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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

# Possible roles of a tumor suppressor gene *PIG11* in hepatocarcinogenesis and $As_2O_3$-induced apoptosis in liver cancer cells

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**Abstract**
*Background* Our previous studies demonstrated that p53-induced gene 11 (*PIG11*) was involved in arsenic trioxide ($As_2O_3$)-induced apoptosis in human gastric cancer MGC-803 cells. Here, we studied further PIG11 expression in human hepatocellular carcinoma (HCC) tissues and cell lines and compared the sensitivity to $As_2O_3$-induced cell apoptosis in HepG2 and L-02 cells.
*Methods* PIG11 expression in human normal liver tissues, HCC tissues, and cell lines was determined by immunohistochemistry and immunocytochemistry methods, using an anti-human PIG11 antibody. Cell viability was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diplenyltetrazolium bromide (MTT) assay. Cell apoptosis was determined by flow cytometry. Reverse-transcriptase polymerase chain reaction (RT-PCR) and Western blotting were performed to analyze PIG11 mRNA and protein expression in cells. Protein intensity was calculated by comparison with the intensity of $\beta$-actin, using densitometry. PIG11 was knocked down using small interfering RNA (siRNA).
*Results* We found that PIG11 expression was significantly downregulated in HCC tissue and the cell lines (Bel-7402, SMMC-7721, HepG2 cells). Further, HepG2 cells were more sensitive to $As_2O_3$-induced apoptosis than L-02 cells. The expression of PIG11 mRNA and protein was upregulated to a greater extent in HepG2 than in L-02 cells. In the presence of actinomycin D or cycloheximide, the amount of PIG11 protein expression did not increase. Likewise, the inhibition of PIG11 by siRNA decreased $As_2O_3$-induced PIG11 protein expression by more than 85% and partially prevented $As_2O_3$-induced apoptosis in both HepG2 and L-02 cells.
*Conclusion* The above results demonstrated that the *PIG11* gene may be involved in $As_2O_3$-induced apoptosis in HepG2 cells and suggested that the adaptive response of PIG11 expression is one of the important factors in enhancing cell sensitivity to $As_2O_3$-induced apoptosis.

**Keywords** p53-induced gene 11, PIG11 protein, hepatocellular carcinoma, HepG2 cells · L-02 cells, Apoptosis · Arsenic trioxide ($As_2O_3$)

## Introduction

p53-induced gene 11 (*PIG11*) is one of the set of PIGs initially reported by Polyak et al. [1]. PIGs are direct downstream targets that can be activated by p53 and that trigger apoptosis in the p53-dependent apoptosis pathway [2]. Most PIGs encode proteins that generate or respond to oxidative stress [3–5]. *PIG11* was localized to human 11p11.2 PIG11, and displays an unusual structure, apparently containing only one exon. Some investigators demonstrated that PIG11 was significantly induced by wild-type p53 in HI299 cells [6] and in ECV-304 cells [7]. Recently, *PIG11* has been identified as one of three liver tumor-suppressor genes: *PIG11*, *MTCH2*, and *PRDM11*. The experimental analysis of four human hepatocellular carcinoma (HCC) cell lines (HepG2, Hep3B, SNU398, and SNU449) showed the transcript for PIG11 was lost or

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significantly decreased in the HepG2 and Hep3B cell lines, suggesting *PIG11* may be a candidate tumor-suppressor gene [8]. However, the role of PIG11 protein during apoptosis or the inhibition of cell growth remains unknown.

Arsenic trioxide ($As_2O_3$) was identified in vitro and in vivo to be an effective drug in the treatment of acute promyelocytic leukemia (APL). Numerous clinical trials of this drug are under way for the treatment of hematopoietic malignancies and solid tumors [9–11]. Our previous work demonstrated that PIG11 was markedly upregulated in the $As_2O_3$-induced apoptosis of MGC-803 cells, and that transiently transfected PIG11 induced cell apoptosis at a low level and enhanced the apoptotic effects of $As_2O_3$ in human transformed embryonic kidney cells 293 (HEK 293 cells) [12, 13]. A full understanding of PIG11 expression and HCC cell sensitivity to $As_2O_3$ could promote the application of $As_2O_3$ in the clinical setting.

In the present study, we further investigated PIG11 expression in human normal liver tissues, HCC tissue, and cell lines by immunohistochemistry and immunocytochemistry methods, using an anti-human PIG11 antibody. According to the above observations, we selected two kinds of cells: HepG2 cells (lower PIG11 expression) and human embryo hepatocytes L-02 (higher PIG11 expression) to compare their sensitivity to $As_2O_3$-induced cell apoptosis and their gene expression profiles following PIG11 knockdown, using RNA interference. Our results revealed that the *PIG11* gene may be involved in $As_2O_3$-induced apoptosis in HepG2 cells.

