

Integrin $\beta 1$ subunit overexpressed in the SMMC-7721 cells regulates the promoter activity of p21^{CIP1} and enhances its transcription

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Abstract Evidence has been emerging to suggest that integrin could induce growth inhibition in some cell types. Some of the molecular mechanisms underlying growth arrest have been elucidated. We reported here that overexpression of integrin $\beta 1$ imposed a growth inhibitory effect on the hepatocellular carcinoma cell line SMMC-7721, and this phenomenon was mainly attributed to the cyclin-dependent kinase inhibitor p21^{CIP1}. Furthermore, our findings suggested that transcription activity of the p21^{CIP1} gene could be upregulated in the integrin $\beta 1$ -overexpressing cells, and possibly controlled by the *cis*-elements in the core region of the p21^{CIP1} promoter.

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Key words: Integrin; p21^{CIP1}; Hepatocellular carcinoma cell line; Transcriptional regulation; Promoter activity

1. Introduction

Integrins are a large family of cell receptors binding to the components of the extracellular matrix (ECM), which generally activate growth-promoting signaling pathways, such as activation of extracellular signal-regulated protein kinase, the c-Jun NH₂-terminal kinase [1,2], and phosphatidylinositol 3'-kinase/protein kinase B [1,3]. However, many studies have demonstrated that integrins give rise to growth inhibition rather than growth stimulation [4–6]. It seems to be apparent from these studies that integrin signaling may play a major role in negative control of cell growth, which may be lost in some cancer cells.

A member of the CIP/KIP family, p21^{CIP1} (here referred to as p21), the first cyclin-dependent kinase (CDK) inhibitor to be identified [7,8], participates in activation control of various cyclin/CDK protein kinases [9,10], and subsequently plays an important role in growth arrest, cellular differentiation, DNA repair, cell senescence and apoptosis [10,11]. The proximal region of the p21 promoter containing the Sp1 sites was shown to be the minimal area for mediating up-regulation

of the p21 gene by various external signals derived from the milieu, such as p53-dependent DNA damage, transforming growth factor- β (TGF- β), phorbol ester, okadaic acid, progesterone, geranylgeranyltransferase I inhibitor and others [12–18]. Some factors, such as phospholipase D1/2 [19], lead to down-regulation of the p21 promoter activity. Meanwhile, p21 gene expression is also regulated by several transcription factors of importance for growth inhibition, such as p53, VDR, RAR, C/EBP α , STATs, Smads, Sp1/Sp3, AP2/AP4, Myc/Max, Myc/Miz-1, etc. [16,20–30].

In our previous studies, it had been demonstrated that overexpression of $\beta 1$ subunit or $\alpha 5\beta 1$ could inhibit cell proliferation because of the unoccupied $\beta 1$ -class integrins, or relative lack of its ligands [31,32]. This phenomenon may be involved in the CDK inhibitor p21 [32]. Since growth inhibition was induced in integrin-overexpressing cells, we hypothesized that interference with p21 expression may be one mechanism by which integrins can arrest cell growth. We report here that overexpression of integrin $\beta 1$ subunit in SMMC-7721 cells could up-regulate transcription activity of the p21 gene, which may be controlled by the *cis*-elements in the core region of the p21 gene promoter via a certain integrin signaling pathway.

2. Materials and methods

2.1. Cell lines, plasmids and stable transfections

The human hepatic cancer cell line SMMC-7721 was obtained from the Liver Cancer Institute in Zhongshan Hospital (Shanghai, China). Mammalian expression vector pcDNA3- $\alpha 5$ was a gift from Dr. Sue E. Craig (School of Biological Sciences, University of Manchester, Manchester, UK). The pcDNA3- $\beta 1$ plasmid was constructed as described previously [31]. Stable transfections were performed using LipofectamineTM 2000 reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions as described previously [32]. Integrin $\alpha 5$ - and/or $\beta 1$ -overexpressing cells were designated $\alpha 5$ -7721, $\beta 1$ -7721 and $\alpha 5\beta 1$ -7721, respectively. We also selected cells with different $\alpha 5$ protein levels such as $\alpha 5.1$ and $\alpha 5.3$, and cells with different $\beta 1$ protein levels such as $\beta 1.1$ and $\beta 1.3$. Basic culture medium consisted of RPMI 1640 medium supplemented with 10% calf bovine serum, 100 IU/ml penicillin and 100 μ g/ml streptomycin. These integrin-overexpressing cell lines were maintained in the same medium as above plus 500 μ g/ml geneticin (Gibco BRL). These cells were incubated at 37°C, 5% CO₂.

2.2. Survival and growth curves of integrin $\alpha 5/\beta 1$ -overexpressing cells

Polyhydroxy-ethylmethacrylate (poly-HEMA, Sigma)-coated 96-well plates were prepared as described previously [33]. Fibronectin (FN)-coated culture plates were also prepared. Briefly, 100 μ l FN in phosphate-buffered saline (PBS; 15 μ g/ml) was added into each well of 96-well plates. These plates were incubated for 1 h at 37°C, and

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Abbreviations: CDK, cyclin-dependent kinase; ECM, extracellular matrix; FN, fibronectin; PBS, phosphate-buffered saline; poly-HEMA, polyhydroxy-ethylmethacrylate

washed with PBS extensively. Cells were then seeded onto poly-HEME- or FN-coated plates at a density of 2×10^4 cells/well in 200 μ l RPMI 1640 medium, and grown for 0, 2, 4, 6 or 8 days, respectively. Meanwhile, an equal amount of fresh medium was added into wells without cells, as control. For each assay, 20 μ l of MTT was added, and these plates were maintained in culture for 4 h. Following incubation, culture medium was discarded and 150 μ l dimethyl sulfoxide was added to each well. These plates were vibrated gently for 10 min, which was followed by detection in the universal microplate reader at 490 nm.

