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A P53 target gene, PIG11, contributes to chemosensitivity of cells to arsenic trioxide

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Abstract The tumor suppressor p53 regulates the expression of various genes that promote apoptosis. PIG11 (P53-induced gene 11), also referred to as TP53I11 (tumor protein p53 inducible protein 11), is a direct p53 target gene. Recent data demonstrated that PIG11 was up-regulated markedly in arsenic trioxide induced apoptosis by DDRT-PCR, suggesting a new class of p53 target genes that sensitize cells to the effects of chemotherapeutic agents. In this study, through the construction of a recombinant GFP-PIG11 expression vector and transfection of HEK293 cells with GFP or GFP-PIG11, the role of PIG11 in apoptosis was analyzed. Results demonstrated that the percentage (11.38%) of apoptotic cells with GFP-PIG11 transfection was higher than that (7.28%) of with only GFP transfection ($P < 0.05$). At 24 h after 1 μM of arsenic trioxide treatment, apoptotic cells exhibited a significant increase in the expression of GFP-PIG11 (36.67% ± 2.78), in contrast, 10.50% ± 2.03 only GFP and 5.25% ± 0.96 vehicle control ($P < 0.01$). In addition, we showed that intracellular content of reactive oxygen species (ROS) was 9.66 ± 0.52 in GFP-PIG11 transfection, higher than 5.21 ± 0.08 in GFP only and 5.99 ± 0.45 in vehicle control ($P < 0.01$). The above results suggest that overexpression of PIG11 could induce cell apoptosis in the low levels and enhanced the apoptotic effects of arsenic trioxide. The process could be involved in intracellular generation of ROS.
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Keywords: p53 target gene; PIG11 (P53-induced gene 11); Overexpression of PIG11 gene; Apoptosis; Arsenic trioxide (As_2O_3); Reactive oxygen species

1. Introduction

PIG11 (P53-induced protein 11), also referred to as TP53I11 (tumor protein p53 inducible protein 11), is a p53 target gene [1]. Mutations that inactivate the p53 tumor suppressor protein are the most common genetic aberrations known to occur in human cancers. The best-described biological functions of p53 are the induction of cell cycle arrest and apoptosis in response to cellular stresses [2–4]. It is known that p53 is a transcription factor that binds to specific sequences in DNA and activates the transcription of target gene [5]. In recent years, a number of p53 target genes have been identified that mediate apoptosis including Fas, DR5/Killer, PERP, *Bax*, *Noxa*, *PUMA*, *Bid*, P53AIPI, Pidd, Apaf-1, PEG3/Pw1, IGFBP3, PIGs, Scotin, AIF, p53RDL1,PAC1, and Siah-1b [6–10]. Polyak et al. [1] initially reported the p53-induced genes (PIGs) using the SAGE technique following p53 overexpression. These PIGs were given numbers 1–13. In general, the set of PIGs encode proteins that generate or respond to oxidative stress. Such as PIG1 (galectin-7) which belongs to the galectin family, members of which can stimulate superoxide production. Expression of PIG1 results in enhanced cytochrome *c* release during apoptosis [11]. PIG6 is a homologue of proline oxidoreductase [12]. PIG8 may be the tumor suppressor genes as suggested by its involvement in p53-induced apoptosis [13]. PIG12 is a novel member of the microsomal glutathine-*S*-transferase family of gene [1]. The PIGs are direct downstream targets that can be activated by p53 and trigger the apoptosis in p53-dependent apoptosis pathway. Recently, some investigators demonstrated that PIG11 were significantly induced by wild-type p53 in H1299 cells [14] and in ECV-304 cell [15]. But the function and structure of PIG11 remain to be determined. Our previous studies demonstrated that PIG11 was upregulated markedly in As_2O_3 induced- apoptosis of MGC-803 cells using differential display reverse transcription PCR (DDRT-PCR) and the apoptosis can partially be inhibited by means of anti-PIG11 oligonucleotide treatment [16]. Thus, it is of interest for us to further study about PIG11.

