# A mutant P53 can activate apoptosis through a mechanism distinct from those induced by wild type P53

Ming He[a], Paul S. Rennie[a,c], Visia Dragowska[b], Colleen C. Nelson[a,c], William Jia[b,c,*]

[a] *The Prostate Centre at Vancouver General Hospital, Vancouver, BC, Canada*
[b] *Department of Advanced Therapeutics, British Columbia Cancer Research Centre, Vancouver, BC, Canada*
[c] *Department of Surgery, University of British Columbia, Vancouver, BC, Canada*

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**Abstract A common mutation in P53 protein occurs at amino acid residue 281 in the DNA binding domain ($P53^{gly(281)}$), which results in loss of transcriptional regulation of P53 target genes and has been reported to gain pro-oncogenic functions. In the present study, we investigated the activity of $P53^{gly(281)}$ in P53-null PC3 human prostate cancer cells and found that the $P53^{gly(281)}$ induced apoptosis as efficiently as the wild-type P53 (wtP53). However, in contrast to wtP53-induced apoptosis, the $P53^{gly(281)}$-induced apoptosis was insensitive to overexpression of bcl-2. Thus, our findings indicate that while a mutation in the DNA binding domain of p53 may result in a more oncogenic form of the protein, it may also paradoxically result in the 'gain' of a new, alternative pathway for apoptosis.** © **2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

*Key words:* P53; PC3 cell; Apoptosis; Mutation

## 1. Introduction

*P53* is a well-characterized tumor suppressor gene and *p53* mutations are closely associated with most advanced malignant tumors [1,2]. Functions of P53 protein include growth arrest, differentiation and apoptosis, depending on the cellular context [3–9]. It is generally accepted that P53 induces cell arrest by increasing P21 at the transcriptional level [10,11]. However, the mechanism of P53-induced apoptosis may be more complicated. P53 can induce apoptosis by increasing the expression of *Fas* or *bax* genes through binding to the P53-specific binding sequence in the regulatory region of these genes [12]. However, some early work showed that P53-induced apoptosis may not necessarily be dependent on transcriptional activity since P53-dependent apoptosis could occur in the presence of inhibitors of transcription or translation [13,14]. Recently, it was reported that the transcription-deficient P53 with mutations at the 22–23 amino acid residues [15] are still capable of inducing apoptosis [16,17]. In contrast, others have shown that the same p53 mutant was unable to induce apoptosis [18–20]. Most recently, the results from studies of murine p53 gene mutations also supported the observation that transcriptional activity is required for P53-induced apoptosis [21,22].

The transactivation domain of P53 protein requires 42 amino acids at the amino terminus, especially residues 22 and 23, which interact with the basal transcriptional elements of target genes to regulate transcriptional machinery of the cell [15,23–28]. The sequence-specific DNA binding domain (the core DNA binding domain) is located in the middle of the protein between amino acids 102 and 292 and binds to the regulatory region of its target genes [29–32]. More recent studies have demonstrated that the C-terminus is involved in regulating both the DNA binding and apoptosis activities of P53 [33,34].

It is believed that P53 protein with mutations at certain 'hot-spots' such as amino acid residue 248 (Arg→Trp) or 281 (Asp→Gly) fail to bind to the P53-specific binding region of the target gene and lose the original transactivation function [12]. Interestingly, the $P53^{gly(281)}$ mutant has been known to have a gain of function phenomenon in a few P53-null cell lines. In particular, this mutant transcriptionally upregulates expression of certain genes such as multidrug resistant gene type-1 [35] and HIV long terminal repeat [36]. It also enhances the tumorigenic potential of some cells [35]. Thus, it is generally believed that this mutant P53 protein not only antagonizes wild type p53 (wtP53) to interfere with its normal functions, it is also more oncogenetic due to a gain of abnormal functions [37–39]. In the present study, we investigated this mutant in another P53-null cell line, the prostate cancer PC3 cells, and found that the $P53^{gly(281)}$ mutant possesses a gain of apoptotic pathway distinct from that of wtp53.

## 2. Materials and methods

### 2.1. Cell culture

The human prostate cancer PC3 cell line was obtained from American Type Culture Collection (Rockville, MD, USA). The cell line was maintained in RPMI 1640 medium (Gibco BRL, Burlington, ON, Canada) supplemented with 5% fetal bovine serum containing antibiotic mixture at 37°C under a humidified 5% $CO_2$ atmosphere.

