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# PIG11 protein binds to DNA in sequence-independent manner *in vitro*

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**Abstract**

PIG11 (p53-induced protein 11), one of early transcriptional targets of tumor suppressor p53, was up-regulated in the induction of apoptosis or cell growth inhibition by multiple chemopreventive agents. However, its biological role remains unclear. Here, we expressed $His_6$-tagged PIG11 protein in *Escherichia coli* and demonstrated the recombinant $His_6$-tagged PIG11 protein could bind to supercoiled and relaxed closed circular plasmid DNA or linear DNA with different length using gel retardation assays *in vitro*. The interaction between DNA and PIG11 protein was sequence-independent and related to charge effect. The reducing thiol group in PIG11 protein was involved in the binding activity of PIG11 to DNA. Furthermore, the images of atomic force microscopy directly confirmed the binding of DNA and PIG11 protein and showed the PIG11–DNA complex formed a beads-on-a-string appearance in which PIG11 protein associated with DNA as polymer. These findings suggest that PIG11 protein may play an important role by interaction with other biological molecules in the regulation of apoptosis and provided us a novel angel of view to explore the possible function of PIG11 *in vivo*.


*Keywords:* PIG11 protein; DNA binding; Sequence-independent; Gel retardation assay; Atomic force microscopy

PIG11 (p53-induced protein 11) is one of tumor suppressor p53-induced protein (PIGs), first reported and named by Polyak et al. [1], which are early transcriptional targets of p53 detected by SAGE technique after overexpression of p53 and before the onset of morphological signs of apoptosis. Recent investigations suggested PIG11 may be one of candidate tumor suppressor gene from chromosome 11p11.2 [2] and was up-regulated in the induction of apoptosis or cell growth inhibition by multiple chemopreventive agents. The expression of PIG11 was lost or decreased in human liver tumor HepG2 and Hep3B cells [2]. And PIG11 was down-regulated in ovarian endometrial cysts compared to the corresponding eutopic endometrial tissues [3]. PIG11 was one of apoptotic genes induced by curcumin in human breast cancer MCF-7 cell [4]. Chiba et al. also found PIG11 exhibited substantial induction in trichostatin A-induced cell growth inhibition on multiple hepatoma cell lines [5]. Our previous studies demonstrated PIG11 was up-regulated markedly in arsenic trioxide-induced apoptosis of MGC-803 cells and overexpression of PIG11 could induce cell apoptosis in a low level and enhanced the apoptotic effects of arsenic trioxide in HEK293 cells [6,7]. However, the biological role of PIG11 protein product in apoptosis remains to be determined.

It is interesting that analysis for sequences homology of PIG11 by BLAST demonstrated that similar sequences had not been found, and no domain, repeats, motifs or features could be predicted with confidence using SMART analysis [7]. These suggested PIG11 may be a member of a novel gene family involved in the regulation of apoptosis and may play its role by interaction with other biological molecules, such as DNA, RNA, protein, etc. As one of p53-induced genes, we assumed whether PIG11 could bind to DNA like some apoptosis-related protein with sequence-independent DNA-binding activity, such as p53 [8], PRG3 (p53-responsive gene 3) [9], AIF (apoptosis-inducing factor) [10], etc.

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In this work, we expressed $His_6$-tagged PIG11 protein in *Escherichia coli* and demonstrated the recombinant $His_6$-tagged PIG11 protein can bind to double-stranded DNA from different source in a sequence-independent manner using gel retardation assays *in vitro*. Furthermore, we employed atomic force microscopy (AFM) to observe and confirm the interaction between DNA and PIG11 protein.

## Materials and methods

*Construction of recombinant PIG11 protein prokaryotic expressing vector.* The sequence coding PIG11 (GenBank/EMBL/DDBJ, Accession No. BC003010.1) was amplified by polymerase chain reaction (PCR) from the recombinant GFP–PIG11 expression vector [7]. For recombinant expression in *E. coli*, *Bam*HI (5′-end) and *Hin*dIII (3′-end) were added during PCR amplification, and the corresponding amplification products were subcloned in the pET30a vector (Novagen, UK) which contains a 6× His tag at N-terminus. The DNA sequence of construct was confirmed by sequencing with ABI 377 automated fluorescence-based sequencer (Shanghai Gene Core Biotechnologies Co., Ltd., China).