In this study, a recombinant GFP-PIG11 expression vector was constructed and transiently transfected into HEK293 cells. It further demonstrated that there is a p53 consensus binding site in the promoter of the PIG11 gene. PIG11 protein is a cytoplasmic protein. The overexpression of PIG11 could induce cell apoptosis in the low levels and enhanced the apoptotic effects of arsenic trioxide in HEK293 cells. The process was involved in intracellular generation of ROS.

2. Materials and methods

2.1. Construction of a recombinant GFP-PIG11 expressing vectors

PIG11 cDNA encoding amino acids were obtained by reverse transcription-PCR (RT-PCR) of total RNA from MGC-803 cells with As_2O_3 1 μM treatment for 24 h. The PCR product was purified and subcloned into pcDNA3.1/NT-GFP mammalian expression vector using GFP Fusion TOPO® TA Expression Kits (Invitrogen), according to the manufacturer's instruction. Subcloned PCR fragments were confirmed by sequencing with ABI 377 automated fluorescence-based sequencer (Shanghai Gene Core Biotechnologies Co., Ltd., China). A corrected nucleotide sequence for PIG11 is available from GeneBank/EMBL/DDBJ, Accession No. BC003010.1.

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2.2. Cell culture, transfection, and detection of GFP-PIG11 fusion protein

HEK293 cells (Human transformed primary embryonic kidney cells, ATCC No. CRL-1573) were grown at 37 °C with 5% CO_2 in PRMI 1640 culture medium with 10% FCS, 100 μg/ml streptomycin and 100 U/ml penicillin. Cells were seeded onto grass coverslips placed in 6-well plates. The cells were cultured in fresh medium for 24 h and were then transiently transfected with expression vector encoding GFP-PIG11 or GFP only using Lipofectamine™ 2000 (Invitrogen) following the manufacturer's recommendation. At 24 h after transiently transfection, the cells were observed by fluorescence microscope (Olympus, IX-71. AQUACOSMOS 2.0, Analysis software). GFP was excited at 488 nm and fluorescence emission was detected through a 505 nm long pass filter. Average transfected efficiency was analyzed through flow cytometry (FACS).

2.3. Apoptosis assays

HEK293 cells were transiently transfected with GFP or GFP-PIG11 24 h later, and 1 μM of arsenic trioxide (As_2O_3, Sigma Chemical St. Louis, MO, USA) for 24 h or untreatment. The cells were harvested by trypsinization, washed in PBS, and then fixed in 70% ethanol. After washing cells twice with PBS, DNA was stained with propidium iodide (50 μg/ml) containing 250 μg/ml ribonuclease A, followed by flow cytometry analysis (Coulter EPICS XL flow cytometry). Percentage of cells in sub-G1 phase was regarded as the percentage of apoptotic cells. Morphologic charge of apoptotic cells was visualized and photographed under fluorescence microscope after cells were stained with Hoechst 33342.

2.4. Measurement of ROS

Superoxide production was determined by measuring the conversion of dihydroethidium (DHE) to ethidium [17]. Briefly, cells were transiently transfected with GFP or GFP-PIG11 for 24 h. Cells were washed in PBS, resuspended in 1 ml PBS, and DHE was added to each sample (final concentration 2 μM). Samples were immediately vortexed and incubated at 37 °C in the dark for 30 min. After incubation, cells were washed with PBS, protected from light, and analyzed by FACS within 10 min. Excitation peak of ethidium is at 518 nm, fluorescence emission peak is at 605 nm.

2.5. Sequence analysis

The genomic sequence of PIG11 in human chromosome 11 (GI: 22038623) was analyzed for the p53 responsive element using the PATCH software (www.gene-regulation.com_cgibin_pub_programs_patch_bin_patch.cgi). The characteristic was predicted through the SMART, SOSUI, PSORT and Predotar prediction program.

2.6. Statistics

All data are expressed as means ± S.D. (standard deviation), statistical significance was assessed by the Student's *t*-test.