2.3. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNAs were isolated using the Trizol system (Watson Biotechnologies, Shanghai, China) according to the manufacturer's guidelines. RT-PCR was performed to quantify the mRNA level of the p21 gene. (dT)₁₅ primer and AMV-RTase were used for first strand synthesis. cDNA products (2 μ l) were mixed with *Taq* DNA polymerase (SABC, Luoyang, China), 50 pmol/l of each appropriate primer, 200 μ mol/l each dNTP in a reaction buffer containing 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 0.01% (W/V) bovine serum albumin (BSA), 2 mmol/l MgCl₂ in a final volume of 100 μ l. The primer pair for p21 was described previously [34]. Primers for β -actin were also described [35], which were used as the internal control. The expected product sizes were as follows: p21, 159 bp; β -actin, 412 bp. The samples were amplified for 32 cycles at cyclic temperatures of 94°C 45 s, 60°C 45 s, 72°C 45 s. PCR products were analyzed through 1% agarose gel electrophoresis and following ethidium bromide staining. The band area of p21 was measured and normalized by that of β -actin, and then the specific p21 mRNA level was estimated.

2.4. Western blot analyses of integrin β 1 subunit and p21

Cells were plated at a density of 5×10^5 cells/8 ml of medium in flasks and grown for 24 h. Cultured cells were then harvested with trypsinization and centrifugation and rinsed twice in ice-cold PBS, and lysed in 1 \times sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 μ g/ml phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin and 5 mM Na₃VO₄) for 10 min on ice. The samples were boiled and clarified by centrifugation at 12000 $\times g$ for 8 min at 4°C. The supernatants were transferred to a microcentrifuge tube and stored at -20°C. Protein lysates were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride membranes. The membranes were blocked in 5% non-fat dry milk in PBST (PBS+0.05% Tween 20) and incubated with mouse anti-human integrin β 1 subunit McAb (BD Transduction Laboratories, 1:500–1:1000) or rabbit anti-human p21 polyclonal antibody (Santa Cruz, 1:500) diluted in 5% milk in PBST overnight at room temperature. Following three washes in PBST, the blots were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Calbiochem) diluted in 1:500. Finally, these blots were washed three times in PBST, and developed by enhanced chemiluminescence (Amersham).

2.5. Flow cytometry analysis for cell membrane surface integrin β 1 subunit

Cell surface integrin β 1 protein levels were detected as described previously [31,36]. Briefly, cells were plated at a density of 1×10^5 cells/8 ml of medium in flasks and grown for 24 h. They were collected by detachment with 2 mmol/l EDTA and centrifugation at 300 $\times g$ for 5 min, and washed twice with ice-cold PBS. Cells were then blocked with 1 mg/ml BSA in PBS, and incubated with primary antibody α 5-mAb and β 1-mAb against human α 5 β 1 expressed onto the cell plasma membrane for 1–4 h at 4°C, and then rinsed twice with PBS. Cells were incubated with a secondary antibody conjugated with fluorescein for 1 h at 4°C, washed twice with PBS, and resuspended in 0.5 ml of PBS, then analyzed on the FACS (Becton Dickinson). A suspension of 1×10^4 cells was analyzed for each sample, and each experiment was repeated at least twice.

2.6. p21 promoter deletion-luciferase reporter constructs

Human p21 promoter region (1908 bp) was obtained from SMMC-7721 genomic DNA by PCR with the primer pair Up3/Down1, which harbored *Kpn*I and *Hind*III sites at the 5'-end of each primer. PCR products were digested with the two enzymes and cloned into *Kpn*I and *Hind*III sites of pGL3-control (designated pGL3-1908). Based on

pGL3-1908, we used the primer pairs Up2/Down1, Up1/Down1, and Up0/Down1 to amplify different length fragments of p21 promoter with identical 3'-ends. These products were digested with *Kpn*I and *Hind*III and cloned back into *Kpn*I and *Hind*III sites of pGL3-control, giving pGL3-600, pGL3-287, pGL3-217, respectively. These p21 promoter-luciferase reporter plasmids were incorporated with different genomic fragments from the human p21 promoter to drive expression of luciferase. Sequences of primers used in this study were described previously [37], and are listed below: Down1: 5'-GCC AAGCTT CCG GCT CCA CAA GGA ACT GA-3', Up0: 5'-GAC GGTACC CTG GCC TGC TGG AAC T-3', Up1: 5'-GAC GGTACC ACC AAC GCA GGC GAG GGA CT-3', Up2: 5'-GAG GGTACC GGT GTC TAG GTG CTC CAG GT-3', Up3: 5'-GTC GGTACC CGT GGT GGT GGT GAG CTA GA-3' (double