*Expression and purification of $His_6$-tagged PIG11 protein.* The PIG11 prokaryotic expression vector, named pETPIG11, was transformed into *E. coli* BL21 (DE3) cells. Transformers were grown in LB medium containing 30 μg/ml kanamycin overnight at 37 °C for small-scale culture. The overnight culture (10 ml) was transferred to 1 l of the same medium as above and the bacteria were grown in a rotatory shaker to mid-logarithmic phase ($A_{600} = 0.6$) at 37 °C. Protein production was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 3 h. Cells were harvested by centrifugation at 4000*g* for 30 min at 4 °C, and then resuspended in 40 ml of Buffer A (20 mM Tris–HCl, pH 8.0, 500 mM NaCl) and frozen at −80 °C until use. To purify $His_6$-tagged PIG11 protein, the frozen cell pellets were thawed at 37 °C followed by ultrasonication on ice for 15 × 10 s to break the cells. The precipitation was recovered after the lysed bacteria were centrifuged at 16,000*g* for 30 min at 4 °C. The pellet was resuspended in 30 ml Buffer B (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 2 M urea, 2% Triton X-100) and sonicated on ice for 4 × 10 s and centrifuged at 16,000*g* for 15 min at 4 °C. After a second round of urea wash as above, the pellet was resuspended in 30 ml Buffer C (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 6 M guanidine hydrochloride, 2 mM 2-mercaptoethanol) and stirred for 1–2 h in room temperature. The homogenate was centrifuged at 16,000*g* for 20 min at 4 °C to remove insoluble materials. The solubilized proteins were subjected to metal chelate affinity chromatography using Chelating Sepharose Fast Flow (Amersham Biosciene) column which was charged with $Ni^{2+}$ according to manufacturer's instruction. After the column (5 ml) was equilibrated with Buffer C, the protein sample was loaded, and the column was washed with Buffer C, then changed the washing buffer to Buffer D (20 mM Tris–HCl, pH8.0, 500 mM NaCl, 6 M urea, 2 mM 2-mercaptoethanol). Protein folding was performed by the use of a linear gradient from 6 to 0 M urea at a flow rate of 0.5 ml/min in a total volume of 40 column volumes. The recombinant protein was eluted by increasing the imidazole concentration from 40 to 500 mM. The protein samples were analyzed by using 15% SDS–PAGE and visualized by staining with Coomassie brilliant blue R250 (Sigma).

*Agarose gel retardation assays.* The linear dsDNA with different length (λDNA, 1141 and 378 bp) were incubated with PIG11 protein at different PIG11/DNA molar ratios in 20 mM Tris–HCl (pH 8.0) at room temperature for 30 min. To examine whether interaction of PIG11 with DNA was related to charge effect and the redox state of cysteine residues in PIG11 protein respectively, different amounts of NaCl (500, 750, 1000, and 1250 mM) and DTT (0.1, 1, 5, 10, and 100 mM) were, respectively, added to the mixture of PIG11 and DNA fragment and incubated at room temperature for 30 min. All samples were loaded onto agarose gel in 0.5× TBE buffer after mixing with reaction product loading buffer (10×) containing 60% 0.25× TBE buffer and 40% glycerol or free DNA control loading buffer (10×) containing 60% 0.25× TBE buffer, 40% glycerol, and 0.2% (w/v) brompherol blue and electrophoresed at 80 V for approximately 0.5–1 h.

*Atomic force microscopy (AFM).* For pure DNA or protein experiments, samples were diluted to desired concentration in water with 5 mM $MgCl_2$, which was added to enhance the adsorption of DNA to mica surface. For the binding experiment, PIG11 protein (0.5 μg/ml final concentration) was mixed with λDNA (5 μg/ml final concentration) in the buffer containing 20 μM Tris–HCl (pH 8.0), 0.5 mM NaCl, and 5 mM $MgCl_2$. The mixture was kept at room temperature for 5 min. A 10-μl drop of sample was pipetted onto freshly cleaved mica, and kept in air for 3 min at room temperature. The mica was wash with ultrapure water for 3 times to remove unbound DNA and/or protein. After drying the surface with a gentle stream of $N_2$, the sample was applied to AFM imaging. A multimode SPM (NanoScope IIIa Multi-Mode AFM, Veeco Instruments, USA) operated in tapping mode was employed. The AFM images were initially scanned at a 5 μm scan size with a scan rate of 1–2 Hz and height of 5 or 20 nm and then zoomed in as necessary. The capture images were analyzed using NanoScope version 5.12b48 Software. The height of the particles bound to DNA was measured using cross section analysis. For each assay, the height of particles were averaged and given a calculated standard deviation.