## Materials and methods

### Cell culture and drug treatments

Human embryo hepatocytes L-02 (Shanghai Institute Of Cell Biology, Chinese Academy of Science, Shanghai, China) and three human HCC cell lines: Bel-7402, SMMC-7721, and HepG2 cells (Cancer Research Institute, Xiangya School of Medicine, Central South University, Changsha, China) were cultured in RPMI 1640 medium (Gibco-BRL, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (Sijiqing Co. 1, Hangzhou, China), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% $CO_2$ at 37°C. After seeding for 6–8 h, cells were treated with $As_2O_3$ (Sigma Chemical Company, St. Louis, MO, USA), actinomycin D (Act-D; Calbiochem, San Diego, CA, USA), or cycloheximide (CHX; Sigma Chemical Company, St. Louis, MO, USA) as described in the figure legends. The viability of cells was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diplenyltetrazolium bromide (MTT) assay.

### Human tissue collection

Human normal liver tissues samples (from six patients with hepatolithiasis, three patients with chronic hepatitis, and one patient with liver rupture) and corresponding tumor tissues (the clinical and pathological information on the HCC tissue samples is given in Table 1) were obtained from the Department of Pathology, First Affiliated Hospital, Nanhua University. All HCC samples were from surgical operations

**Table 1** Clinical and pathological information on HCC tissue samples

| Number of cases | Sex | Age (years) | Tumor size | Histological differentiation grade | TNM stage |
|---|---|---|---|---|---|
| 1 | Female | 50 | 12 × 9 × 6 $cm^3$ | Poorly differentiated | Stage IV |
| 2 | Male | 52 | 2 × 2 × 1 $cm^3$ | Well-differentiated | Stage II |
| 3 | Male | 55 | 7 × 8 × 6 $cm^3$ | Well-differentiated | Stage III |
| 4 | Male | 62 | 3 × 2.5 × 3 $cm^3$ | Well-differentiated | Stage III |
| 5 | Male | 38 | 10 × 10 × 10 $cm^3$ | Poorly differentiated | Stage III |
| 6 | Male | 40 | 3 × 2 × 3 $cm^3$ | Poorly differentiated | Stage II |
| 7 | Female | 39 | 6.5 × 5 × 5 $cm^3$ | Poorly differentiated | Stage II |
| 8 | Female | 11 | 16 × 11 × 10 $cm^3$ | Well-differentiated | Stage II |
| 9 | Male | 48 | 5 × 5 × 5 $cm^3$ | Well-differentiated | Stage III |
| 10 | Male | 36 | 3 × 2 × 3 $cm^3$ | Well-differentiated | Stage II |
| 11 | Male | 52 | 3 × 3 × 3 $cm^3$ | Poorly differentiated | Stage III |
| 12 | Male | 36 | 8 × 6 × 6 $cm^3$ | Poorly differentiated | Stage IV |
| 13 | Male | 61 | 2.5 × 1.5 × 2.5 $cm^3$ | Well-differentiated | Stage II |
| 14 | Male | 47 | 6 × 6 × 5 $cm^3$ | Well-differentiated | Stage II |
| 15 | Male | 47 | 10 × 8 × 8 $cm^3$ | Poorly differentiated | Stage III |
| 16 | Male | 26 | 4 × 3 × 4 $cm^3$ | Well-differentiated | Stage III |

in HCC patients. The diagnosis of HCC was based on histopathological examination. The patients and healthy individuals signed an informed consent form for the study which was reviewed by the Institutional Review Board.

### Immunohistochemistry and immunocytochemistry investigation

The streptavidin-peroxidase (S–P) method was used to detect the expression of PIG11 protein in human normal liver tissues, para-carcinoma tissues, HCC tissues, and L-02, Bel-7402, SMMC-7721, and HepG2 cells. A polyclonal rabbit anti-human PIG11 antibody (antiserum titer, 1:1000–1:5000) was prepared by our laboratory [14]. The expression of of PIG11 protein was determined with an UltraSensitive S–P (Mouse/Rabbit) kit (MaiXin, Bio, Fuzhou, China), according to the manufacturer's protocol. Phosphate-buffered saline (PBS) was used instead of PIG11 as the negative control, and $\beta$-actin protein was used as the control. Semiquantitative analysis was performed as follows: images (magnification 400×) were obtained on a PIPS-2020 tall acuity color pathology image analysis system (Chongqinghaitian Armarium, Chongqing, China). Each slice photographed two to three random fields. Optical densities (ODs) of the immunohistochemistry images were also estimated by this system. The mesenchymal OD (MOD, at least five random points) and the parenchymal OD (POD, at least ten random points) were detected for each picture. Relative OD (ROD) = MOD − POD.

### Apoptosis assays

The cells, treated or untreated with 12 μM $As_2O_3$, were harvested by trypsinization, washed in cold PBS, and then fixed in 70% ethanol. After the cells had been washed twice with PBS, DNA was stained with propidium iodide (5 μg/ml) containing 250 μg/ml RNaseA, followed by flow cytometry analysis (Beckman Coulter Epics Ultra flow cytometry, Beckman Coulter, Inc. Fullerton, CA, USA). The percentage of cells in the sub-G1 phase was regarded as the percentage of apoptotic cells. Total DNA was prepared using a DNA apoptosis cell ladder isolation kit (Beijing Biodev-Tech. Scientific and Technical, Beijing, China), according to the manufacturer's protocol. The cell DNA was run on a 2% agarose gel for the DNA ladder. DNA fragments were checked under ultraviolet light in comparison with a DNA marker (DL2000: TaKaRa Bio Inc., Ohtsu, Japan).