3. Results

3.1. Identification of a putative p53 consensus site in the PIG11 gene and characteristic prediction of PIG11 protein

P53 is a transcription factor that binds to DNA in a sequence-specific manner to activate transcription of target genes. The consensus DNA-binding sequence for p53 consists of two repeats of the 10 bp motif 5′-PuPuPuC(A/T)(A/T)GPyPyPy-3′ separated by o-10 bp [18]. To determine whether the PIG11 gene is a direct transcriptional target of p53 [1,19], 20 kb of the region of human chromosome 11 containing the PIG11 locus was analyzed by using the PATCH software. A putative p53 consensus binding site was identified in the promoter region comprising nucleotides -1085/-1063, 5′-AcACAAGaCTatAAACAAGCCC-3′. This DNA element consists of the two decamers with 2 bp apart (see Fig. 1).

PIG11 protein is consisted of 121 amino acids. Molecular weight 12.904 kDa and PI 8.24. Analysis for sequences homology 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. SOSUI Analysis indicated that these amino acid sequences are of a soluble protein. Possibility of located nucleon is 43.5%, cytoplasma 34.8%, mitochondrion 13.0%, cytoskeleton 8.7% through PSORT II prediction. We constructed a GFP–PIG11 fusion protein in this study, the results showed that the majority of GFP–PIG11 fusion protein was present in the cytoplasm, as control, GFP alone was evenly distributed in the GFP–PIG11 fusion protein and the nucleus (Fig. 2). It suggests that PIG11 could be a soluble cytoplasm protein.

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GGCAGTGTCA  ATGGTGGGCG  GCGACAGAGG  AAAGTGGTAC  CCCGGACACA  -1300
CACCCCTCCT  CTTTCCGCAT  AGGTGAGGAA  AAACACCTAC  TCCTTGATTT
CGGTCCCCCG  CATCCTCCCA  TCAAACCGGA  CCTTTACTGT  AGTGTGAGTG  -1200
ATTTCTGGCG  GTTATCGTTG  AAAACGTCAA  AAAAAAGGGT  TACGCTGTAC
CATATGACGG  AGGGATCAAC  TAGGCCAGAT  GTCGGTGTAA  TAAGTGTCTG  -1100
GGGTGTCAAA  CTTTGAAcACA AGaCTATAAA  CAAGCCCACG  AAATAAAATT
CTGTGGTCCT  CCGAGTTTTT  ACCCTCCACT  GGGCCCCTGT  AGGGGCGTGA  -1000
GACGGAGACC  GTCGGGACCG  ACCCCGTCCT  CCCACTCTAA  ACGGACCCCG
GTGTCCCGTG  ACTCTGTCGT  CCCAGGGGAC  GATCTCCCGG  GTCACCGAAG  -900
ACTGGGACAG  ACATCTACCC  CTACCGAAAA  GACGGAACGG  TCTCCCACCC
ACGTTCACCC  TTACTCTAAG  ACCTTCACCA  GGCTCCATCC  TTCACCGTGC  -800
TGGCAATAAG  TGGAACCAAC  CGTAACTTTG  GGGCCCTCGA  ACCAAAGGAG
TAAACGTCGC  ACCGGCAGTT  ATCGCGGGTA  GAACGTCCCG  TCGACACTAA  -700
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Fig. 1. A potential p53-binding site was identified in the promoter of the PIG11 gene. The sequence that is underlined indicates the pair of decamers with a minimal space between both decamers and containing the smallest number of disparities with the consensus sequence (5′-PuPuPuC(A/T)(A/T)GPyPyPy-3′, Pu = G or A, Py = C or T).

3.2. Transfection and detection of GFP-PIG11 fusion protein in HEK293 cells

In order to look for a biological role of PIG11, a fusion gene was constructed consisting of GFP linked to the N-terminal of PIG11 gene coding sequence, and HEK293 cells were transfected with a control GFP vector or a GFP-PIG11 vector. We have tested the MGC-803 cells used in the previous study [16], but the transfection efficiency was very low. Hence, we used HEK293 cells because it can be efficiently transfected with expression vector. Average transfected efficiency at 24 h after transient transfection was analyzed through FACS. The percentages of positive cells were 65.1–71.8% and 60.2–67.8% in GFP only and GFP-PIG11, respectively.