## Results

### *Expression and purification of $His_6$-tagged PIG11 protein*

Expression of the $His_6$-tagged PIG11 gene in *E. coli* was analyzed by SDS–PAGE. Fig. 1 showed the 15% SDS–PAGE pattern of the cell lysate from *E. coli* cells harboring mock vector and cells harboring pETPIG11 before and after induction with IPTG for 3 h. A very intensely staining protein band can be observing in the induced total cell lysate from cells harboring pETPIG11 (Fig. 1, lane 4), but not cells harboring mock vector and uninduced cells harboring pETPIG11 (Fig. 1, lanes 1–3). The result indicated the majority of the overexpressed recombinant

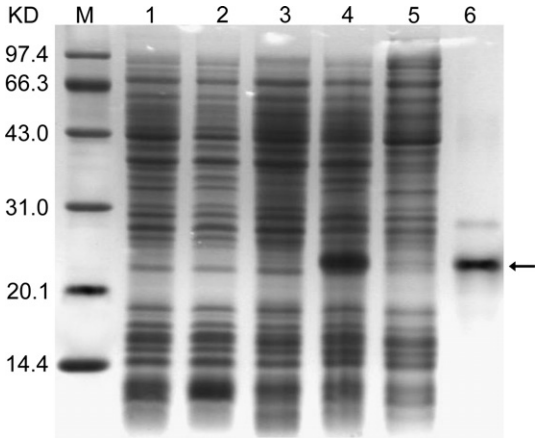


Fig. 1. SDS–PAGE analysis of the purification of $His_6$-tagged PIG11 protein. Proteins were separated on 15% acrylamide gel and stained with Coomassie brilliant blue R250. Lane M, molecular mass markers; lane 1, total protein extract of the cells carrying mock vector before IPTG induction; lane 2, total protein extract of the cells carrying mock vector after IPTG induction; lane3, total protein extract of the cells carrying the vector with the insert before IPTG induction; lane 4, total protein extract of the cells carrying the vector with the insert after 1 mM IPTG induction for 3 h; lane 5, supernatant of the induced cell carrying the vector with the insert after lysis; lane 6, major peak of the chelating affinity chromatography after renaturation. The arrow indicates PIG11 protein.

$His_6$-tagged pig11 protein was insoluble, which was almost not expressed in solube supernatant (Fig. 1, lane 5). After purification and renaturation on metal chelate affinity chromatography charged with $Ni^{2+}$, a nearly 90% purified protein was obtained (Fig. 1, lane 6). The apparent molecular weight was about 22 kDa.

*PIG11 protein binds to DNA in a sequence-independent manner*

To detect whether PIG11 protein interact with DNA, the gel retardation assay was used. The double-stranded DNA with different length was retarded in agarose gel, with the increasing molar ratio of PIG11/DNA (Fig. 2A, panels 1–3), while no retardation was detected in the gel when dsDNA was incubated with BSA (Fig. 2A, panel 4) at different BSA/DNA molar ratios. Similar result was obtained when PIG11 protein was incubated with plasmid pcDNA3.1/NT-GFP (Fig. 2B). The results suggested PIG11 interacted with dsDNA, either supercoiled, relaxed closed circular or linear DNA with different length, and the binding might not be a sequence-dependent manner because of no obvious sequence similarity among the dsDNA and high PIG11/DNA molar ratio.