### Total RNA isolation

After $As_2O_3$ treatment for 24, 48, and 72 h, total RNA was isolated from the HepG2 and L-02 cell lines using TRIzol reagent (Bio.Basic, Shanghai, China) extraction, according to the manufacturer's protocol. RNA was precipitated with isopropanol, washed with 75% ethanol, and dissolved in diethylpyrocarbonate (DEPC)-treated water. The integrity and purity of total RNA were identified by 1% agarose gel electrophoresis and with an ultraviolet spectrophotometer, respectively.

### Reverse-transcriptase polymerase chain reaction (RT-PCR)

The SuperScript First-Strand Synthesis System for RT-PCR kit (Promega Corp., Madison, WI, USA) was used to synthesize cDNA, according to the kit instructions. Total RNA (2 μg) and 1 μl 0.5 μg/μl Oligo (dT) 15 (Promega Corp., Madison, WI, USA) was heated at 70°C for 5 min and quickly chilled on ice. Moloney Murine Leukemia virus Reverse transcriptase H-200U, 5 × RT buffer 4 μl, 10 mM dNTPs 1 μl, and RNase ribonuclease inhibitor 20 U were added to a total volume of 50 μl, incubated at 25°C for 5 min and 42°C for 1 h, and inactivated at 70°C for 15 min. The PIG11 primers used were: upstream primer, 5′-TAC AGT TGG GAA GGA GGC-3′; downstream primer, 5′-GAA TAA ATA GTG ACC GTG AAG A-3′. $\beta$-Actin designed by the primer analysis software primer 5.0 as an internal control was used in independent reactions. The $\beta$-actin primer consisted of: upstream primer, 5′-ACA CTG TGC CCA TCT ACG AGG GG-3′; downstream primer, 5′-ATG ATG GAG TTG AAG GTA GTT TCG TGG AT-3. A 10-μl aliquot of the RT mix was amplified in the presence of 100 μM dNTP mixture, 2 μM primer, 5 U Taq DNA polymerase, and 4 μl 10 × PCR buffer. The cycling parameters were: 94°C for 1 min, 48.5°C for 1 min, 72°C for 2 min; 30 cycles, following a final extension for 7 min at 72°C. The PCR product was separated on 2% agarose gels and stained with ethidium bromide. The expression of PIG11 was compared with that of $\beta$-actin—a housekeeping gene.

### Western blotting

HepG2 and L-02 cells treated with 12 μM $As_2O_3$ were harvested, rinsed twice with cold PBS, and incubated in lysis buffer containing 10 mM Tris-Hcl (pH 7.6), 100 mM NaCl, 1 mM ethylene diamine tetraacetic acid (EDTA; pH 8.0), 100 μg/ml phenylmethylsulfonylfluoride (PMSF), and 1 μg/ml aprotinin on ice for 1 h. Following centrifugation at 12 000*g* at 4°C for 15 min, the supernatants were stored at −70°C until assay. The amount of protein in the supernatant was determined using bicinchoninic acid (bca) protein assay reagent (Pierce Biotechnology, Inc., Rockford, IL, USA). An equal amount of protein sample was completely vortexed with 5 × sodium dodecyl sulfate (SDS)-gel buffer, and boiled for 5 min at 100°C to dissolve the bound proteins. The samples were segregated on 100 or

150 g/L SDS-polyacrylamide gels, transferred onto a Polyvinylidene Fluoride (PVDF) membrane (Amersham, Buckinghamshire, UK) in glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% methanol (v/v)] and blocked with 50 g/L defatted milk for 2 h, then probed with different primary antibodies. Anti-mouse or anti-rabbit IgG conjugated peroxidase was used as a secondary antibody. The filters were then incubated in SuperSignal enhanced chemiluminescence-Horseradish Peroxidase (ECL-HRP) detection reagent (Pierce Biotechnology, Inc., Rockford, IL, USA) for 1 min, followed by exposure to X-ray film. To measure the expression of each gene, the relative intensity was calculated by comparing it with the intensity of $\beta$-actin, using densitometry (Alphazmager 2200, NatureGene Corp, Medford, NJ, USA).

### PIG11 knockdown by transfection of PIG11 siRNA

The PIG11 siRNA and nonspecific control (NSC) siRNA were both chemically synthesized by GenePharma (Shanghai, China). The siRNA sequence targeting PIG11 is 5-CAGGUCUUAGGCAAUGAAATT-3. Cells ($2 \times 10^5$ per well) were seeded in six-well plates overnight and then were transfected with either PIG11-specific siRNA (100 nM), NSC siRNA, or mock transfected, using 9 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) per well in 1.5 mL of RPMI 1640 medium containing 10% PBS. The transfection medium was replaced with fresh culture medium 6 h later. After another 18 h, cells were treated with arsenic, as indicated in the figure legends. The expression of PIG11 was assayed by Western blotting using rabbit anti-PIG11 polyclonal antibody 72 h after transfection.