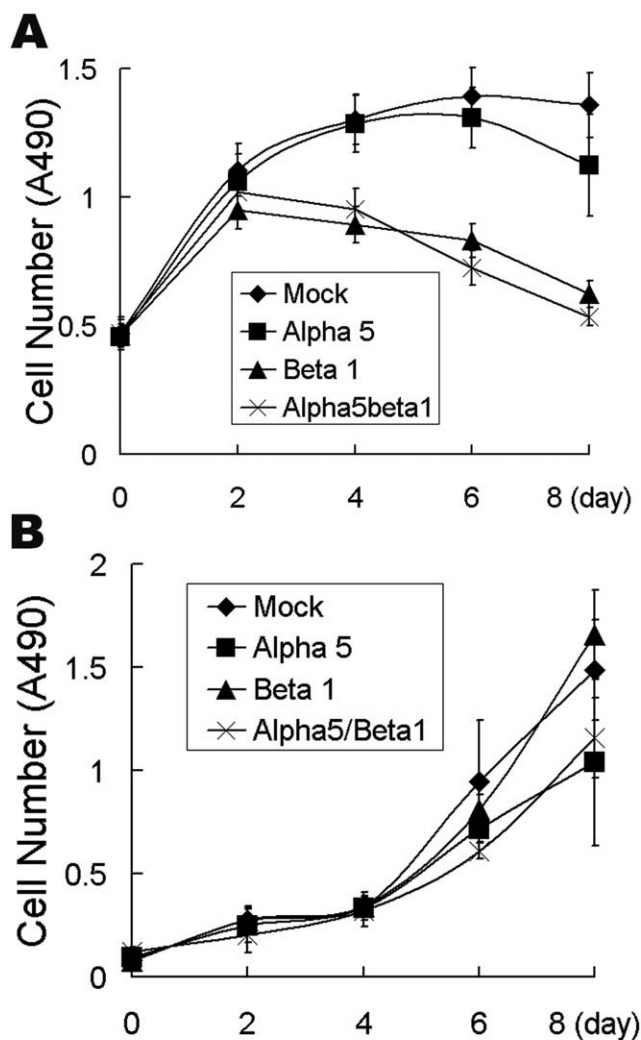


Fig. 1. Survival course of α 5 and/or β 1 integrin-transfected SMMC-7721 cells. Cell suspensions of the mock and integrin α 5/ β 1-transfected cells were seeded onto 96-well plates, and cultured for 0, 2, 4, 6 or 8 days. Subsequently, cells were quantified with the MTT assay. The mock cells (Mock) represent the parental SMMC-7721 cells transfected with empty vector pcDNA3. Other types of cells were α 5-7721 (Alpha 5), β 1-7721 (Beta 1) and α 5 β 1-7721 (Alpha5beta1). A: Cells were seeded onto poly-HEME-coated 96-well plates and grown for the indicated times. From the 4th day of culture, surviving cells declined significantly in β 1-7721 and α 5 β 1-7721 cells compared with the mock cells (for one sample, $t=4.207$, $P=0.006$). B: Cells were grown on fibronectin-coated 96-well plates. The four cell types had no statistical difference in growth rate even after day 6. But on the 8th day, β 1-7721 and the mock cells grew more rapidly than the two others ($t=3.204$, $P=0.008$).

underline, *Hind*III site; single underline, *Kpn*I site). These constructs were sequenced and compared with the GenBank database.

2.7. Transient transfections and luciferase assays

SMMC-7721 cells were seeded onto 6-well plates, and grown to over 90% confluence. Each p21 promoter deletion–luciferase reporter construct was co-transfected with pGFP- β -gal plasmid (a gift from Houyan Song, Department of Molecular Genetics, Shanghai Medical College, Fudan University, Shanghai, China) using Lipofectamine[®] 2000 reagent according to the manufacturer's instructions. Lysates were harvested at 48 h post transfection. To prepare lysates, cells were washed twice with ice-cold PBS, and then 300 μ l of 1 \times luciferase cell culture lysis reagent (25 mmol/l Tris-phosphate, 2 mmol/l dithiothreitol, 2 mmol/l 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid, 10% glycerol, 1% Triton X-100) was added directly to the cells. Cells were scraped and transferred to a microcentrifuge tube, then centrifuged at 14 500 $\times g$ for 5 min at 4°C to pellet insoluble materials. The supernatants were assayed for luciferase activities in triplicate in a luminometer. Luciferase activities of cell lysates were measured according to the manufacturer's recommendations (Promega, Madison, WI, USA). In this assay, each tube contained 20 μ l of lysate and 100 μ l of luciferase assay buffer. Luciferase activity of each sample was normalized by β -gal activity. Transfection efficiency was determined through the positive cells with green fluorescence from the green fluo-

rescent protein (GFP) under the fluorescent microscope. All luciferase assays were carried out at least in triplicate, and the experiments were made repeatedly.

