Asian Journal of Andrology (2009) 11: 669–677
© 2009 AJA, SIMM & SJTU All rights reserved 1008-682X/09 $ 32.00
www.nature.com/aja

Original Article

Exogenous p27KIP1 expression induces anti-tumour effects and inhibits the EGFR/PI3K/Akt signalling pathway in PC3 cells

Jun Chen, Dan Xia, Jin-Dan Luo, Ping Wang

Department of Urology, The First Affiliated Hospital, Medical College of ZheJiang University, Hangzhou 310003, China

Abstract

p27 is a cyclin-dependent kinase inhibitor that regulates the progression of cells from G_1 to S phase of the cell cycle. Loss of p27 has been associated with disease progression and with an unfavourable outcome in prostate cancer. In this study, we investigated whether exogenous p27 expression in the human androgen-independent prostate cancer PC3 cell line had any effect on cell growth, and we studied the molecular mechanisms involved. p27 expression was restored in PC3 cells by plasmid delivery. Cell proliferation and apoptosis were assessed in PC3 cells transfected with *p27*. We also investigated the effects of p27 on the epidermal growth factor receptor (EGFR)/phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathway in PC3 cells. By restoring p27 expression in PC3 cells, we observed that p27 reduced proliferation and induced arrest in G_0/G_1 phase. Moreover, *p27*-transfected PC3 cells underwent apoptosis, as shown by flow cytometric analysis and western blotting analysis of Bcl-2, Bax, Bad, caspase-3 and poly(ADP-ribose)polymerase expression. Furthermore, the p27-induced anti-tumour action correlated with inhibition of the EGFR/PI3K/Akt signalling pathway, as confirmed by western blotting analysis and densitometry of EGFR, PI3K (p85), Akt and p-Akt^{S473} expression. Our results suggest that exogenous expression of p27 inhibits the proliferation of PC3 cells through induction of G_1 arrest and apoptosis, and this process correlates with inhibition of the EGFR/PI3K/Akt signalling pathway.

Asian Journal of Andrology (2009) 11: 669–677. doi: 10.1038/aja.2009.51; published online 7 September 2009.

Keywords: apoptosis, EGFR, p27, prostate cancer cells, signalling pathway

1 Introduction

Prostate cancer is one of the leading causes of death from cancer in developed countries, and its incidence is increasing across the world. Although most cases initially respond to androgen deprivation, eventually this therapy fails, and the patients die of recurrent androgen-independent prostate cancer (AIPC). Currently, there is no effective therapeutic option for this form of disease. The precise molecular mechanisms of AIPC development are poorly understood, and elucidation of these mechanisms is the first step towards improving AIPC treatment outcomes.

Progression toward androgen independence correlates with up-regulation of autocrine and paracrine growth factor loops, including overexpression of epider-

Correspondence to: Dr Ping Wang, Department of Urology, The First Affiliated Hospital, College of Medicine, Zhejiang University, 79 Qing'chun Road, Hangzhou 310003, China.
Fax: +86-571-8723-6594 E-mail: wpzjueduuro@126.com
Received: 3 May2009 Revised: 30 May 2009
Accepted: 20 July 2009 Published online: 7 September 2009

mal growth factor receptor (EGFR). EGFR has a critical role in tumour growth, and prostate tissue becomes more susceptible to the growth-promoting action of EGF family growth factors during androgen withdrawal [1, 2]. The general inhibition of tyrosine kinase signalling pathways provides a therapeutic advantage against prostate cancer metastasis [3]. Therefore, inhibiting the activation of growth factor receptors, especially that of EGFR, may be a promising strategy for the treatment of prostate cancer.

Expression of the cyclin-dependent kinase inhibitor (CDKI) p27 is lost in a large fraction of human prostate cancers, and reduced or absent expression of p27 correlates with poor clinical outcome [4]. Exogenous p27 overexpression results in cell cycle regulation and an increase in cell apoptosis in human prostate carcinoma cell lines [5]. In addition, some studies have shown that EGF-induced stimulation of growth in prostate cancer cells is associated with downregulation of p27 [6, 7]. Based on these theories, we hypothesized that the antitumour action of CDKI p27 may be through the EGF signalling pathway in prostate cancer cells.

The aim of this study was to determine whether restoration of p27 expression in human hormone-independent prostate cancer PC3 cells has a significant effect on proliferation, apoptosis and EGFR signalling in PC3 cells.

2 Materials and methods

2.1 Cell culture

PC3 and LNCaP cells derived from human prostate cancer specimens were obtained from the American Type Culture Collection (Rockville, MD, USA). These cells were maintained at 37ºC in a humidified atmosphere of 5% CO_2/95% air and serially passaged in RPMI-1640 (Hyclone, Utah, USA) supplemented with 10% fetal bovine serum.