### 2.2. DNA and transfection

Plasmids containing wtp53 or mutant p53 cDNA were kindly provided by Dr. Levine (Department of Molecular Biology, Princeton University). The plasmid $p53^{gly(281)}$ consists of mutant p53 cDNA at residue 281, changing aspartic acid to glycine, which is in the sequence-specific DNA binding domain. PC3 cells ($5\times10^5$) were seeded in 60 mm culture dishes the day before transfection. Cells were transfected with a total of 3.5 μg DNA plasmids purified with Qiagen maxi-preparation kit (Mississauga, ON, Canada)

### 2.3. Hypodiploid DNA apoptosis analysis

Samples containing $10^6$ of cells/60 mm dish were harvested following brief trypsin digestion (Gibco BRL, Burlington, ON, Canada).

*Corresponding author. Present address: Department of Ophthalmology, University of British Columbia, 2550 Willow Street, Vancouver, BC, Canada V5Z 3N9. Fax: (1)-604-875 4232.
*E-mail address:* wjia@interchange.ubc.ca (W. Jia).

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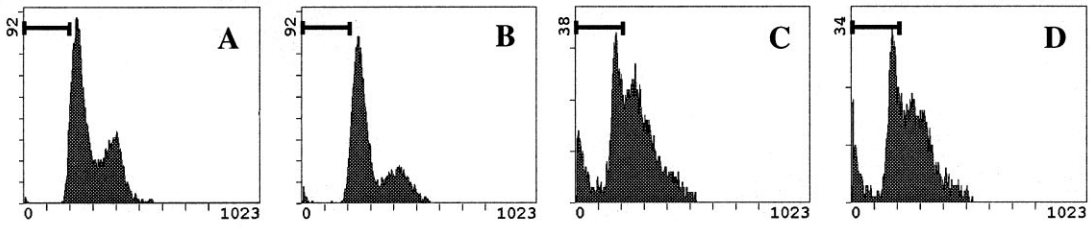


Fig. 1. Representative flow cytometry results for PC3 cells showing apoptotic effect following transfection with wtp53 or mutant p53. The subpopulation of apoptotic cells was represented by the area for cells with sub-diploid DNA content. The marker on the top of each plot represents the cut-off point for calculating percentage of this cell population. A: Untransfected; B: vector control; C: wtp53; D: $P53^{gly(281)}$.

The cells were fixed with 70% ice-cold ethanol on ice for 30 min followed by treatment with RNaseA (1 mg/ml) and propidium iodide (PI) (50 μg/ml). DNA analyses were determined using an Flow cytometer (Epics Elite ESP, Beckman Coulter) and a DNA content log-histogram was generated. [40,41].

### 2.4. Determination of Fas and FasL expression by flow cytometric analysis

The total amount of expression of Fas antigen in PC3 cells in total and that on the cell surface were determined by flow cytometry. For cell surface staining, the cells ($2\times10^5$) were pretreated with human IgG (ICN Immunobiologicals, Lisele, IL, USA, 50 μg/ml) for 10 min on ice, and then incubated 30 min on ice with R-Phycoerythrin (R-PE)-conjugated mouse anti-human Fas monoclonal antibody or R-PE-labeled mouse IgG1 monoclonal isotype control (Pharmingen, Canada), then washed twice and resuspended in PBS containing 1 μg/ml of PI (Sigma Chemical Co., St. Louis, MO, USA). For total staining, $2\times10^5$ cells were fixed in 2% paraformaldehyde for 15 min at room temperature and then incubated in 10% Tween-20 for another 15 min to permeabilize the cells. The staining procedure was the same as for surface staining, except fluorescein isothiocyanate (FITC)-conjugated mouse anti-human monoclonal antibody and FITC-labeled mouse IgG1 monoclonal isotype control (Pharmingen Canada) were used.

## 3. Results

### 3.1. $p53^{gly(281)}$ mutant can activate apoptosis in PC3 cells

*3.1.1. P53-induced apoptosis.* Expression of the P53 proteins was confirmed in transfected cells by Western blotting using a P53-specific monoclonal antibody that recognized both wild type and mutant P53 (data not shown). Flow cytometry results demonstrated a typical hypodiploid peak as an indication of apoptosis in cells transfected with either *wtp53* or $p53^{gly(281)}$, respectively (Fig. 1). Nearly one third of the cells ($30.8\%\pm6.1$, $n=6$) were apoptotic 48 h after *wtp53* transfection relative to the untreated control samples. Interestingly, transfection with $p53^{gly(281)}$ cDNA also resulted in apoptosis ($22.1\%\pm3.0$, $n=6$), which was at a level similar to that induced by *wtp53* ($P=0.124$, Student's *t*-test). To rule out the variation caused by difference in transfection efficiency among cell cultures, a construct carrying the gene of green fluorescent protein (GFP) was co-transfected with the above *p53* gene constructs. No significant difference in GFP expression was found with flow cytometry across all the cell cultures examined.