3. Results

3.1. Overexpression of integrin β 1 subunit inhibits proliferation of SMMC-7721

In previous studies, we showed that overexpression of integrin α 5 β 1 or β 1 subunit affects cell growth negatively [31,32]. To further elucidate the negative effects of integrin β 1 overexpression on cell proliferation, we employed poly-HEME to block attachment of the cells to the ECM. Two to four days later, growth inhibitory effects of integrin β 1 overexpression were observed in the hepatocellular carcinoma-derived SMMC-7721 cells (Fig. 1A). On day 6, growth of α 5 β 1-7721 and β 1-7721 cells was inhibited to near 53% and 60% of the control level, respectively. Moreover, the great extent of inhibitory effects even lasted to the 8th day. Nevertheless, these transfectants grew at a similar rate when plated onto the FN-coated plates, even if these cells were grown for 6 days (Fig. 1B). Thus, these results suggest that the inhibitory effects of integrin β 1 overexpression might be attributed to a lack of ECM, such as FN.

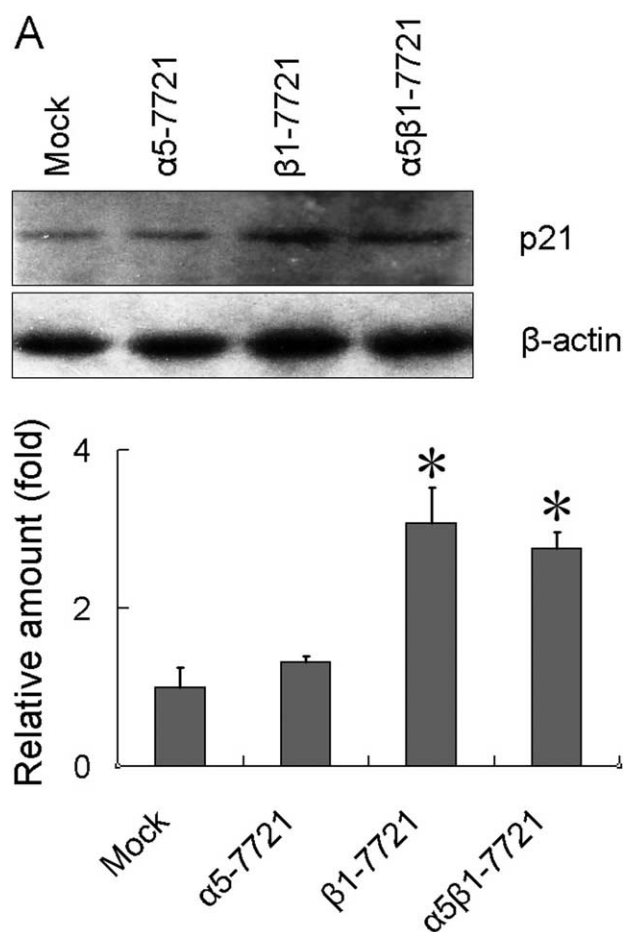


Fig. 2. mRNA and/or protein levels of the cell cycle regulatory p21 gene were increased in β 1-7721 and α 5 β 1-7721 transfectants. A: Immunoblot assays showed p21 protein level was increased in the β 1-7721 and α 5 β 1-7721 cells. The protein level of β -actin was detected to determine the loading amount in each well in the SDS-PAGE gel. B: mRNA level of p21 was assessed by RT-PCR, and normalized by that of β -actin. It was apparent that its mRNA level was increased in the β 1- and α 5 β 1-transfected cells. Here shown are representative data from three independent experiments (* P < 0.01, compared with the mock cells).

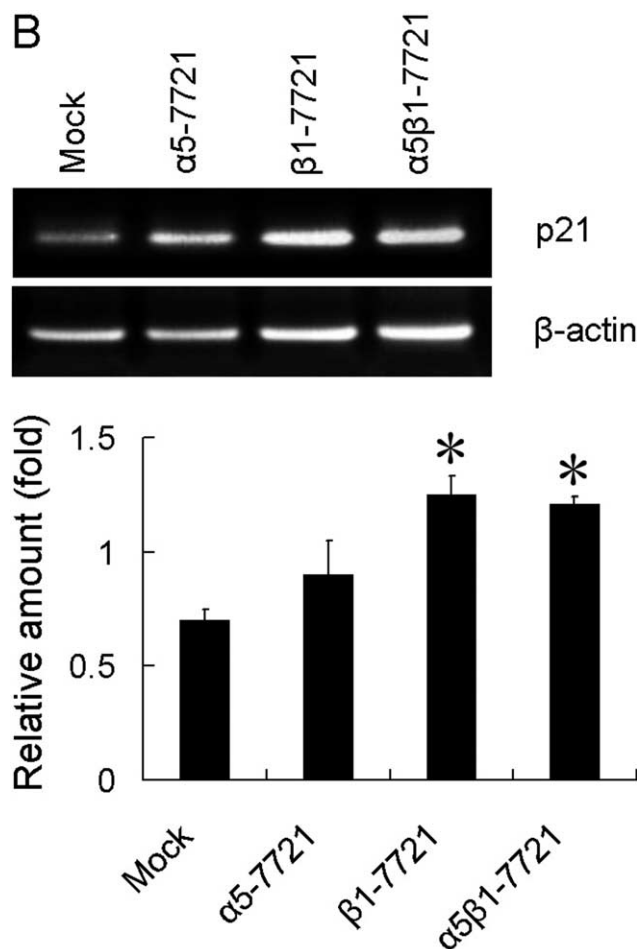


Fig. 2 (Continued).