*Effects of NaCl and DTT on the interaction of PIG11 protein with DNA*

As shown in Fig. 2C, the reduction of PIG11–DNA complex in agarose gel was observed by incubating

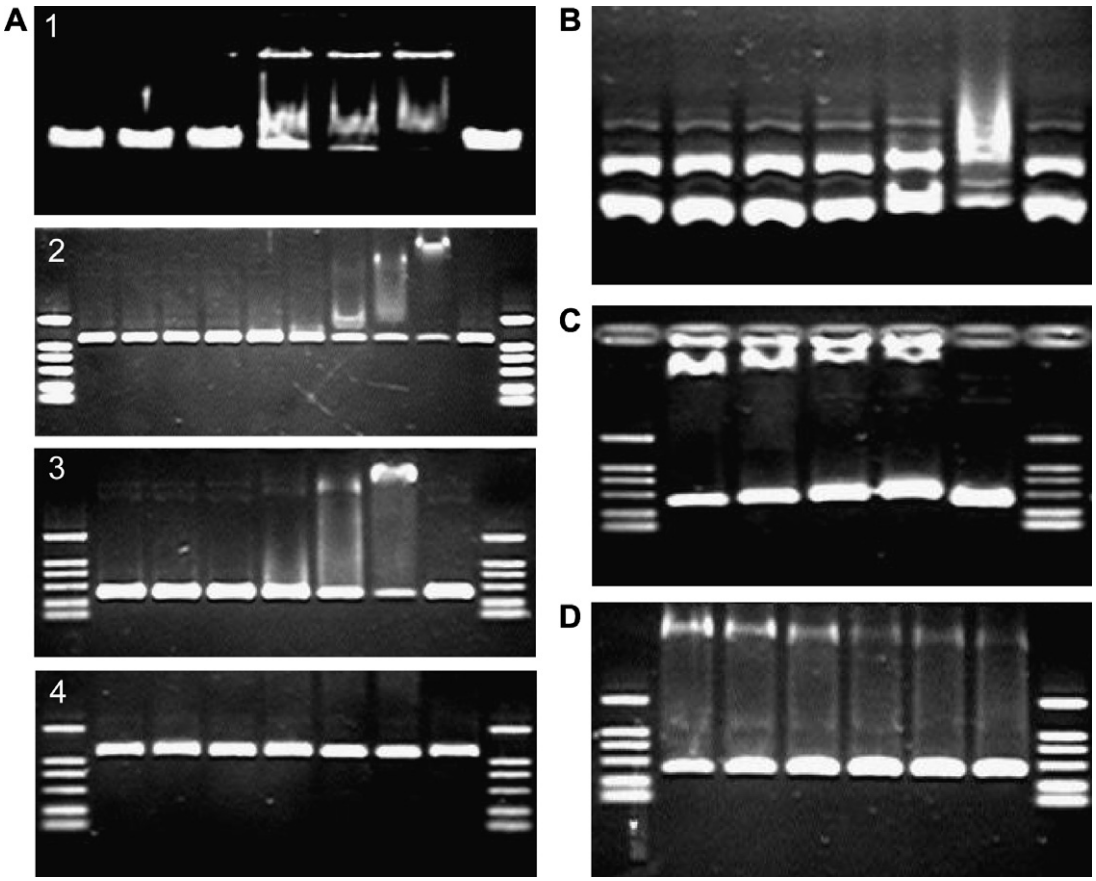


Fig. 2. Gel retardation assay of protein–DNA binding. Recombinant $His_6$-tagged PIG11 protein or BSA was incubated with dsDNA at different protein/DNA molar ratios in 20 mM Tris–HCl (pH 8.0) at room temperature for 30 min. Aliquots were then electrophoresed in agarose gel and stained with ethidium bromide. DNA molecular marker is DL2000. (A) Interaction of recombinant $His_6$-tagged PIG11 protein or BSA with linear dsDNA. 1, interaction of recombinant $His_6$-tagged PIG11 protein with λDNA. Lanes from left to right represent different PIG11/DNA molar ratios (0, 100, 1000, 10,000, 20,000, 40,000, and 0, respectively); 2, interaction of recombinant $His_6$-tagged PIG11 protein with dsDNA fragment with 1141 bp. Lanes from left to right represent different PIG11/DNA molar ratios (0, 10, 20, 100, 200, 500, 1000, 2000, 5000, and 0, respectively); 3, interaction of recombinant $His_6$-tagged PIG11 protein with dsDNA fragment with 378 bp. Lanes from left to right represent different PIG11/DNA molar ratios (0, 1, 10, 100, 200, 500, and 0, respectively); 4, interaction of BSA with dsDNA fragment with 1141 bp. Lanes from left to right represent different BSA/DNA molar ratios (0, 10, 100, 1000, 3000, 10,000, and 0, respectively). (B) Interaction of recombinant $His_6$-tagged PIG11 protein with plasmid pcDNA3.1/NT-GFP. Lanes from left to right represent different PIG11/DNA molar ratios (0, 10, 100, 1000, 2000, 6000, and 0, respectively). (C) Interaction of recombinant $His_6$-tagged PIG11 protein with dsDNA fragment with 378 bp in the presence of different concentrations of NaCl. Lanes from left to right represent PIG11 protein with 378-bp dsDNA fragment (molar ratio 500:1) in the presence of different concentrations of NaCl (500, 750, 1000, and 1250 mM, as final concentration, respectively). Lane 6 is DNA without protein. (D) Interaction of recombinant $His_6$-tagged PIG11 protein with dsDNA fragment with 378 bp in the presence of different concentrations of DTT. Lanes from left to right represent PIG11 protein with 378-bp dsDNA fragment (molar ratio 500:1) in the presence of different concentrations of DTT (0, 0.1, 1, 5, 10, and 100 mM, as final concentration, respectively).