To confirm the apoptotic nature of the cell death, nuclear fragmentation was seen in cells stained with Hoechst 33342 under a fluorescent microscope 24–48 h after transfection (data not shown).

### 3.2. The $P53^{gly(281)}$ mutant was not able to transcriptionally upregulate Fas and BAX synthesis

To verify that the apoptotic activity seen in $P53^{gly(281)}$ was not through the same mechanism as wtP53 in PC3 cells, we first tested the ability of this mutant P53 to upregulate Fas or BAX expression in PC3 cells, since the two genes are the well-known targets that are transcriptionally regulated by wtP53.

*3.2.1. Fas expression in p53-transfected cells.* Transient expression of P53 into PC3 cells caused increased levels of Fas as measured by flow cytometry (Fig. 2a). To minimize variation caused by transfection efficiency, we co-transfected a

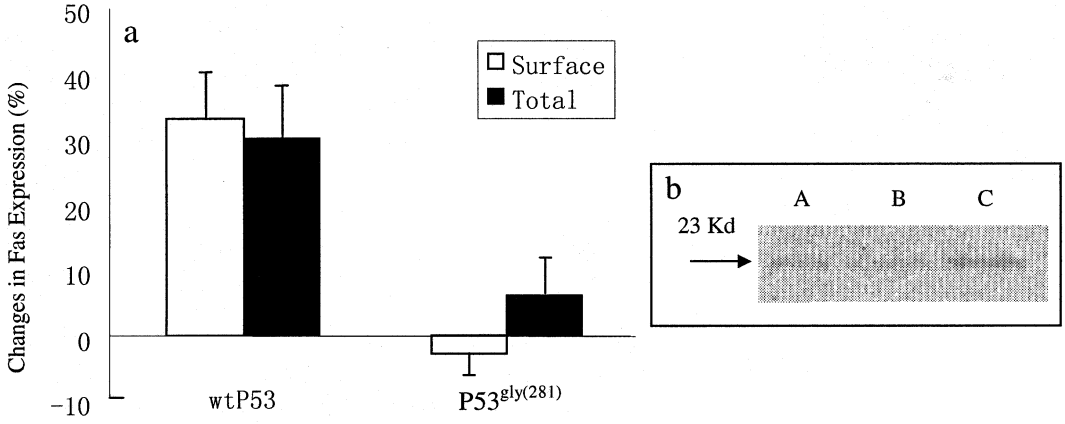


Fig. 2. a: Effects of P53 on Fas expression in PC3 cells. Cells were transfected with either *wtp53* or $p53^{gly(281)}$ mutant constructs and the Fas expression levels were measured with flow cytometry. The base line expression level was determined with cell cultures transfected with control plasmid pGL2 and the results shown are the mean differences (±S.E.M.) between the *p53*-transfected cells and control from two experiments. b: Effects of P53 on levels of Bax in PC3 cells. Cells were transfected with control vector or *wtp53* or $p53^{gly(281)}$ mutant constructs and the Bax expression levels were measured with Western blotting. A: control; B: $p53^{gly(281)}$ transfected; and C: *wtp53* transfected.

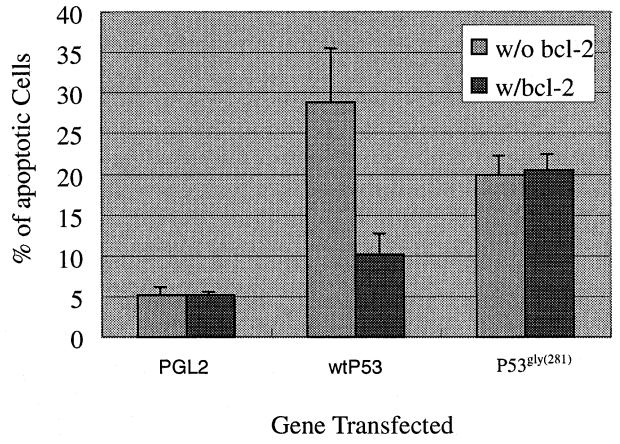


Fig. 3. Effects of *bcl-2* co-transfection on P53-induced apoptosis of PC3 cells. PC3 cells were co-transfected with either the *wtp53* or the $p53^{gly(281)}$ or control plasmid pGL2 and *bcl-2* gene constructs. Apoptosis was measured with flow cytometry 48 h later. Data points represent means (±S.D.) of three independent experiments each with duplicated samples.