3.3. Overexpression of PIG11 gene enhanced As_2O_3 induced-apoptosis in HEK293 cells

The transfected HEK293 cells with GFP or GFP-PIG11 were stained with Hoechst 33342. The observation of fluorescence microscope showed that HEK293 cells with overexpression of PIG11 exhibited a significant increase in apoptotic cells (Fig. 3A, 1–3). Apoptotic cells were quantified by FACS as the proportion of cells that had a DNA content of less than 2N (sub-G_1 DNA content) (Fig. 3B). The flow cytometry scans analysis indicated that 11.38% of the HEK293 cells population was undergoing apoptosis after transfection with the GFP-PIG11 vector. In contrast, 7.28% and 3.21% of the HEK293 cells were undergoing apoptosis with only GFP vector and vehicle control, respectively ($P < 0.05$) (Fig. 3B and C). The above results indicate that PIG11 fusion protein influenced cell apoptosis in HEK293 cells.

HEK293 cells were transiently transfected with GFP or GFP-PIG11 24 h later 1 μM of As_2O_3 was added to the above

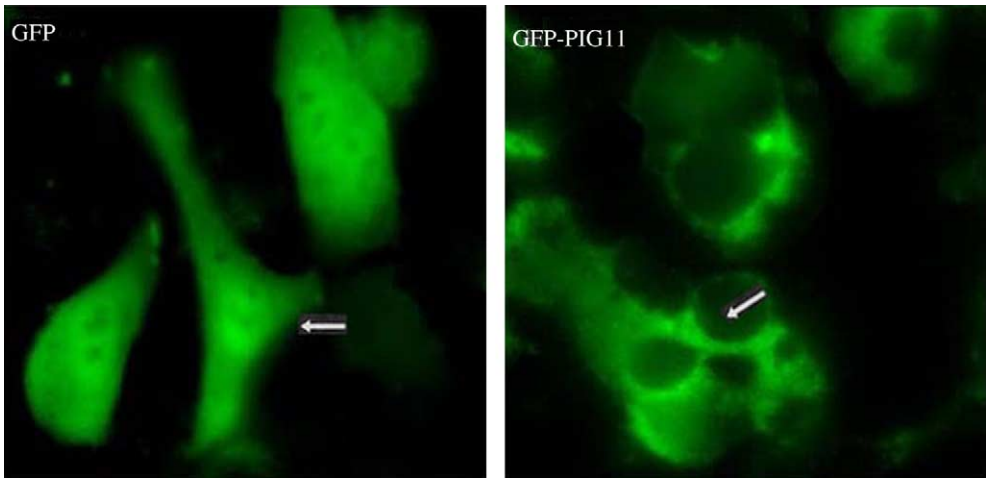

Fig. 2. Distribution of GFP-PIG11. Cells were photographed under a fluorescence microscope at 24 h after GFP or GFP-PIG11 transient transfection. GFP-PIG11 was distributed in the cytoplasma (right panel, arrow), while GFP was the homogenous distribution in cytoplasma and nucleus (left panel, arrow). Magnifications 400×.