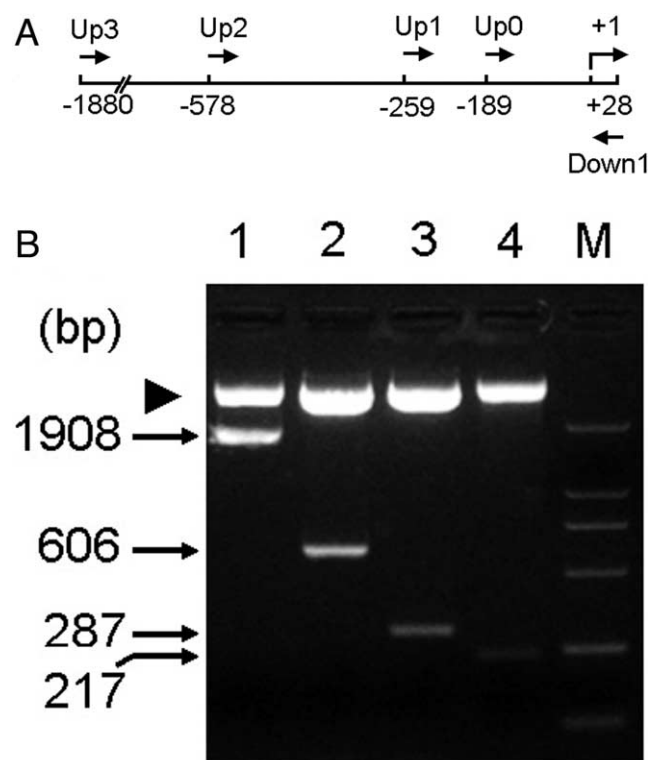


Fig. 3. Construction diagram of p21 promoter-luciferase reporter vectors. A: Schematic representation of the human p21 gene promoter. Primers used are indicated as Up3, Up2, Up1, Up0 and Down1. PCR products from the 5'-flanking region of the p21 gene were amplified from the genomic DNA in SMMC-7721 cells. B: Identification of p21 promoter deletion-luciferase reporter constructs. The four constructs of the p21 promoter region were digested with the same two restriction enzymes *KpnI/HindIII* and resolved by 0.8% agarose gel electrophoresis. pGL3-control was cleaved 240 bp off by *KpnI* and *HindIII* enzymes (arrowhead). The sizes (bp) of inserts are indicated at the left of lanes. Lane 1, pGL3-1908; lane 2, pGL3-600; lane 3, pGL3-287; lane 4, pGL3-217; M, DL-2000 DNA marker (TaKaRa).

3.2. p21 expression levels were increased in the integrin $\beta 1$ transfectant cells

To investigate whether CDK inhibitor p21 is involved in growth inhibition induced by integrin $\beta 1$ overexpression in

SMMC-7721 cells, protein and mRNA levels of p21 were examined in integrin $\beta 1$ transfectant cells. A marked induction of p21 protein and mRNA levels was observed in integrin $\beta 1$ -overexpressing cells, such as $\beta 1$ -7721 and $\alpha 5\beta 1$ -7721. As shown in Fig. 2A, a significant increase (two-fold) of p21 protein levels was found in $\beta 1$ -7721 and $\alpha 5\beta 1$ -7721 compared with the mock or $\alpha 5$ -7721 cells. Meanwhile, the p21 mRNA level was also increased in integrin $\beta 1$ -overexpressing cells (Fig. 2B). Consequently, these findings implied that p21 gene expression was not only modulated at the post-translational level, but also regulated at the transcriptional level. Additionally, we also observed notable induction of another CDK inhibitor, p27^{KIP1}, in integrin $\beta 1$ -overexpressing cells [32]. However, only the protein level of p27 was increased in the integrin $\beta 1$ transfectants. Therefore, induction of p21 gene expression could be one of the major reasons for growth inhibition in the integrin $\beta 1$ -transfected cells.

3.3. Core region of p21 promoter identified in SMMC-7721 cells

To determine whether p21 mRNA transcription is mediated by overexpressing integrin $\beta 1$ subunit, we did experiments to clarify whether integrin overexpression could stimulate the activity of the p21 gene promoter. We amplified the p21 gene promoter region extending from -1880 to +28 bp by PCR, and constructed p21 promoter deletion-luciferase reporter constructs (Fig. 3A). These plasmids contain different fragments of the p21 gene promoter, with pGL3-1908 extending from -1880 to +28 bp (1908 bp), pGL3-600 from -578 to +28 bp (606 bp), pGL3-287 from -259 to +28 bp (287 bp), pGL3-217 from -189 to +28 bp (217 bp) (Fig. 3B). To elucidate the core region of the p21 gene promoter, we performed transient transfections with the promoter deletion-luciferase reporter constructs in SMMC-7721 cells. The transfection efficiency was controlled by the GFP fluorescence of the co-plasmid pGFP- β -gal (Fig. 4). The p21 gene promoter activity was evaluated by the use of relative luciferase units, whose expression was driven by the inserted different fragments of the p21 promoter. Among the four constructs, pGL3-1908 containing the region of the p21 promoter between -1880 and +28 bp and pGL3-217 between -189 and +28 bp were shown to have stronger luciferase activities compared with control vector (Fig. 5). However, luciferase activity driven