GFP-expressing plasmid with the *wtp53* or $p53^{gly(281)}$. Only GFP-positive cells were gated for the measurement of Fas levels on the cell surface. As shown in Fig. 2a, after transfection of the *wtp53* gene, Fas expression was increased both in total and on the cell surface by 33 ± 7.1% and 30 ± 8.0%, respectively ($n = 4$, $P < 0.005$). Since the amount of increased Fas expression on the cell surface was similar to that in the total (Fig. 2a), the increased Fas antigen on the cell surface following the expression of wtP53 was likely due to increased newly synthesized Fas rather than redistribution due to a translocation mechanism. Thus, the increased Fas level on the cell surface was mainly caused by enhanced transcriptional activity of fas gene regulated by the transfected *p53*. In contrast, transfection with the $p53^{gly(281)}$ had no effect on the Fas expression in PC3 cells ($n = 4$, $P > 0.05$) (Fig. 2a).

Results of P53-induced Bax expression were shown in Fig. 2b. While the level of Bax was substantially upregulated by transfected *wtp53* at 24 h, Bax level in cells transfected with $p53^{gly(281)}$ remained low.

In separate experiments (data not shown), we also found that in PC3 cells, transfection with $P53^{gly(281)}$ could not induce *p21* gene expression nor G1 arrest, which are other hallmark measures for the normal function of P53 [44]. It is thus apparent that the mutation in the DNA binding domain in $P53^{gly(281)}$ disabled P53 function of transcriptional regulation in these cells.

### 3.3. *Bcl-2 can inhibit wtP53 but not* $P53^{gly(281)}$ *activation of apoptosis*

As Fas ligand was not required in wtP53-induced apoptosis of PC3 cells, it was apparent that the Fas pathway was not involved. Another possible P53-induced apoptotic pathway is through activating Bax [42,43]. Since Bcl-2 can form heterodimers with Bax, overexpression of Bcl-2 can block apoptosis induced by Bax. We examined the effect of Bcl-2 by co-transfecting a *bcl-2* cDNA with the above p53 genes. As shown in Fig. 3, co-transfection with Bcl-2 significantly reduced the apoptosis induced by wtP53 ($P < 0.05$, *t*-test). This suggested that the P53-induced apoptosis in PC3 cells was mediated primarily through the Bax/Bcl-2 pathway. In contrast, overexpression of Bcl-2 had no significant effect on the apoptosis induced by the $p53^{gly(281)}$ ($P > 0.05$). In addition, transfection with $p53^{gly(281)}$ did not induce upregulation of Bax levels in PC3 cells examined by Western blotting (Fig. 2b). This indicated that the mutant non-transcriptionally active form of P53 had acquired a mechanism distinct from that of wtP53 to elicit an apoptotic response.

## 4. Discussion

In the present study, we demonstrated that, despite the lack of P53-specific transactivation function, the $P53^{gly(281)}$ mutant was able to induce apoptosis to an extent similar to that seen with wtP53 (Fig. 1). To distinguish the mechanism for the mutant P53-induced apoptosis from that of wtP53, we first confirmed that the $P53^{gly(281)}$ had lost P53-specific transactivation activity in PC3 cells by demonstrating that $P53^{gly(281)}$ failed to induce Fas expression, which is a known transcriptionally regulated target gene of wtP53. Although overexpression of Bcl-2 blocked wtP53-induced apoptosis to near control levels, it had no effect on the activity of the $P53^{gly(281)}$ mutant.

The reported effects of gain of function for $P53^{gly(281)}$ in transfected cells have been to cause the cells to become either more oncogenic or more resistant to apoptosis [37–39]. This is the first time to our knowledge that an apoptotic function of the $p53^{gly(281)}$ has been demonstrated. Based on the observations that $P53^{gly(281)}$ has lost the ability to bind to a P53-specific DNA binding sequence [45] and that the $P53^{gly(281)}$-induced apoptosis was insensitive to Bcl-2, we speculate that $P53^{gly(281)}$ may activate another apoptotic pathway distinct from the mechanisms of wtP53. Thus, the phenomenon of $P53^{gly(281)}$-induced apoptosis is essentially a 'gain of pathway', which may act through binding directly to the regulatory region of a new target gene or by interacting with a new target protein. Recently, it has been suggested that the C-terminal basic domain and parts of the N-terminal, including the proline-rich domain, are required for apoptotic activity of P53 [33,34,46]. It will be interesting to investigate the roles of these regions in $P53^{gly(281)}$ in the induction of apoptosis in PC3 cells. The results reported in the present study indicate the complexity of P53 mutations, where a mutant can be anti-apoptotic in some cancer cells while pro-apoptotic in others.

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