2.2 Expression and reporter plasmid construction

Total RNA was isolated from normal prostate tissue using TRIzol™ (Invitrogen Inc, CA, USA) according to the manufacturer's instructions. Two micrograms of total RNA was used for cDNA synthesis (SuperScript II Reverse Transcriptase; Invitrogen), and one-tenth of the obtained cDNA was used for PCR to amplify *p27* and *p27-HAtag*. The conditions of PCR for each individual gene were optimized to analyse the amplified product in the linear range of amplification by adjusting the amplification cycles for each set of primers. The nucleotide sequences of the primers used to amplify the *p27* and *p27-HAtag* genes were as follows: *p27*, sense primer 5′-CGC GAA TTC GCG ATG TCA AAC GTG CGA GTG TCT-3′ and antisense primer 5′-CCG GGA TCC GGC GTT TGA CGT CTT CTG AGG CC-3′; *p27-HAtag*, sense primer 5′-CGC GAA TTC GCG ATG TCA AAC GTG CGA GTG TCT-3′ and antisense primer 5′-GCC GGA TCC TTA AGC GTA ATC CGG AAC ATC GTA TGG GTA CGT TTG ACG TCT TCT GAG GCC-3′.

The amplified *p27-HAtag* and *p27* DNA were cloned into a pGEM-T Easy vector (Promega,Wisconsin, USA), following the protocol provided by the manufacturer. The DNA was digested with *Eco*RIand *Bam*H1 restriction enzymes, and the fragments representing *p27-HAtag* and *p27* cDNA were excised from a 1% agarose gel. The DNA was purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The resulting *p27-HAtag* fragment was directionally cloned into the *Eco*RIand *Bam*H1 restriction sites of plasmid pcDNA3.1(–) (Invitrogen). *p27* was cloned into pEGFP-N1 (Clontech, California, USA) at *Bam*H1 and *Eco*R1 sites. The success of the cloning was confirmed by sequencing the plasmid.

2.3 Transient transfections

For transfections, plasmid DNA was prepared using a Qiagen plasmid midi kit (Qiagen GmbH, Hilden, Germany). A total of 5×10^5 cells were plated in six-well plates for 24 h. Plasmid DNA was delivered into cells with Lipofectamine™ 2000 (Invitrogen) following the manufacturer's protocol. Briefly, 4 μg of tested plasmid DNA was mixed with 10 μL of Lipofectamine™ 2000 at room temperature before addition to a well containing 2 mL of serum-free RPMI 1640 medium. After 30 min, DNA–Lipo complexes were added to the well and incubated for 4–6 h at 37ºC in a CO_2 incubator. The DNA–Lipo-containing medium was then replaced.

2.4 Reporter gene assays

PC3 cells were transfected with pEGFP-N1-*p27*. At 24 and 48 h after transfection, reporter gene expression was monitored by fluorescence microscopy, and GFP fusion protein was quantified by flow cytometry (Becton Dickinson, FACScan, San Diego, CA, USA).

2.5 Cell proliferation assays

Cells were plated in 96-well plates at a density of 1.0 ×

10^4 cells per well containing 100 μL of culture medium; after 24 h of cultivation, the cells were transfected with plasmid DNA. The control group was transfected only with pcDNA3.1 (black, vehicle alone). After a period of incubation, cell viability was evaluated by 3-(4,5′-dimethylthiazol-2-yl)-2,5′-diphenyl tetrazolium bromide (MTT) assay. All experiments were performed in triplicate. In a parallel experiment, we also determined cell viability by trypan blue dye exclusion assays. Trypsinized cells were stained with trypan blue, and the total number of cells and the number of viable cells in each well were counted.

2.6 Flow-cytometric analysis of cell cycle and apoptosis

The cells transfected with various plasmid DNAs for 24 or 48 h were assayed for cell cycle progression by the propidium iodide (PI) staining method. Briefly, PC3 cells were harvested in saline-EDTA, fixed in cold 70% ethanol and stored at 4ºC for 24 h. After storage, the cells were pelleted at 800 × *g*, aspirated and re-suspended in RNase A (Invitrogen) in PBS and incubated for 30 min at room temperature. PI was added after RNase digestion, and cells were incubated for an additional 30 min in the dark. Cells were then analysed for cell-cycle distribution using a Becton-Dickinson FACScan.

Annexin V (Annexin V–FITC) and PI double staining was used to determine apoptosis. Annexin V binds to cells that express phosphatidylserine on the outer layer of the cell membrane, and PI stains the cellular DNA of cells with a compromised cell membrane. Cells that stain positive for Annexin V–FITC and negative for PI are undergoing apoptosis. Cells that stain positive for both Annexin V–FITC and PI are either in the end stage of apoptosis or are already dead. Cells that stain negative for both Annexin V–FITC and PI are alive and not undergoing measurable apoptosis. Cells were collected and washed twice with cold PBS and then resuspended in 1 × binding buffer at a concentration of 1×10^6 cells mL^{-1}. Cells were double stained with FITC-conjugated annexin V and PI for 15 min at room temperature. Annexin V and PI were added according to the manufacturer's recommendations (BD pharMingen, San Diego, CA, USA). Samples were immediately analysed by flow cytometry.