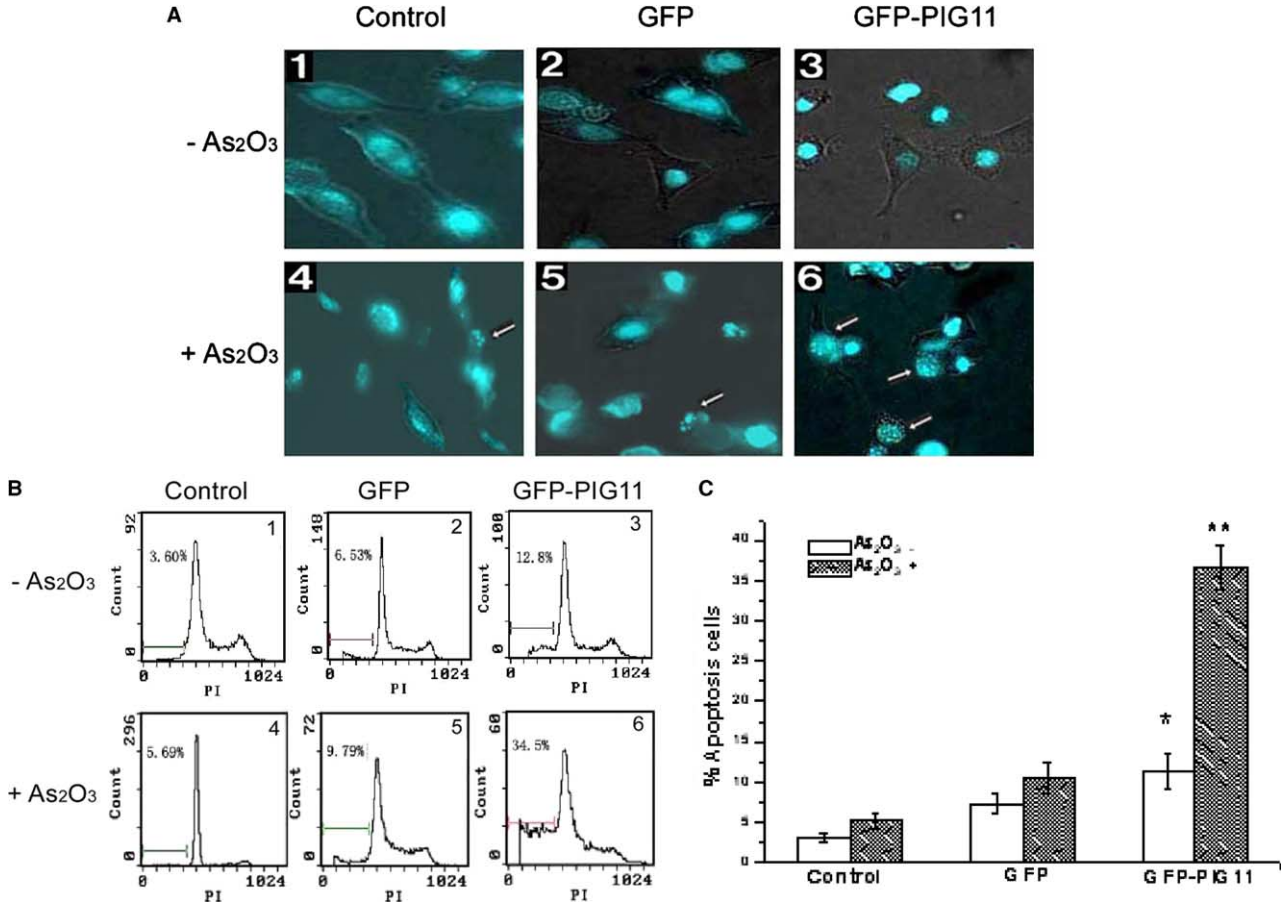

Fig. 3. Overexpression of PIG11 gene involved apoptosis in the transfected HEK293 cells and enhanced As_2O_3 induced-apoptosis. The transfected HEK293 cells with GFP or GFP-PIG11 were treated with 1 μM. of As_2O_3. Apoptotic cells were evaluated by FACS at 24 h after As_2O_3treatment. (A) Apoptotic cells were determined by Hoechst 33342 staining (1: GFP-/As_2O_3-; 2: GFP+/As_2O_3-; 3: GFP-PIG11+/As_2O_3-; 4: GFP-/As_2O_3+; 5: GFP +/As_2O_3+; 6: GFP-PIG11+/As_2O_3+). The typical apoptotic cells (see arrow in figure) showed nuclear condensation and fragment. (B) The sub-G1 DNA content in GFP-positive cells represents apoptotic cells. (1: GFP-/As_2O_3- ; 2: GFP+/As_2O_3-; 3: GFP-PIG11+/As_2O_3-; 4: GFP-/As_2O_3+ ; 5: GFP +/As_2O_3+; 6: GFP-PIG11+/As_2O_3+). (C) Data are presented as the means ± S.D. of the percentage of apoptotic cells in three independent experiments. $^{**}P < 0.01$ vs. other groups. $^{*}P < 0.05$ vs. GFP or control.