PIG11 protein with dsDNA at different NaCl concentration, ranging from 500 to 1250 mM. The interaction was not totally inhibited even at 1250 mM of NaCl. It suggested the interaction of PIG11 protein with DNA was related to charge effect.

The effect of the reducing reagent DTT on the interaction of PIG11 protein with dsDNA was detected by incubating PIG11 protein with dsDNA at different concentration of DTT, ranging from 0.1 to 100 mM. A little PIG11–DNA complex was detected at a very high concentration of DTT (100 mM), as shown in Fig. 2D. Therefore, the reducing thiol groups in PIG11 were involved in the binding activity of PIG11 protein to DNA.

*PIG11 protein binds to DNA as polymer*

The binding of DNA and PIG11 protein was directly confirmed by atomic force microscopy (AFM). As shown in Fig. 3A and B, λDNA appeared in strings on the mica surface, while pure PIG11 protein exhibited ellipsoid-like structure. The average height of λDNA was about $0.62 \pm 0.28$ nm (Fig. 3A, panel 3), which suggested λDNA was composed of double strands. The average height of PIG11 protein was about $3.74 \pm 0.58$ nm (Fig. 3B, panel 3), and PIG11 protein automatically assembled larger particle with the protein concentration increasing. However, when PIG11 protein was incubated with λDNA, the