### Statistical analysis

Results were analyzed with the SPSS 11.5 statistical software package (SPSS Inc. Chicago, IL, USA). All data values are expressed as means ± SD. Comparisons between different groups were made by one-way analysis of variance (ANOVA), and $P < 0.05$ was taken as statistically significant. At least three independent experiments were carried out.

## Results

### Expression of PIG11 protein in human normal liver tissues, para-carcinoma tissues, and HCC tissues

Figure 1 shows cells with intense staining in normal liver tissues; because of the many brown-stained particles visualized in the cytoplasm, intense positive expression of PIG11 was indicated (Fig. 1A-a). In para-carcinoma tissues, staining of cells was relatively weak compared to normal liver tissues, suggesting weak positive expression (Fig. 1A-b), while staining of cells was negative in HCC tissues; few brown-stained particles were seen in the cytoplasm (Fig. 1A-c). The relative ODs of human normal liver tissues, para-carcinoma, and HCC tissues were 65.15 ± 8.64, 41.83 ± 4.69, and 40.69 ± 12.92,

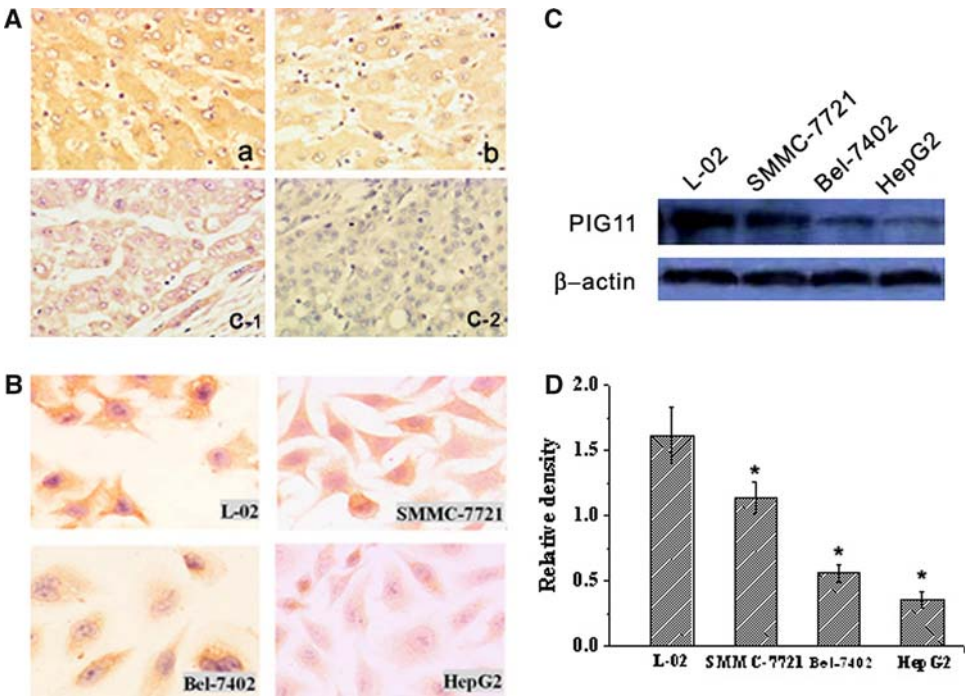


**Fig. 1** **A** PIG11 expression in human normal liver tissue, para-carcinoma tissue, and hepatocellular carcinoma (HCC) tissue. *a* Normal liver tissue; *b* para-carcinoma tissues; *c-1* well-differentiated HCC tissue; *c-2* poorly differentiated HCC tissue. **B** PIG11 expression in HCC cell lines (HepG2, Bel-7402, and SMMC-7721) and L-02 cells; **C** Western blotting of PIG11 protein in HepG2, Bel-7402, and SMMC-7721, and L-02 cells. **D** Semiquantitative analyses of PIG11 proteins in HepG2, Bel-7402, and SMMC-7721, and L-02 cells. Data shown were compiled from five independent experiments. * $P < 0.01$ versus other cells. **A** and **B** 400×

respectively, indicating the PIG11 expression was downregulated significantly in HCC tissue, compared to paracarcinoma and normal tissues ($P < 0.01$).

Immunohistochemistry results also showed the distribution of PIG11 was tissue-specific and correlated to the level of cellular differentiation. Analytical results of immunohistochemistry showed that PIG11 expression was weakly positive in well-differentiated HCC tissues (Fig. 1A-c-1), while it was negative in poorly differentiated HCC tissue (Fig. 1A-c-2). The relative ODs of the well-differentiated and poorly differentiated HCC tissues were 46.64 ± 10.83 and 33.04 ± 11.81, respectively. The PIG11expression was downregulated in poorly differentiated HCC tissues, compared to well-differentiated HCC tissues ($P < 0.05$).