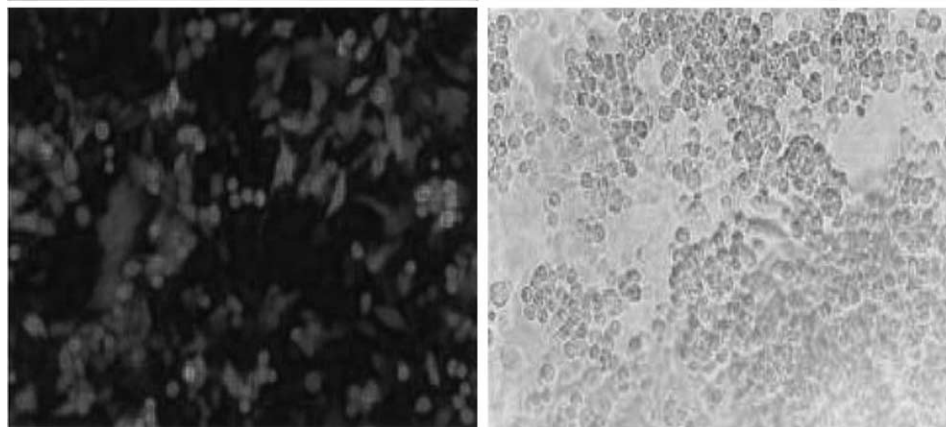


Fig. 4. Transient transfection efficiency of the p21 promoter constructs. The constructs of the p21 promoter along with pGFP- β -gal were transiently transfected into the parental SMMC-7721 and its derivatives. The efficiency of transfection was determined by the ratio of GFP-positive cells to the total in some random visual fields. Samples with more than 30% positive cells were regarded as validly transiently transfected.

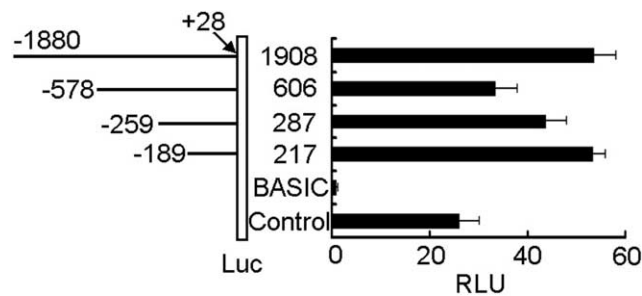


Fig. 5. Schematic representation of p21 promoter and activities of p21 promoter serial deletion constructs. SMMC-7721 cells were transiently transfected with serial deletion reporter constructs. pGL3-1908 is a construct containing the full-length sequence of the p21 promoter region amplified from the genomic DNA of SMMC-7721 cells. pGL3-basic (denoted by BASIC) is a firefly luciferase reporter vector lacking promoter activity, and pGL3-control (denoted by Control) plasmid was regarded as a positive control, which contains the SV40 promoter and enhancer. Transient transfections and luciferase assays were performed twice or in triplicate, and the data were normalized to the activity of bacterial β -galactosidase and expressed as means \pm S.D. RLU, relative luciferase units; 1908, pGL3-1908; 606, pGL3-600; 287, pGL3-287; 217, pGL3-217.

by the different inserts, either from -578 to $+28$ bp or from -259 to $+28$ bp, was decreased to some extent. So, some repressive elements could exist in the p21 gene promoter region extending from -578 to -189 bp, which is consistent with a previous report [23], and the proximal region (between -189 and $+28$ bp) might harbor the core elements that controlled p21 gene expression in hepatic cancer cells.

3.4. Activity of p21 core promoter was increased with overexpression of integrin $\beta 1$ subunit

As mentioned above, the p21 mRNA level was increased in integrin $\beta 1$ -overexpressing $\beta 1$ -7721 and $\alpha 5\beta 1$ -7721 cells, and the proximal region of the p21 gene promoter was responsible for its expression. So we hypothesized that the important elements might reside in the proximal region and be regulated by the integrin $\beta 1$ overexpression. To investigate this effect of integrin $\beta 1$ overexpression on p21 promoter activity, we examined the luciferase activity of the pGL3-217 construct in integrin $\beta 1$ transfectant cells. It was noted that its luciferase activity was stronger in integrin $\beta 1$ -overexpressing cells than in cells with a lower level of integrin $\beta 1$ expression (Fig. 6D). Meanwhile, we evaluated the luciferase activities driven by four different inserts in an integrin $\beta 1$ -overexpressing cell strain ($\beta 1.3$). Apparently, luciferase activity from the pGL3-217 construct was still maintained at higher levels. However, only the promoter activity of pGL3-287 (from -259 to $+28$ bp) was significantly decreased (Fig. 6E). So it occurred to us from these hints that p21 promoter activity could be up-regulated in integrin $\beta 1$ -overexpressing cells, which possibly acted through the proximal region (between -189 and $+28$ bp) of the p21 gene promoter, and that repressive element(s) might be present in the insert of pGL3-287, especially from -259 to -189 bp.