2.7 Western blot analysis

Treated cells were washed with cold PBS and lysed in protein extraction buffer containing protease inhibitors. The protein concentration of each sample of the tumour cell lysates was quantified using Coomassie protein assay reagent (Pierce, Rockford, IL, USA). An equal amount of protein was subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane by blotting overnight at 30 V/35 mA. Membranes were blocked for 2 h at room temperature in 5% (w/v) non-fat milk/Tris-buffered saline with 0.1% (v/v) Tween-20. Western blotting was carried out overnight at 4ºC using the following antibodies: p27 monoclonal antibody (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), HA-Tag monoclonal antibody (1:500; New England Biolabs, Ltd, Ipswich, MD, USA), Bax monoclonal antibody (1:200; Santa Cruz Biotechnology, Inc.), Bcl-2 monoclonal antibody (1:200; Santa Cruz Biotechnology, Inc.), Bad monoclonal antibody (1:500; Cell Signaling), caspase-3 monoclonal antibody (1:200; Santa Cruz Biotechnology, Inc.), poly(ADP-ribose)polymerase (PARP) monoclonal antibody (1:200; Santa Cruz Biotechnology, Inc.), EGFR polyclonal antibody (1:200; Santa Cruz Biotechnology, Inc.), phosphatidylinositol 3-kinase (PI3K) (p85) (1:1000; Cell Signaling), Akt and phosphor-$Akt^{Ser\text{-}473}$ (1:500; New England Biolabs) and glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (1:5 000; Acris Antibodies GmbH, Hiddenhausen, Germany). Blots were then incubated with a 1:2 000 dilution of goat anti-rabbit IgG peroxidase-conjugated secondary antibody (Sunnyvale, CA, USA) or a 1:2 000 dilution of goat anti-mouse IgG peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.), washed and developed with enhanced chemiluminescence (Pierce).

2.8 Statistical analysis

For all groups, data are presented as the mean ± SD of the mean. Results were analysed by one-way analysis of variance (ANOVA) and unpaired *t*-test to identify significant differences between groups. The level of statistical significance was set at $P < 0.05$, and all statistical analysis were done using SPSS 11.0 software (version11.0; SPSS, Inc., Chicago, IL, USA).

3 Results

3.1 Characterization of the reporter gene in PC3 cells

PC3 cells were transfected with pEGFP-N1-*p27*. At 24 and 48 h post-transfection, reporter gene expression was monitored by fluorescence microscopy, and

the fluorescence signal was quantified by flow cytometry. Many brightly fluorescent cells were found among the transfected PC3 cells. The GFP fusion protein was readily detectable by flow cytometry, and the transfection efficiency was about 53%–55% at 24 h and about 45%–48% at 48 h.

3.2 Plasmid-mediated p27-HA expression in PC3 cells

To address the question of p27-HA expression after transfection into PC3 cells, western blotting was performed using cell lysates at 24 and 48 h after transfection. Figure 1 shows the results for anti-p27 and anti-HA. There was a basal level of p27 expression in LNCaP cells. No p27 protein was detectable in untransfected or pcDNA3.1-transfected PC3 cells, whereas *p27-HA* delivery resulted in strong expression of p27 protein. To further assess the p27–HA fusion protein, western blotting was performed using anti-HA. The results indicated that the expression of HA was in accordance with that of p27.

3.3 The antiproliferative effect of p27 was associated with G_0/G_1 arrest in PC3 cells

Table 1a shows the effects of p27 and the vehicle on the proliferation of PC3 cells. Proliferation status was assessed by MTT assay. Transfection of PC3 cells with *p27* produced a significantly lower absorbance compared with the control group ($P < 0.05$) at the 48-h time point, but it failed to produce a measurable difference at the 24-h time point. In parallel, trypan blue assays were performed to study cell viability. Table 1b and c show that the expression of p27 led to significantly lower numbers of viable cells and higher numbers of dead cells.

DNA content cell-cycle analysis was performed on PC3 cells transfected with control plasmid and p27. Cells were harvested after transfection at the 24- and 48-h time points. Cell-cycle distribution analysis revealed the average percentage of cells in G_0/G_1, S and G_2/M after transfection. The proportion of cells in the different phases of the cell cycle were analysed by flow cytometry using PI staining. Representative histograms and the mean percentage of cells in each cell-cycle phase derived from multiple experiments are shown in Figure 2. Consistent with the cell proliferation studies, transfection of PC3 cells with *p27* caused cells to accumulate in G_0/G_1 and reduced the number

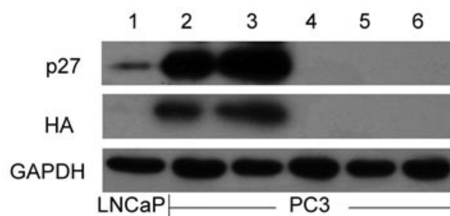

Figure 1. Plasmid-mediated p27-HA expression in PC3 cells. Cells were harvested and lysed for Western blot analysis at 24 and 48 h after transfection. There was a low level of p27 expression in LNCaP cells. Control plasmid-transfected PC3 cells and PC3 cells did not show any detectable p27, whereas *p27*-HA-transfected PC3 cells were positive for p27 staining. The expression of HA was in accordance with p27 expression in transfected PC3 cells. GAPDH was used as a loading control. Lane 1, LNCaP cells; lane 2, p27-PC3 cells 24 h; lane 3, p27-PC3 cells 48 h; lane 4, pcDNA3.1-PC3 24 h; lane 5, pcDNA3.1-PC3 48 h; lane 6, PC3 cells.