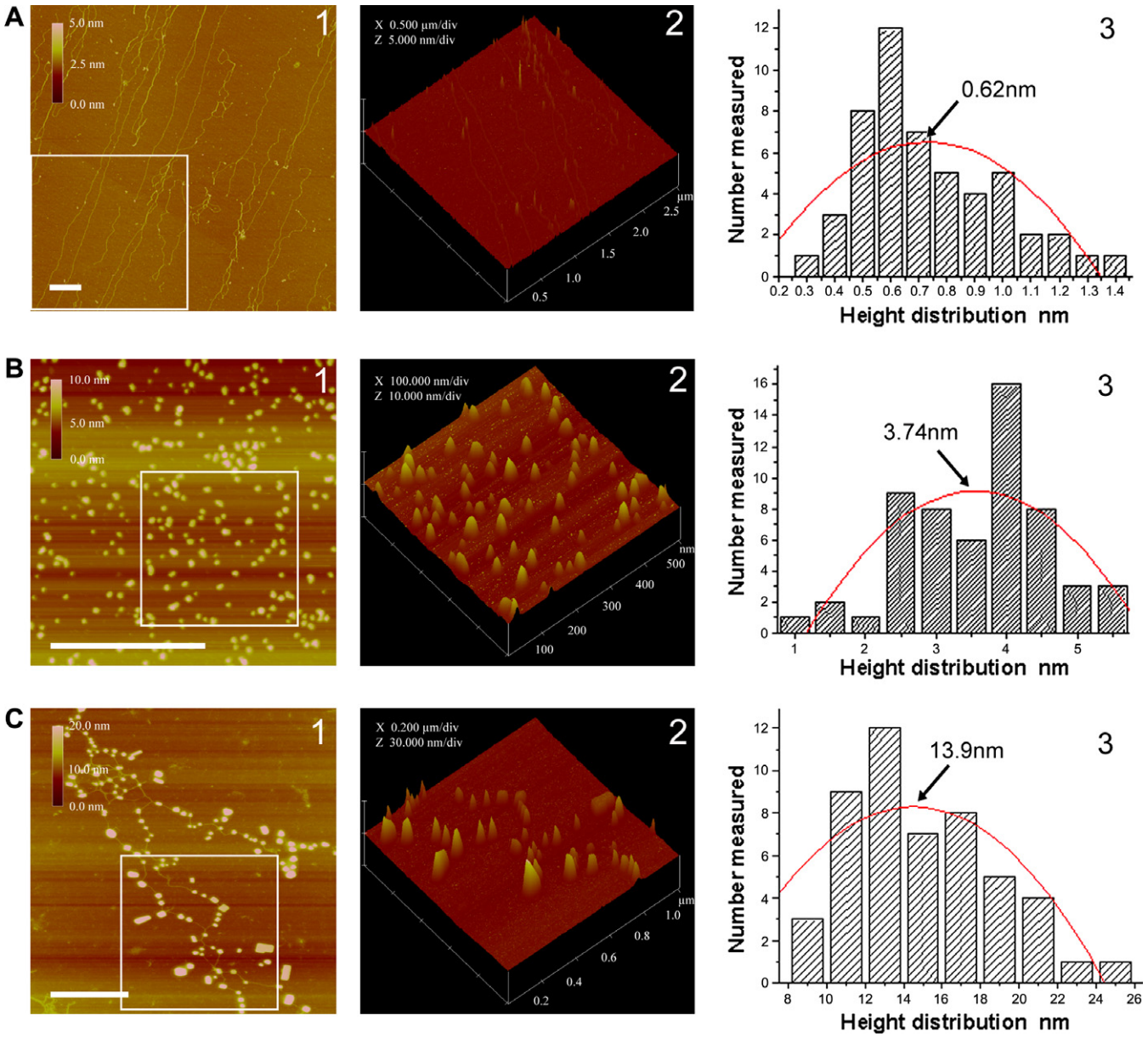


Fig. 3. Visualization of PIG11–λDNA complex by atomic force microscopy. For the binding experiment, λDNA (5 μg/ml final concentration) was incubated with PIG11 protein (0.5 μg/ml final concentration). (A) AFM image of λDNA alone. 1, AFM image of λDNA; 2, the frame in 1 is displayed as 3D surface plot; 3, the height distribution of sample 1. (B) AFM image of PIG11 protein alone. 1, AFM image of PIG11 protein; 2, the frame in 1 is displayed as 3D surface plot; 3, the height distribution of sample 1. (C) AFM image of PIG11–λDNA complex. 1, AFM image of PIG11–λDNA complex; 2, the frame in 1 is displayed as 3D surface plot; 3, the height distribution of sample 1. Size bars correspond to 500 nm.

resultant complex had a beads-on-a-string appearance. The average height of the bound PIG11 protein became $13.9 \pm 2.98$ nm (Fig. 3C, panel 3), suggesting that PIG11 protein could be polymers binding to λDNA and each bound PIG11 particle probably bound 15-bp nucleotide.