Figure 1B shows the PIG11 protein expression in the three HCC cell lines (HepG2, Bel-7402, and SMMC-7721) and in L-02 cells. The level of PIG11 expression was compared with that of $\beta$-actin (Fig. 1C), and the relative density of PIG11 protein in the three HCC cell lines (HepG2, Bel-7402, and SMMC-7721) and the L-02 cell line was 0.36 ± 0.06, 0.56 ± 0.07, 1.14 ± 0.12, and 1.62 ± 0.21, respectively (Fig. 1D). These findings indicated that the expression of PIG11 protein was positive in L-02 cells, while it was significantly downregulated in SMMC-7721, Bel-7402, and HepG2 cells ($P < 0.01$).

## Growth inhibition and apoptosis induction in HepG2 and L-02 cells by $As_2O_3$

According to the above observations, we selected two kinds of cells: HepG2 cells (lower PIG11 expression) and human embryo hepatocytes L-02 (higher PIG11 expression) to study further their sensitivity to $As_2O_3$-induced cell apoptosis. As shown in Fig. 2, after the cells were treated with 0.625–40 μM of $As_2O_3$, the cell growth was slightly inhibited in L-02 cells, while it was inhibited significantly in HepG2 cells, in a dose- and time-dependent manner. In cells treated with 10 μM $As_2O_3$ for 72 h, the growth inhibition ratio (%) was 47.11 ± 3.31 in HepG2 cells and 13.68 ± 1.91 in L-02 cells. The 50% inhibitory concentration (IC50) of HepG2 cells was 12.8 μM.

Flow cytometry analysis revealed that, in HepG2 cells treated with 12 μM $As_2O_3$ for 24, 48, and 72 h, respectively, the percentages of apoptotic cells were 9.74 ± 1.2, 21.89 ± 2.14, and 42.72 ± 0.83%. The apoptosis rates were 5.81 ± 1.00, 8.65 ± 2.05, and 13.15 ± 1.06% in L-02 cells treated with the same way (Fig. 3a, b). Marked DNA ladders were observed after HepG2 cells were exposed to $As_2O_3$ for 48 h and 72 h, but the changes were not obvious in L-02 cells (Fig. 3c). These results indicated that HepG2 cells had higher sensitivity to $As_2O_3$-induced apoptosis than L-02 cells.

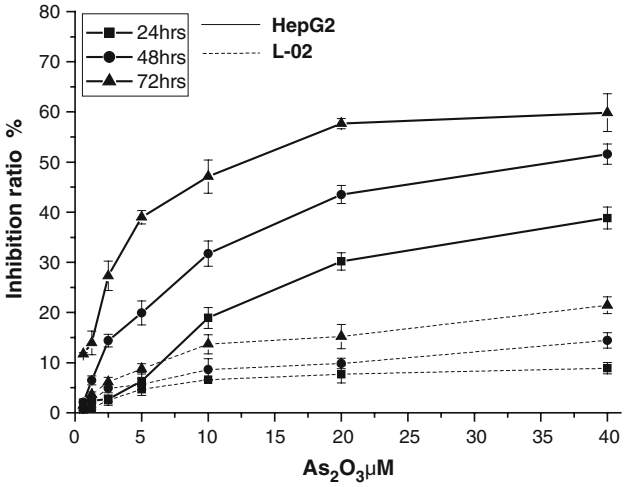


**Fig. 2** Effects of $As_2O_3$ on cell growth inhibition in HepG2 and L-02 cells. The cellular growth inhibition ratio was measured using the MTT assay. Data values are the means ± SD of three independent experiments

## Upregulation of PIG11 mRNA and protein in $As_2O_3$-induced apoptosis

As shown in Fig. 4a, B, PIG11 mRNA and proteins were markedly upregulated in $As_2O_3$-induced apoptosis in HepG2 cells. The expression of PIG11 mRNA in HepG2 cells was significantly increased, to 2.00 and 4.06 times the initial level, in 48 and 72 h ($P < 0.01$). These values in L-02 cells were 1.38 and 1.89 times the initial level. PIG11 protein was increased to 1.48 and 1.72 times the initial level in HepG2 cells after 48 and 72 h treatment with $As_2O_3$ ($P < 0.05$), whereas at these times, PIG11 protein was increased to 1.19 and 1.30 times the initial level in L-02 cells. These findings indicated that the upregulation of PIG11 mRNA and protein was greater in $As_2O_3$-induced HepG2 apoptosis than in $As_2O_3$-induced L-02 apoptosis.

## Effects of cycloheximide and actinomicin D on $As_2O_3$,-inducible PIG11 protein

Actinomycin D binds to DNA at the transcription initiation complex and inhibits RNA synthesis, while CHX is an inhibitor of protein synthesis, which blocks the translational elongation on ribosomes. Figure 5a shows that Act-D reduced constitutive PIG11 levels in HepG2 cells by twofold, and $As_2O_3$ increased PIG11 levels in HepG2 cells by 1.9-fold and in L-02 cells by 1.4-fold. However, there was no statistically significant difference in PIG11 levels between cells treated with Act-D alone and cells treated with $As_2O_3$ and Act-D. CHX inhibited the constitutive expression of PIG11 in HepG2 cells by more than 60% (Fig. 5b). In a similar manner, the $As_2O_3$-mediated induction of PIG11 levels in HepG2 cells was higher than