Table 1. Effect of p27 transfection on the growth of PC3 cells.

Time (h)	Control (Mean ± SD)	p27 (Mean ± SD)
(a) MTT assays		
0	0.668 ± 0.042	0.678 ± 0.038
24	0.893 ± 0.055	0.732 ± 0.068
48	1.380 ± 0.083	0.963 ± 0.057[a]
(b) Trypan blue dye assays of viable cells ($\times 10^4$)		
0	1.45 ± 0.29	1.42 ± 0.31
24	2.92 ± 0.42	2.08 ± 0.38[b]
48	4.38 ± 0.47	3.17 ± 0.50[a]
(c) The proportion of dead cells (%)		
0	4.98 ± 0.53	5.03 ± 0.47
24	5.26 ± 0.64	7.78 ± 0.73[b]
48	5.88 ± 0.60	8.76 ± 0.79[c]

Cell proliferation was assessed at 0, 24 and 48 h after transfection using the MTT assay. PC3 cell viability was analysed by trypan blue dye exclusion assays. Significance levels pertain to unpaired *t*-test from one-way ANOVA.
[a]$P < 0.05$, [b]$P < 0.05$ and [c]$P < 0.01$ relative to the corresponding control groups at the same time point.

Table 2. Quantitative analysis of apoptotic cells (%) by means of Annexin V–FITC analysis.

Time (h)	Control (Mean ± SD)	p27 (Mean ± SD)
24	6.76 ± 0.52	18.59 ± 1.24[a]
48	6.90 ± 0.46	21.60 ± 1.56[b]

[a]$P < 0.01$ and [b]$P < 0.01$ relative to the corresponding control groups at the same time point.

of cells in S and G_2/M. These results indicate that the antiproliferative effect of p27 was associated with cell-cycle arrest in G_0/G_1.

3.4 *Effect of p27 on apoptosis and apoptosis-related proteins in PC3 cells*

To clarify whether p27 induces apoptosis in PC3 cells, an alternative evaluation of apoptosis was done using Annexin V–FITC analysis. Through this assay, apoptotic and necrotic subpopulations can be distinguished. The apoptotic rates of PC3 cells after control plasmid or *p27* transfection were quantified by Annexin V and PI double staining followed by flow cytometric analysis. The loss of plasma membrane asymmetry is an early event in apoptosis that results in the exposure of phosphatidylserine residues at the outer plasma membrane leaflet. Annexin V, a phospholipid-binding protein, specifically binds to phosphatidylserine residues. The results from an annexin V–FITC binding assay showed that higher proportions of annexin V-positive cells were observed in the *p27*-transfected group at the 24- and 48-h time points, with values of 18.59% and 21.60%. These values were greater than those of the control-plasmid-transfected group (Table 2).

To provide further confirmation that p27 inhibits cell growth owing to the induction of apoptosis, we examined the expression of the Bcl-2 family proteins caspase-3 and PARP after transfection. As shown in Figure 3, the effect of p27 on cellular apoptosis corresponded to lower caspase-3 protein levels (caspase-3 activation is represented by the loss of its pro-form), concurrent with lower Bcl-2 levels and higher Bax and Bad levels in transfected cells. Caspase-3 is one of the executioner caspases that responds to the activation of the intrinsic mitochondrial apoptotic pathway, which can be triggered by blockade of ErbB signalling [8, 9]. When caspase-3 is activated, the intact 33-kDa form is cleaved into an activated form of 17/19 kDa, which in turn cleaves PARP [9]. Consistent with the caspase-3 activation results, p27 markedly increased the level of cleaved PARP. The results suggest that cellular apoptosis induced by p27 was triggered by activation of caspase-3 and regulated by Bcl-2 proteins.

3.5 *Exogenous expression of p27 inhibits EGFR/PI3K/Akt signalling pathway in PC3 cells*

EGFR signalling might be one of the most critical signalling mechanisms for cancer cells, including prostate cancer cells [1, 3, 10]. To investigate the effects of EGFR on PC3 cells, we analysed signalling molecules

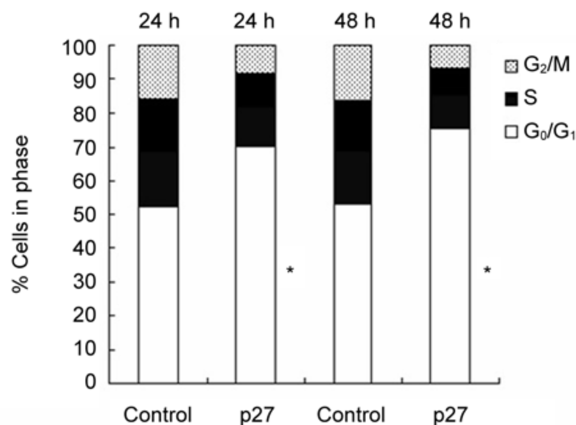

Figure 2. Effect of *p27* transfection on cell cycle progression. Cells were transfected with control plasmid and *p27*. Cells were harvested 24 and 48 h after transfection. Cell-cycle analysis of PC3 cells treated with a control plasmid or *p27*. Cells were exposed to propidium iodide and analysed by flow cytometry as described in Materials and methods. Histograms show DNA quantitation. $^*P < 0.05$, compared with the control groups.