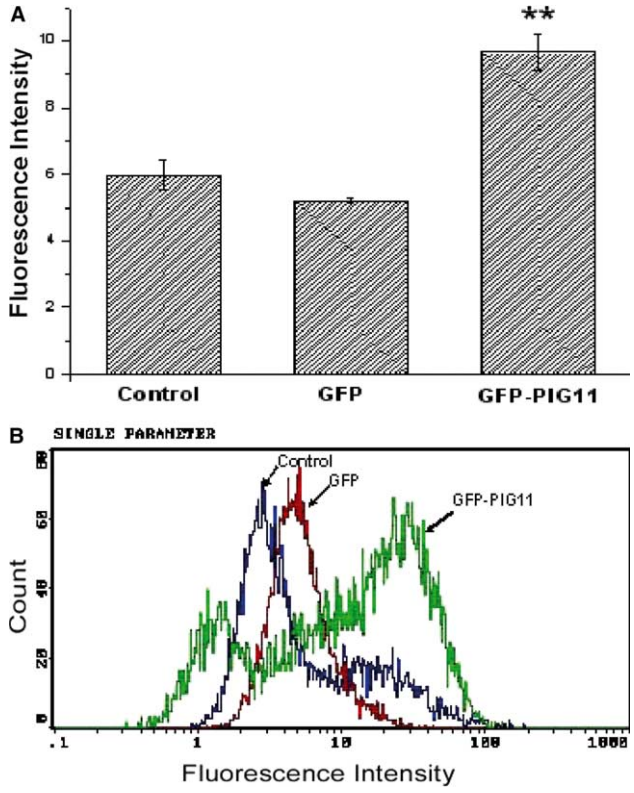

Fig. 4. GFP-PIG11 fusion protein stimulated ROS production in HEK293 cells. HEK293 cells were transiently transfected with GFP or GFP-PIG11 for 24 h, and then were stained with DHE and analyzed by FACS in total GFP-positive cells. The data were presented as the means ± S.D. of DHE fluorescence from three experiments. $^{**}P < 0.01$ vs. GFP or control. (A) Fluorescence intensity for DHE in HEK293 cells without and with GFP or GFP-PIG11. (B) Single parameter in HEK293 cells without and with GFP or GFP-PIG11.

samples. At 24 h after treatment, HEK293 cells with overexpression of PIG11 exhibited a significant increase than the samples without As_2O_3 treatment in apoptotic cells (Fig. 3A, 4–6). Apoptotic cells were quantified by FACS: 36.67% ± 2.78 GFP-PIG11 vs. 10.50% ± 2.03 only GFP and vs. 5.25 % ± 0.96 vehicle control ($P < 0.01$) (Fig. 3B and c). The results indicated that the expression of PIG11 protein increased the sensitivity of As_2O_3-mediated apoptosis in HEK293 cells.

3.4. Overexpression of PIG11 gene increased the generation of ROS in HEK293 cells

Since the set of PIGs generate or respond to oxidative stress, we examined whether PIG11 could influence the production of ROS. Intracellular content of ROS was evaluated by the oxidation of redox-sensitive fluorescence probes DHE. As shown in Fig. 4, an increase of fluorescence intensity at 24 h after transient transfection with GFP-PIG11 vector was observed. Intracellular content of ROS was 9.66 ± 0.52 in HEK293 cells. In contrast, 5.21 ± 0.08 in GFP only and 5.98 ± 0.45 in vehicle control ($P < 0.01$) (Fig. 4A).

4. Discussion

The PIG11 gene was localized to human 11p11.2 and displays an unusual structure, apparently containing only one exon. A potential p53-binding site was identified in the promoter of PIG11 gene, according to our previous analysis of known p53-binding sites (5′-PuPuPuC(A/T)(A/T)GPyPyPy-3′) [19]. This DNA element consists of two decamers, there is a minimal space between both decamers. Recently, several candidate liver tumor suppressor genes from this region had been identified, using a functional model of tumor suppression. It was reported that the transcript for PIG11 was lost or significantly decreased in some human hepatocellular carcinomas (HepG2 and Hep3B) [20]. It suggests that PIG11 as a candidate liver tumor suppressor gene was existed in this chromosome region.