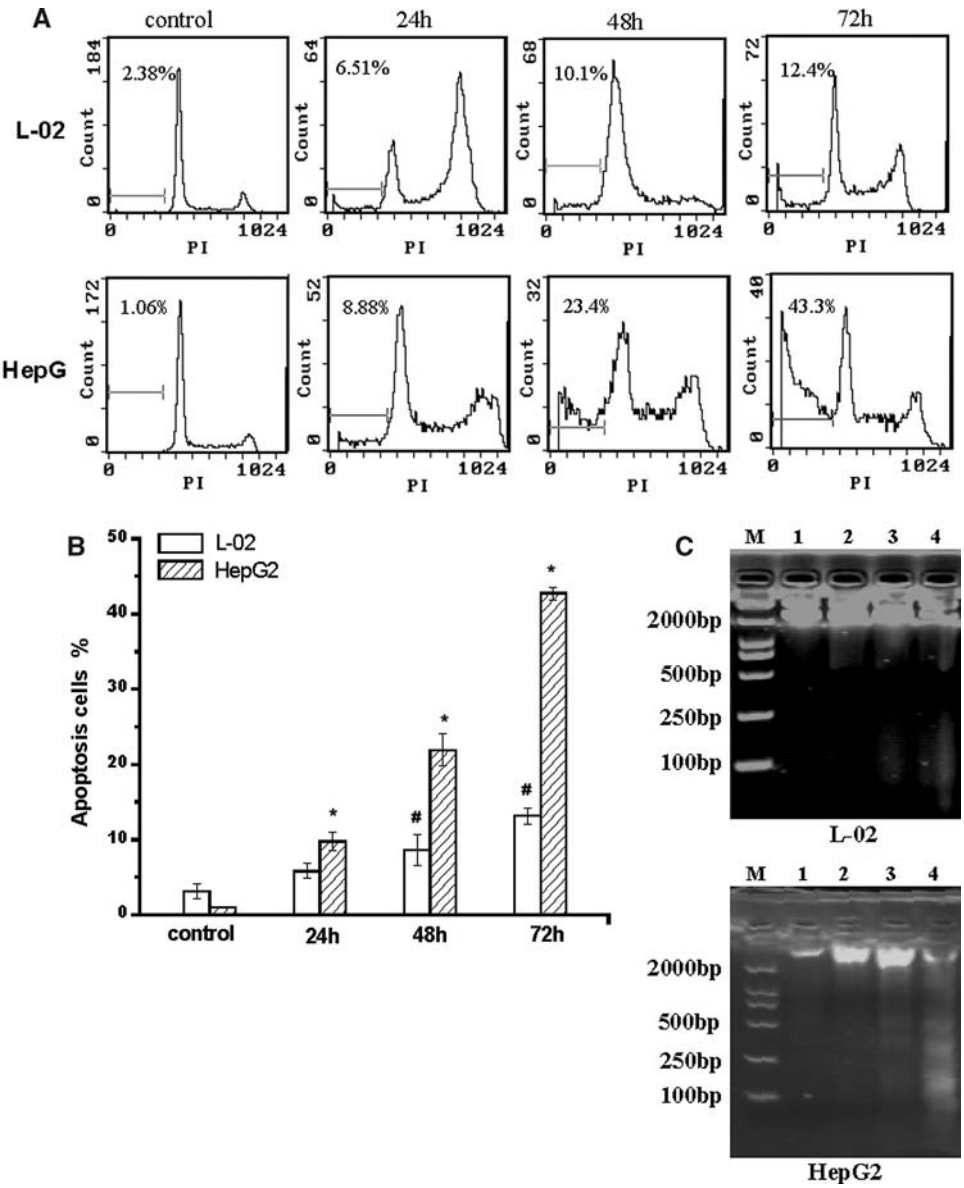


**Fig. 3** **a** Percentages of apoptotic cells in sub-G1 phase. L-02 and HepG2 cells were treated with 12 μM $As_2O_3$ for 0–72 h. **b** The apoptotic rates of cells in HepG2 and L-02 cells. Data values are presented as the means ± SD of the percentage of apoptotic cells in three independent experiments. * $P < 0.01$ versus control; #$P < 0.05$ versus control. **c** DNA fragmentation induced by $As_2O_3$ in HepG2 and L-02 cells. *Lane M*, DNA marker; *Lane 1*, control (untreated with $As_2O_3$); *Lanes 2–4*, cells treated with $As_2O_3$ for 24, 48, and 72 h, respectively

that obtained in L-02 cells with CHX, the levels being higher than those obtained in cells treated with CHX alone and those from cells treated with $As_2O_3$ and CHX. Thus, $As_2O_3$-mediated induction of apoptosis requires new protein synthesis.