Reportedly, there are several transcription factor binding sites in the proximal region of the p21 gene promoter. On the basis of these reports, we employed the GenoMatix Suite/MatInspector software [38] to analyze the potential binding sites in this proximal region (between -189 and $+28$ bp). As shown in Fig. 7, there are a variety of binding sites for Sp1, AP2, AP4, E2F and Myo D. In particular, six

Sp1/Sp3 binding sites existed in this area. As is well known, Sp1 is a kind of general transcription factor involved in transcription control of many target genes. Moreover, some evidence showed that Sp1 could regulate expression of the downstream genes (including p21) responsible for a variety of cellular processes (growth arrest, cell differentiation) together with co-activators, such as pRB, Smad, p300/CBP [23,39,40]. So Sp1, along with its co-activator, might be implicated in the p21 expression induced by integrin $\beta 1$ overexpression.

4. Discussion

Although at least two structurally distinct, but functionally related, variants of the p21 gene from discrete promoters in the p21 locus have been identified [41], p21 is an important regulator of cell cycle progression, senescence, and differentiation. Integrin signals are also necessary for cells to regulate differentiation and proliferation [1]. In this study, we observed growth inhibition in $\beta 1$ -overexpressing cells in the presence of poly-HEME, which was released when these cells were grown on FN-coated plates. So unoccupied integrin $\alpha 5\beta 1$ was suggested to be the reason for the growth inhibition.

Additionally, p21 gene expression was induced by overexpression of integrin $\beta 1$ subunit and relative to growth inhibition of $\alpha 5\beta 1$ -7721 and $\beta 1$ -7721 cells. Actually, except for the relative lack of integrin-ECM interaction described here, there are plenty of signals from the cellular microenvironment to regulate p21 gene expression. For example, genotoxic stress leads to activation of the tumor suppressor p53 and subsequently to induction of p21. Moreover, a number of transcription factors are involved in p21 gene promoter activation. Recently, experimental evidence has shown that p21 transcription could be controlled in a p53-dependent or -independent manner, such as by TGF- β [12], okadaic acid [13], flavone [42], and sodium butyrate, which is an inhibitor of histone deacetylase [43].

Several studies report that there are two positive regions in the p21 gene promoter extending from -2100 to -1600 bp and from -124 to -61 bp, which cooperatively regulated p21 gene promoter activation in HepG2 cells [23]. Moreover, the activity of the proximal promoter region (from -119 to $+16$ bp) is similar to that of the full-length promoter of the p21 gene (between -2330 and $+16$ bp) in the Caco-2 colon adenocarcinoma cell line [21]. In our study, the two essential promoter regions were present in the 1.9-kb promoter used, so the minimal p21 promoter-luciferase reporter construct pGL3-217 containing the promoter region between -189 and $+28$ bp functioned as efficiently as a 1.9-kb p21 promoter construct. Although this proximal region was covered in the four constructs used in this research, the luciferase activity was still decreased in either pGL3-600 or pGL3-287, especially the latter, in integrin $\beta 1$ -overexpressing cells. So the repressive elements might be present in these regions, especially from -259 to -189 bp. And the proximal region (from -189 to $+28$ bp) was suggested to be a core region and responsible for its up-regulation mediated by integrin $\beta 1$ overexpression.

How to regulate the p21 gene transcription by overexpression of integrin $\beta 1$ subunit? This problem was not well known yet. We hypothesized that some transcription factors whose binding sites existed in the core promoter region might be involved, and these transcription factors may be modulated