In our previous studies, we found that PIG11 was upregulated markedly by As_2O_3 in MGC-803 cells employing the technique of differential display reverse transcriptase PCR (DDRT-PCR) and the apoptosis in MGC-803 by As_2O_3 can partially be inhibited by means of anti-PIG11 oligonucleotide treatment [16]. It indicated that in As_2O_3-induced apoptosis in MGC-803 cells PIG11, as a downstream target of p53, may play roles in this process. In this work, a recombinant green fluorescence protein PIG11 expressing vector was constructed using GFP Fusion TOPO® TA Expression system. When examined by fluorescence microscope, GFP-PIG11 fusion protein was seen to be localized to a web of cytoplasmic filaments. By 24 h following transfection, cells overexpressing the GFP-PIG11 fusion protein showed substantial levels of cell apoptosis that was morphologically consistent with apoptosis, showing nuclear fragmentation, but proportion of apoptotic cells is of relatively low level.

In recent years, a number of p53 target genes have been identified that mediate apoptosis; however, the induction of some genes may be enough to initiate cell death, the induction of other genes by themselves will not cause the cell to undergo apoptosis or induce apoptosis to low levels. But recent data suggest a new class of p53 target genes that sensitize cells to the effects of chemotherapeutic agents [21], for example, bid-deficient mouse embryonic fibroblasts (MEFs) treated with increasing doses of adriamycin or 5-fluorouracil were significantly more resistant to apopotosis than wild-type MFFs. The p53-induced Bid could help to sensitize the cells to toxic effects of chemotherapeutic drugs [22]. Our results demonstrated that PIG11 fusion protein influenced apoptosis in HEK293 cells, but proportion of apoptotic cells is relatively low level. Moreover, the increase of PIG11 protein enhanced the sensitivity of As_2O_3-mediated apoptosis in HEK293 cells. These results suggest that PIG11 is considered as a P53-chemosensitization gene and overexpression of PIG11 could be one of the many factors in cell apoptosis.

On the other hand, As_2O_3 has become an interesting experimental therapeutics for a wide variety of hematological as well as solid tumors [23–25]. The sensitivity of cells to As_2O_3 may result from their biochemical or cellular background. Modulation of the biochemical environment of the cells might increase the efficacy of arsenic trioxide [26,27]. Some investigated that the redox system of the cell and capacity to eliminate reactive oxygen species (ROS) are involved in the efficacy of As_2O_3[16,28–32]. ROS, such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$), superoxide anion radicals (O_2^-), are known mediators of intracellular signaling cascades. It was demonstrated that ROS and the resulting oxidative stress play an important role in

apoptosis [28]. Generation of ROS could lead to apoptosis, mediated through loss of mitochondrial membrane potential with subsequent changes of outer mitochondria membrane permeability, release of cytochrome *c*, and the activation of caspases [29,30]. Several in vitro investigations showed that both decreases the intracellular GSH content as well as lead to increased intracellular H_2O_2 levels lead to a significant synergistic effect with As_2O_3[31,32]. Ascorbic acid (AA) in combination with As_2O_3 has a potentiating effect [33]. AA widely heralded as an antioxidant. However, AA can also act as oxidizing agent that increases the production of ROS [34,35]. Its capacity to undergo autooxidation results in the formation of H_2O_2 that enhances the effect of As_2O_3 [36].

To augment the apoptosis effects of As_2O_3 in resistant tumor cells to As_2O_3, potential strategies include increase of intracellular ROS production [37]. We show that PIG11 expression increased sensitivity of As_2O_3-mediated apoptosis in HEK293 cells and also enhanced intracellular ROS production. It is possible that PIG11 might be valuable to increase As_2O_3-sensitivity in less sensitive tumor cells may be through involved intracellular generation of ROS. PIG11 was downregulated in ovarian endometriosis (the disease is similar to malignant tumors in some respects) [38]. The function of the protein product of the PIG11 gene (as a negative regulation factor) may have a role in cells apoptosis, mediated through enhanced ROS levels.

In conclusion, PIG11 protein is a cytoplasmic protein, there is a p53 consensus binding site in the promoter of the PIG11 gene. The overexpression of PIG11 could induce cell apoptosis in the low levels and enhanced the apoptotic effects of arsenic trioxide. The process is involved intracellular generation of ROS.

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