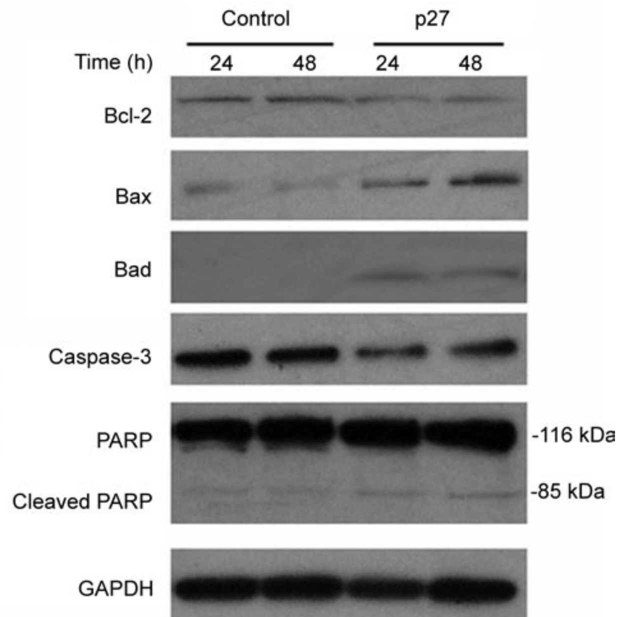

Figure 3. Effects of p27 on apoptosis and apoptosis-related proteins in PC3 cells. Western blot analysis of apoptosis-related proteins expressed in PC3 cells after control plasmid or *p27* transfection.

related to the EGFR pathway. EGFR, PI3K (p85), Akt and p-Akt (S473) were detected by western blotting. As shown in Figure 4A, the expression of EGFR declined after transfection with *p27*; densitometry analysis revealed that EGFR protein levels were reduced by 47% and 62% at the 24- and 48-h time points, respectively.

To analyse downstream signalling events in the EGFR pathway, we next examined the phosphorylation of PI3K (p85), and this also showed noticeable inhibition by the exogenous expression of p27 (Figure 4B). In addition, when we examined the downstream signalling molecules Akt and p-Akt (S473), the level of p-Akt (S473) was reduced by exogenous p27 in PC3 cells (Figure 4C). Densitometry analysis shows that p-Akt (S473) protein levels were reduced by 43% and 50% at 24 and 48 h after transfection, respectively. These data suggest that the exogenous expression of p27 can inhibit EGFR-mediated cell survival signals such as PI3K and Akt.

4 Discussion

Prostate cancer is one of the most common human malignancies, and so far, there is no effective treatment for the hormone-refractory stage of this disease. In this report we show that transfection of PC3 cells with a plasmid expressing *p27* (pcDNA3.1-*p27*) results in high levels of p27 expression. In PC3 cells, this marked increase in p27 levels inhibited growth, caused G1 cell cycle arrest and triggered apoptosis. Importantly, we observed that the EGFR/PI3K/Akt signalling pathway was inhibited by exogenous p27. These results suggest that p27 expression induces antitumour activity at least partly through the inhibition of EGFR signalling in PC3 cells.

p27 is a CDKI, and it has been shown to inhibit the kinase activity of cyclin A–Cdk2, cyclin B–Cdk2, cyclin D–Cdk4 and cyclin E–Cdk2 by preventing Cdk activation [11–13]. In prostate cancer, p27 expression progressively declines with increasing tumour grade and stage [14, 15]. p27 protein has also been shown to have a critical role in promoting apoptosis in breast, renal, lung and prostate cancer cell lines [5, 16–19]. Our findings are therefore in close agreement with those of Katner *et al*. [5], who found that p27 significantly reduced the proliferation of prostate cancer cell lines, causing a visible increase in cellular apoptosis and accumulation of cells at G_0/G_1.