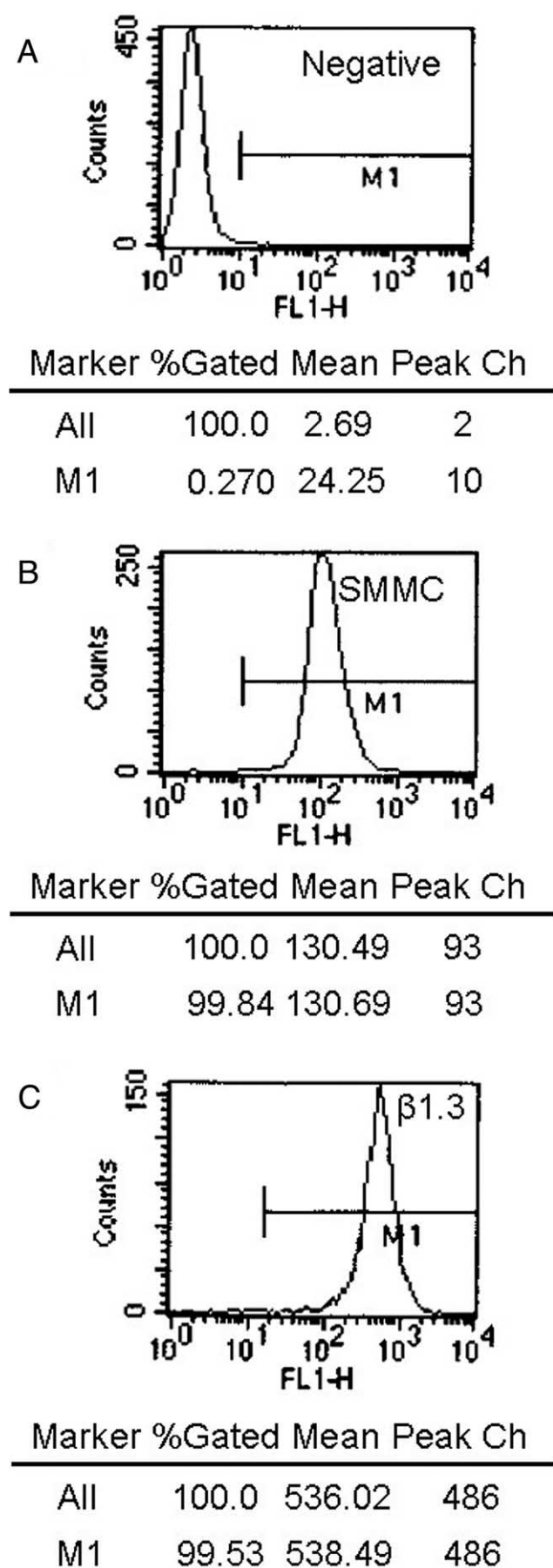


Fig. 6.

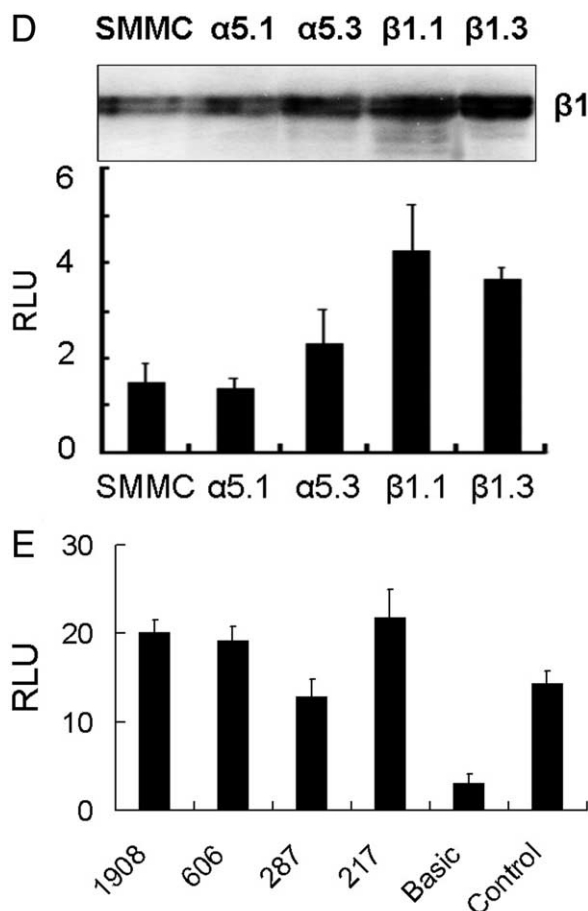


Fig. 6 Activation of p21 gene promoter was up-regulated in integrin $\beta 1$ -overexpressing cells. The cell surface levels of integrin $\beta 1$ subunit were consistent with the protein levels detected by Western blot analysis, and here are shown the surface levels in (A) negative control (Negative), (B) parental SMMC-7721 cells (SMMC) and (C) integrin $\beta 1$ -overexpressing cell $\beta 1.3$. D: The stably integrin $\alpha 5/\beta 1$ -overexpressing cells were transiently transfected with the reporter vector pGL3-217. Cotransfection of pGFP- β -gal, which contains GFP and β -gal, was used to monitor the transfection efficiency. After 48 h in growth medium, cellular extracts were assayed using $1\times$ CCLR. Transient transfections and luciferase assays were performed in triplicate. E: Stably overexpressing cell strain $\beta 1.3$ was transiently transfected with the four p21 promoter constructs, BA-SIC and Control. Data were normalized to the activity of β -galactosidase and are shown as means \pm S.D. RLU, relative luciferase units; 1908, pGL3-1908; 606, pGL3-600; 287, pGL3-287; 217, pGL3-217.

by overexpression of integrin $\beta 1$ in hepatic cancer cells. As is well known, p21 gene expression was controlled by various transcription factors together with some co-activators or co-repressors, especially Sp1/Sp3 and its co-activators [16,20–25]. This might be an important mechanism by which the p21 gene expression was induced in integrin $\beta 1$ -overexpressing cells, and this question remains to be further investigated.

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-189 CTGGCCCTGC TGGAACTCGG CCAGGCTCAG CTGGCTCGGC

-150 GCTGGGCAGC CAGGAGCCTG GGCCCCGGGG AGGGCGGTTC
Sp1

-110 CGGGCGGCGC GGTGGGCCGA GCGCGGGT CC CGCCTCCTTG
Sp1 E2F Sp1

-70 AGGCGGGCCC GGGCGGGGCG GTTGTATATC AGGGCCGTGC
Sp1 Sp1 ▶ +1

-30 TGAGCTGCGC CAGCTGAGGT GTGAGCCAGCT GCCGAAGTCA
E-box (MyoD-type) E-box

+11 GTTCCTTGTG GAGCCGGA
Inr

Fig. 7. Sequence representation of the proximal region of the p21 gene promoter (−189+28). Some putative binding sites for transcription factors regulating the p21 promoter activation are indicated and underlined. The dotted line represents the mutant binding site for E2F transcription factor, and +1 indicates the transcription start site. Inr, initiator.

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