transcriptional corepressor CtBP2. J Biol Chem 281:4183–4189.