To clarify further the mechanism by which p27

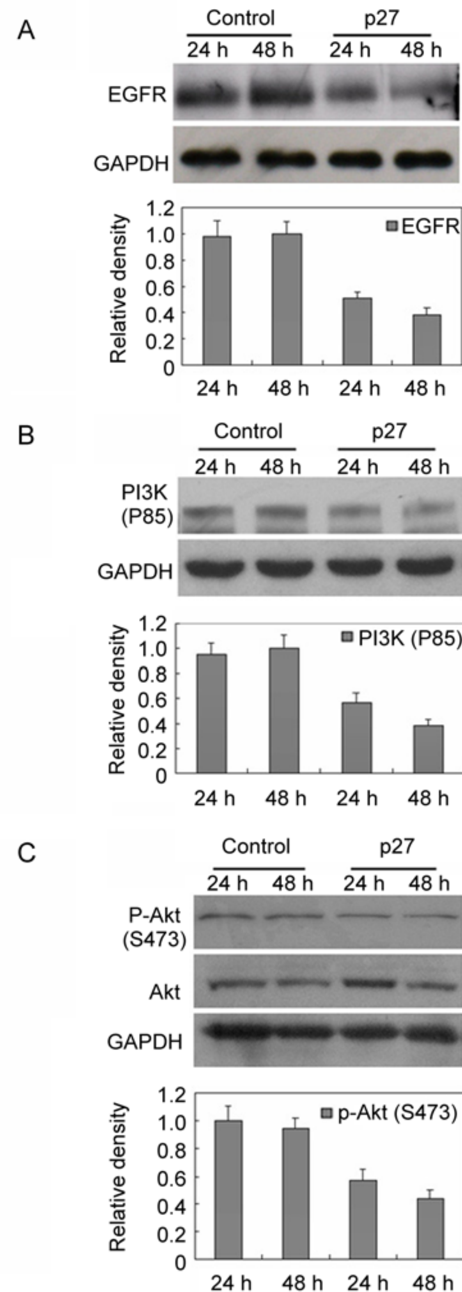

Figure 4. Effects of p27 on epidermal growth factor receptor (EGFR), PI3K (p85), Akt and p-Akt (S473) expression. PC3 cells were transiently transfected with control plasmid and *p27* and then harvested 24 and 48 h after transfection. Cell lysates were analysed for EGFR by Western blot using a specific antibody to EGFR (A), or analysed for PI3K phosphorylation using a specific antibody to PI3K (p85) (B), or analysed for Akt activity using antibodies to Akt (S473) and Akt (C). GAPDH was used a loading control. The western blots were quantified by densitometry using ImageQuant software (Amersham Biosciences, New Jersey, USA). The inhibitory effect of each treatment condition was plotted as a bar graph.

induces apoptosis in PC3 cells, we analysed the expression of apoptosis-related proteins after transfection. A number of pathways are known to lead to apoptosis or inhibition of cell cycle progression. The Bcl-2 family and caspase-3 are important regulators of apoptosis. Several studies have shown that elevated expression of Bcl-2 in prostate cancer cells confers androgen resistance, particularly in advanced disease, and it may facilitate progression to androgen independence [20–22]. Bcl-2 is part of an expanding family of apoptosis-regulatory molecules, which may act as either death antagonists (Bcl-2, Bcl-xl and Mcl-1) or death agonists (Bax, Bak, Bcl-xS, Bad and Bid). The selective and competitive dimerization between pairs of these antagonists and agonists determines how a cell will respond to a given signal [20]. These findings suggest that reducing the expression of the antiapoptotic gene bcl-2 may be a rational strategy for treating AIPC. In our study, we found that p27 induced higher expression levels of Bax and Bad. Conversely, Bcl-2 was markedly downregulated in the *p27* group, more than in the control group. Caspase activation leads to cleavage and inactivation of key cellular proteins, such as poly(ADP-ribose) polymerase [23]. Our results show that transfection of *p27* causes activation of caspase-3, and this coincides with cleavage of PARP. Based on the above information, it is suggested that p27-induced apoptosis occurs predominantly through the activation of intrinsic apoptosis pathways by causing the downregulation of Bcl-2 expression, upregulation of Bax and Bad, and activation of caspase-3 in PC3 cells.

Progression to hormone refractory disease is often associated with overexpression of growth factors and receptors. It has been suggested that upregulation of EGFR is involved in the development of AIPC [24–26]. In addition, the AIPC cell line PC3 was found to express higher levels of EGFR than the LNCaP cell line [1]. These findings provide additional evidence supporting the hypothesis that EGFR expression has a role in the development of AIPC. However, the molecular mechanisms responsible for the activation of EGFR signalling in prostate cancer cells are not clear. Several studies have revealed that inhibition of EGFR signalling, which inhibits the growth of androgen-responsive cells as well as androgen-independent cells, is accompanied by a blockade of the progression of cells from G_1 to S phase owing to the upregulation of p27 protein, which in turn inhibits the CDKs [6, 27]. Notably, experiments in cultured breast cancer cells have implicated p27 as an inhibitor of EGFR signalling [28]. In this study, we found that EGFR levels were lower after *p27* was transfected into PC3 cells. This result suggests that p27 protein acted as an inhibitor of EGFR. We believe that the likely mechanism behind this phenomenon is that p27 negatively regulates EGFR expression in PC3 cells. However, the exact molecular mechanisms require further investigation.

Activated EGFR may induce distinct mitotic cascades, including the MAPK, PI3K/Akt, Shc, NF-κB and phospholipase Cg signalling pathways, which stimulate the proliferation, survival, motility and invasiveness of prostate cancer cells [29–32]. Elevated Akt activity may have a profound role in the progression of human prostate cancer. Akt regulates many of the processes associated with metastatic progression and the emergence of AIPC cells, such as diminished apoptotic response [33] and release from the cell cycle control that follows androgen ablation [34]. Akt can dampen the normal apoptotic response by suppressing the activity of numerous pro-apoptotic proteins, including Bad, caspase-9 and the Forkhead family of transcription factors [35–37]. In this study, we investigated an EGFR-inhibited signalling pathway in *p27*-transfected PC3 cells. Our results suggest that Akt was inhibited by EGFR through inhibition of PI3K (p85) and subsequent phosphorylation of Akt at Ser473. We also found that the reduced Akt activity in these p27-PC3 cells corresponds to enhanced expression of the pro-apoptotic protein Bad. Bad promotes cell death by interacting with anti-apoptotic Bcl-2 members such as Bcl-2 and Bcl-xL, which allows the multidomain pro-apoptotic Bcl-2 family members Bax and Bak to aggregate and cause the release of apoptogenic molecules from the mitochondria to the cytosol, culminating in caspase activation and cell death [38, 39].