## Knockdown of PIG11 prevents $As_2O_3$-induced apoptosis

In order to reach a conclusion on whether PIG11 is involved in carcinogenesis and $As_2O_3$-induced apoptosis, PIG11 was knocked down using siRNA. Figure 6a shows that PIG11 siRNA decreased the PIG11 protein level in HepG2 and L0-2 cells by nearly 86%, demonstrating efficient knockdown. Moreover, NSC siRNA or mock transfection was without effect. In a similar manner, the $As_2O_3$-mediated induction of PIG11 levels was higher in HepG2 cells (about 2.1-fold of control) than in L-02 cells (about 1.5-fold of control). Pretreatment of PIG11 siRNA greatly suppressed the $As_2O_3$-induced PIG11 protein expression, by more than 85% (Fig. 6b), and partially prevented $As_2O_3$-induced apoptosis in both cell lines (Fig. 6c). Moreover, knockdown of the *PIG11* gene did not appear to have affected the basal cell growth without $As_2O_3$ treatment. On other hand, NSC siRNA or mock transfection was without effect, and HepG2 cells were more sensitive to $As_2O_3$-induced apoptosis than L-02 cells, suggesting that PIG11 plays a role in $As_2O_3$-induced apoptosis.

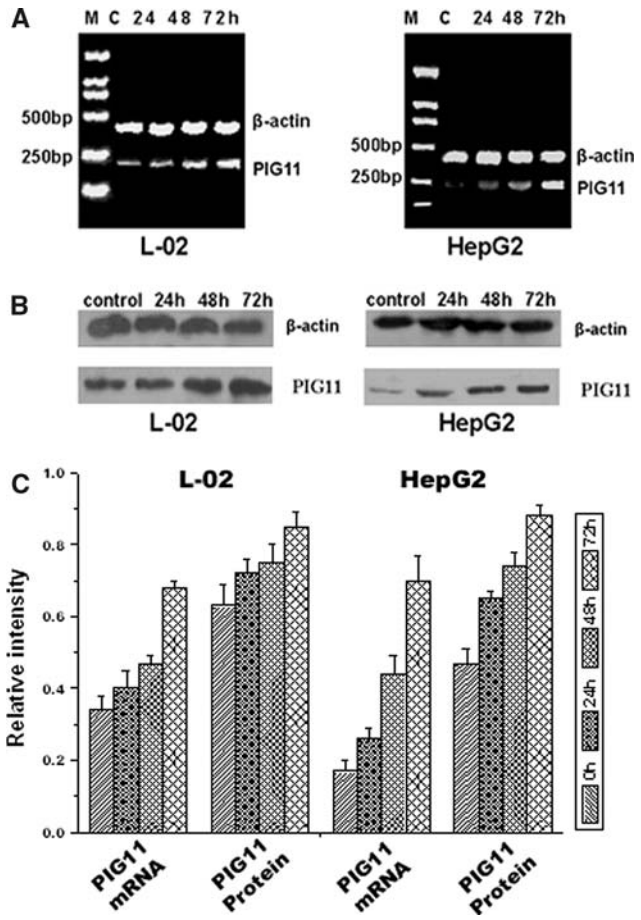


**Fig. 4** **a** Reverse transcriptase polymerase chain reaction (RT-PCR) was performed to analyze the mRNA expression of PIG11. The PCR product of *β*-actin was 450 bp, but that of PIG11 was 227 bp. *Lane M*, DNA marker; *Lane C*, control (treated without $As_2O_3$); *Lanes 24, 48, and 72* represent L-02 and HepG2 cells that were treated with 12 μM $As_2O_3$ for 24–72 h, respectively. **b** Protein expression of PIG11 was assessed using Western blot technology in L-02 cells and HepG2 cells treated with 12 μM $As_2O_3$ for 24–72 h. *Lane control*, treated without $As_2O_3$. *Lanes 24, 48, and 72* represent L-02 and HepG2 cells that were treated with 12 μM $As_2O_3$ for 24–72 h, respectively. **c** Protein expression of PIG11 in L-02 cells and HepG2 cells. Relative intensity of PIG11 expression was calculated by comparing the expression with the intensity of *β*-actin, using densitometry. Data shown were compiled from three independent experiments

## Discussion

In the present study, we found that the expression of PIG11 protein was downregulated significantly in HCC tissues and HCC cell lines ($P < 0.01$). These results are consistent with the rule that the protein expression of tumor suppressor genes, such as *p53* and *p21*, is usually lost or downregulated in malignant tumors. It was reported that the transcript for *PIG11* was lost or significantly decreased in some human HCC cells: HepG2 and Hep3B [8]. Chiba et al. [15] found that eight genes, including PIG11, exhibited substantial induction (ratio >2.0) when trichostatin A (TSA) induced apoptosis in human hepatoma cell lines (HuH7, Hep3B, HepG2, and PLC/PRF/5). The above results further indicate that the *PIG11* gene may be a liver tumor suppressor gene and that *PIG11* gene function may be related to cell apoptosis.