## Discussion

PIG11 is one of 13 genes induced by p53 using the serial analysis of gene expression technique, as demonstrated by the analysis of the p53-transfected colon carcinoma cell line DLD-1 [1]. PIG11 protein is consisted of 121 amino acids. PSORT II prediction indicated possibility of its located nucleus is 43.5%, cytoplasma is 34.8%, mitochondrion is 13.0%, and cytoskeleton is 8.7% [7]. Although we demonstrated the majority of GFP–PIG11 fusion protein was present in the cytoplasm of PIG11-transfected HEK293 cells, GFP–PIG11 partly distributed in the nucleus in other PIG11-transfected cells (data not shown). Therefore, the assumption was proposed that PIG11 may play its role by interacting with DNA in the nuclei. We overexpressed and purified $His_6$-tagged PIG11 protein in *E. coli.* From SDS–PAGE assay, the apparent molecular weight (about 22 kDa) is obviously larger than predicted molecular weight (18.3 kDa, including adapter and hexahistidine amino acid residues), which is probably due to the stretch and loose conformation or structure of $His_6$-tagged PIG11 protein. The expressed insoluble protein was solubilized with guanidine hydrochloride, purified, and refolded by one-step immobilized metal–ion affinity chromatography. The purified PIG11 protein exhibited DNA-binding activity with supercoiled, relaxed closed circular plasmid DNA or linear DNA with different length by gel retardation assays. Although, PIG11 protein binds to different DNA at different PIG11/DNA molar ratios, PIG11 protein binds to them at similar PIG11/DNA (mole/bp) ratios (about 1/5–1/1) considering the difference of DNA length. The high molar ratio of PIG11 protein binding to DNA showed the binding might not be sequence specific [11]. On the other hand, little specificity in the association with the nucleotide sequence was also suggested since the increasing concentration of NaCl affected the binding of PIG11 and DNA. Furthermore, the effect of NaCl on the interaction between PIG11 and DNA demonstrated PIG11 protein may interact with DNA partly by charge effect. It was confirmed that the reducing thiol groups in PIG11 were involved in binding activity of PIG11 to DNA by increasing the reducing reagent DTT concentration to interfere with the amount of PIG11–protein complex. This was consistent with PIG11 protein containing up to seven cysteine residues (7 Cys/121 a.a.). In addition, the N-terminal $His_6$ affinity tag sequence was not involved in the DNA-binding activity of recombinant PIG11 protein because of no DNA-binding activity of other unrelated recombinant $His_6$-tagged protein as control protein validated by gel retardation assays (data not shown).

Atomic force microscopy is commonly used to study the interaction of protein with DNA [12–15]. The remarkable advantage of AFM over conventional techniques is that it allows a direct visualization of the protein–DNA complex on a nanometer scale. It does not require the sample to be stained, shadowed or labeled. We employed AFM to observe the interaction of PIG11 protein with λDNA. When PIG11 was incubated with λDNA, the resultant complex has a beads-on-a-string appearance. Qu et al. observed similar appearance of tau–λDNA complex, in which tau bound to λDNA in a sequence-nonspecific manner, at low tau/DNA mass ratio [16]. The average height of the bound PIG11 protein became much larger compared to the average height of PIG11 protein alone. This suggested that PIG11 could be polymers binding to λDNA. AFM analysis indicated each bound PIG11 probably bound 15-bp nucleotide, which was basically consistent with gel retardation assay that PIG11 protein binds to different DNA at similar PIG11/DNA (mole/bp) ratios (about 1/5–1/1). This further validated PIG11 protein associated with λDNA as polymer.

PIG11 may be one of candidate tumor suppressor gene and is involved in the induction of apoptosis or cell growth inhibition by multiple chemopreventive agents. Our present work implicated PIG11 might play an important biological role by association with DNA in nuclei. Recent investigations found another p53 responsive gene, PRG3 (p53-responsive gene 3), also known as AMID (apoptosis-inducing factor-homologous mitochondrion-associated inducer of death), is a DNA-binding protein that lacks apparent DNA sequence specificity [9]. It also has a potential role in tumor suppression since AMID mRNA levels were down-regulated in tumor tissues compared with matched human normal tissues [17]. AIF (apoptosis-inducing factor), whose amino acid sequence is similar (22% identity) to AMID, translocated to nucleus where it interacts with DNA in a sequence-independent manner in the induction of apoptosis [10]. The physical interaction of AIF with DNA contributes to the compaction of nucleio acid within the apoptotic cells. The DNA-binding activity of AIF is necessary for the apoptosis [18]. When released added to purified nuclei, AIF induced chromatin condensation and large-scale DNA fragmentation to ~50 kb in caspase-independent fashion [19]. It needs to be further investigated whether the DNA-binding activity of PIG11 protein is required for the apoptogenic action. Immunohistochemistry staining using polyclonal PIG11 rabbit antiserum showed many brown-stained particles visualized in the nuclei of chondrosarcoma cells (data unpublished). Since PIG11 does not contain a nuclear localization signal, whether and how PIG11 entered into the nuclei remains unclear.

In conclusion, we demonstrated PIG11 could bind to dsDNA in sequence-independent manner *in vitro*. These results suggested PIG11 protein may play an important role in the nuclei and provided us a novel angel of view to explore the possible function of PIG11 *in vivo*.

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