In summary, we showed that transfection of *p27* plasmid DNA into PC3 cells (which do not express this gene) inhibits their proliferation by causing cells to accumulate in the G_1 phase of the cell cycle and by inducing apoptosis. These effects are due, at least in part, to direct inhibition of the EGFR/PI3K/Akt signalling pathway. Therefore, our results provide a mechanistic explanation for the anti-tumour effects of exogenous p27 expression and, importantly, suggest that the *p27* gene and its regulated pathway are promising targets for AIPC treatment.

Acknowledgment

We thank Professor Rong-Zhen Xu for his expert technical assistance in performing the studies described here.

References

1 Sherwood ER, Van Dongen JL, Wood CG, Liao S, Kozlowski JM, *et al*. Epidermal growth factor receptor activation in androgen-independent but not androgen-stimulated growth of human prostatic carcinoma cells. Br J Cancer 1998; 77: 855–61.
2 Davies P, Eaton CL, France TD, Phillips ME. Growth factor receptors and oncogene expression in prostate cells. Am J Clin Oncol 1988; 11 (Suppl 2): S1–7.
3 Festuccia C, Gravina GL, Angelucci A, Millimaggi D, Muzi P, *et al*. Additive antitumor effects of the epidermal growth factor receptor tyrosine kinase inhibitor, gefitinib (Iressa), and the nonsteroidal antiandrogen, bicalutamide (Casodex), in prostate cancer cells *in vitro*. Int J Cancer 2005; 115: 630–40.
4 Eder IE, Bektic J, Haag P, Bartsch G, Klocker H. Genes differentially expressed in prostate cancer. BJU Int 2004; 93: 1151–5.
5 Katner AL, Hoang QB, Gootam P, Jaruga E, Ma Q, *et al*. Induction of cell cycle arrest and apoptosis in human prostate carcinoma cells by a recombinant adenovirus expressing p27 (Kip1). Prostate 2002; 53: 77–87.
6 Ye D, Mendelsohn J, Fan Z. Androgen and epidermal growth factor down-regulate cyclin-dependent kinase inhibitor p27Kip1 and costimulate proliferation of MDA PCa 2a and MDA PCa 2b prostate cancer cells. Clin Cancer Res 1999; 5: 2171–7.
7 Sgambato A, Camerini A, Faraglia B, Ardito R, Bianchino G, *et al*. Targeted inhibition of the epidermal growth factor receptor-tyrosine kinase by ZD1839 (‘Iressa’) induces cell-cycle arrest and inhibits proliferation in prostate cancer cells. J Cell Physiol 2004; 201: 97–105.
8 Grant S, Qiao L, Dent P. Roles of ERBB family receptor tyrosine kinases, and downstream signaling pathways, in the control of cell growth and survival. Front Biosci 2002; 7: d376–89.
9 Patel T, Gores GJ, Kaufmann SH. The role of proteases during apoptosis. FASEB J 1996; 10: 587–97.
10 Mimeault M, Batra SK. Recent advances on multiple tumorigenic cascades involved in prostatic cancer progression and targeting therapies. Carcinogenesis 2006; 27: 1–22.
11 Martinez LA, Chen Y, Fischer SM, Conti CJ. Coordinated changes in cell cycle machinery occur during keratinocyte terminal differentiation. Oncogene 1999; 18: 397–406.
12 Craig C, Wersto R, Kim M, Ohri E, Li Z, *et al*. A recombinant adenovirus expressing p27Kip1 induces cell cycle arrest and loss of cyclin-Cdk activity in human breast cancer cells. Oncogene 1997; 14: 2283–9.
13 Sgambato A, Zhang YJ, Ciaparrone M, Soh JW, Cittadini A, *et al*. Overexpression of p27Kip1 inhibits the growth of both normal and transformed human mammary epithelial cells. Cancer Res 1998; 58: 3448–54.
14 Tsihlias J, Kapusta LR, DeBoer G, Morava-Protzner I, Zbieranowski I, *et al*. Loss of cyclin-dependent kinase inhibitor p27Kip1 is a novel prognostic factor in localized human prostate adenocarcinoma. Cancer Res 1998; 58: 542–8.
15 Yang RM, Naitoh J, Murphy M, Wang HJ, Phillipson J, *et al*. Low p27 expression predicts poor disease-free survival in patients with prostate cancer. J Urol 1998; 159: 941–5.
16 Katner AL, Gootam P, Hoang QB, Gnarra JR, Rayford W. A recombinant adenovirus expressing p27(Kip1) induces cell cycle arrest and apoptosis in human 786–0 renal carcinoma cells. J Urol; 168: 766–73 .
17 Katayose Y, Kim M, Rakkar AN, Li Z, Cowan KH, *et al*. Promoting apoptosis: a novel activity associated with the cyclin-dependent kinase inhibitor p27. Cancer Res 1997; 57: 5441–5.
18 Hiromura K, Pippin JW, Fero ML, Roberts JM, Shankland SJ. Modulation of apoptosis by the cyclin-dependent kinase inhibitor p27(Kip1). J Clin Invest 1999; 103: 597–604.
19 Wang X, Gorospe M, Huang Y, Holbrook NJ. p27Kip1 overexpression causes apoptotic death of mammalian cells. Oncogene 1997; 15: 2991–7.
20 Raffo AJ, Perlman H, Chen MW, Day ML, Streitman JS, *et al*. Overexpression of bcl-2 protects cancer cells from apoptosis in vitro and confers resistance to androgen depletion in vivo. Cancer Res 1995; 55: 4438 –45.
21 Bauer JJ, Sesterhenn IA, Mostofi FK, McLeod DG, Srivastava S, *et al*. Elevated levels of apoptosis regulator proteins p53 and bcl-2 are independent prognostic biomarkers in surgically treated clinically localized prostate cancer. J Urol 1996; 156: 1511–6.
22 McDonnell TJ, Troncoso P, Brisbay SM, Logothetis C, Chung LW, *et al*. Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. Cancer Res 1992; 52: 6940–4.
23 Thornberry NA, Lazebnik Y. Caspase: enemies within. Science 1998; 281: 1312–6.
24 Kaplan PJ, Leav I, Greenwood J, Kwan PW, Ho SM. Involvement of transforming growth factor alpha (TGFalpha) and epidermal growth factor receptor (EGFR) in sex hormone-induced prostatic dysplasia and the growth of an androgen-independent transplantable carcinoma of the prostate. Carcinogenesis 1996; 17: 2571–79.
25 Gil-Diez de Medina S, Salomon L, Colombel M, Abbou CC, Bellot J, *et al*. Modulation of cytokeratin subtype, EGF receptor, and androgen receptor expression during progression of prostate cancer. Hum Pathol 1998; 29: 1005–12.
26 Di Lorenzo G, Tortora G, D’Armiento FP, De Rosa G, Staibano S, *et al*. Expression of epidermal growth factor receptor correlates with disease relapse and progression to androgen-independence in human prostate cancer. Clin Cancer Res 2002; 8: 3438–44.
27 Mimeault M, Pommery N, Wattez N, Bailly C, Henichart JP. Anti-proliferative and apoptotic effects of anandamide in human prostatic cancer cell lines: implication of epidermal growth factor receptor down-regulation and ceramide production. Prostate 2003; 56: 1–12.

28 Neve RM, Sutterluty H, Pullen N, Lane HA, Daly JM, *et al*. Effects of oncogenic ErbB2 on G1 cell cycle regulators in breast tumour cells. Oncogene 2000; 19: 1647–56.
29 Mimeault M, Pommery N, Henichart JP. New advances on prostate carcinogenesis and therapies: involvement of EGF-EGFR transduction system. Growth Factors 2003; 21: 1–14.
30 Torring N, Dagnaes-Hansen F, Sorensen BS, Nexo E, Hynes NE. ErbB1 and prostate cancer: ErbB1 activity is essential for androgen-induced proliferation and protection from the apoptotic effects of LY294002. Prostate 2003; 56: 142–9.
31 Mimeault M, Moore E, Moniaux N, Henichart JP, Depreux P, *et al*. Cytotoxic effects induced by a combination of cyclopamine and gefitinib, the selective hedgehog and epidermal growth factor receptor signaling inhibitors, in prostate cancer cells. Int J Cancer 2006; 118: 1022–31.
32 Bonaccorsi L, Marchiani S, Muratori M, Forti G, Baldi E. Gefitinib. ('IRESSA', ZD1839) inhibits EGF-induced invasion in prostate cancer cells by suppressing PI3K/AKT activation. J Cancer Res Clin Oncol 2004; 130: 604–14.
33 Isaacs JT. The biology of hormone refractory prostate cancer. Why does it develop? Urol Clin North Am 1999; 26: 263–73.
34 Agus DB, Cordon-Cardo C, Fox W, Drobnjak M, Koff A, *et al*. Prostste cancer cell cycle regulators: reponse to androgen withdrawal and development of androgen independence. J Natl Cancer Inst 1999; 91: 1869–76.
35 Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, *et al*. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 1999; 96: 857–68.
36 Kops GJ, de Ruiter ND, De Vries-Smits AM, Powell DR, Bos JL, *et al*. Direct control of the Forkhead transcription factor AFX by protein kinase B. Nature 1999; 398: 630–4.
37 Sastry KS, Smith AJ, Karpova Y, Datta SR, Kulik G. Diverse anti-apoptotic signaling pathways activated by VIP, EGF and PI3K in prostate cancer cells converge on BAD. J Biol Chem 2006; 281: 20891–901.
38 Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, *et al*. BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK- mediated mitochondrial apoptosis. Mol Cell 2001; 8: 70511.
39 Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, *et al*. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 2001; 292: 727–30.