PIG11 proteins were visualized mainly in the nuclei of chondrosarcoma cells as brown-stained particles (unpublished data). In this work, the majority of PIG11 fusion proteins were present in the cytoplasm of normal liver cells. However, several biologically significant roles for PIG11 localization to the cytoplasm can be envisioned. One role is that PIG11 may be translocated from the cytoplasm to the nucleus at the G1/S transition and is shuttled back to the cytoplasm shortly thereafter. With Atomic Force Microscopy images, we previously confirmed the binding of DNA and PIG11 protein and showed that the PIG11-DNA complex had a beads-on-a-string appearance in which the PIG11 protein associated with the DNA as a polymer [14]. How PIG11 enters the nucleus remains unclear. One possible explanation is that other protein factors may be recruited to the chromosome through interactions with PIG11. Recent investigations have found that another p53 responsive gene, p53-responsive gene 3 (*PRG3*), also known as AMID (apoptosis-inducing factor-homologous mitochondrion-associated inductor of death), is a DNA-binding protein that lacks apparent DNA sequence specificity [16]. It also has a potential role in tumor suppression.

Although the effect of $As_2O_3$ on the cell cycle has been addressed, its precise mechanisms are still much in question. In the present study, treatment with $As_2O_3$ appeared to induce not only apoptosis but also cell-cycle arrest at the G2/M phase. The following possible mechanisms can be advanced: (1) $As_2O_3$ may be linked to the activation of cyclin B-dependent kinase and the accumulation of cyclin B. There are data showing that $As_2O_3$ causes the activation of cyclin B-dependent kinase and the accumulation of cyclin B in association with mitotic arrest [17]. (2) Interference by arsenite with signal transduction pathways involving nuclear transcription factor kappa B (NF$\kappa$B) has been reported [18]. Studies have demonstrated the activation of NF-$\boldsymbol{\kappa\beta}$ as a consequence of increased reactive oxygen species (ROS) production attributable to the oxidative stress generated by $As_2O_3$ [19]; (3) we found that PIG11 in cytoplasm was obviously increased after $As_2O_3$ treatment. This abundant PIG11 may bind other proteins, such as ATM kinase. If damaged DNA cannot be repaired immediately, single breaks can accumulate, and this could specifically block the DNA damage response and lead to the above observed results of G2/M arrest. DNA damage has been shown to trigger p53-independent apoptosis or growth arrest.

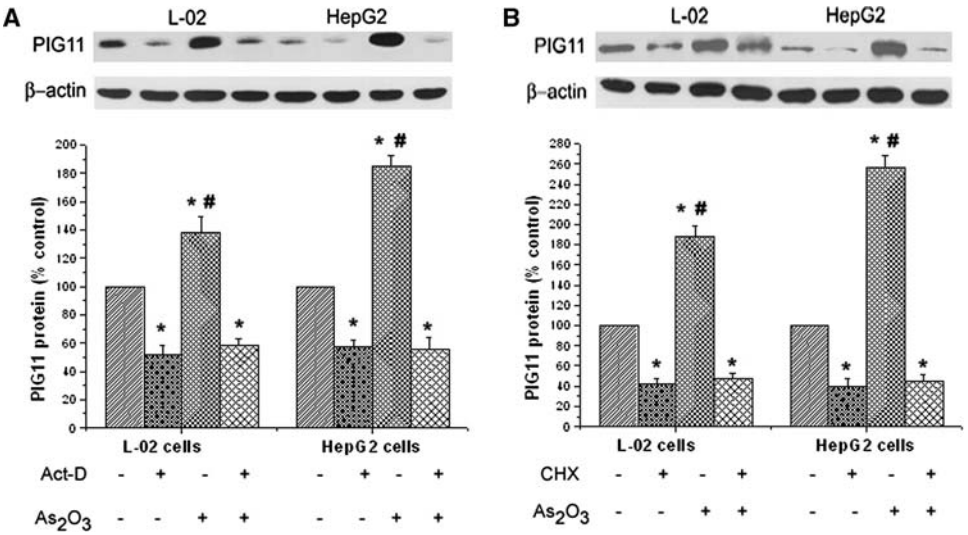


**Fig. 5** **a**, **b** Effects of inhibitors on $As_2O_3$-induced PIG11 expression. **a** Cells were pretreated with 5 μg/ml of actinomycin D (*Act-D*), to inhibit further RNA synthesis, immediately before treatment with 12 μM $As_2O_3$. **b** Cells were pretreated with 10 μg/ml of cycloheximide (*CHX*), to inhibit further protein synthesis, immediately before the addition of 12 μM $As_2O_3$. Data are means ± standard deviation of three independent experiments. * $P < 0.01$, compared to control; #$P < 0.01$, compared to respective Act-D or CHX-alone treatment

Our experimental data showed that the constitutive level of PIG11 protein in L-02 cells was higher than that in HepG2 cells. The marked DNA ladder revealed that the percentage of apoptotic cells in HepG2 cells treated with $As_2O_3$ was obviously higher than that in L-02 cells treated in the same way. The apoptotic rate of HepG2 cells treated for 72 h was 3.26 times higher than that of L-02 cells. These data suggest that HepG2 cells were more sensitive to $As_2O_3$-induced apoptosis than L-02 cells. The lower expression of PIG11 could account for the higher sensitivity to $As_2O_3$ treatment in HepG2 cells compared with L-02 cells. Western blot analysis indicated that the upregulation of PIG11 mRNA and protein was greater in $As_2O_3$-induced apoptosis in HepG2 cells than in L-02 cells. Therefore, we can compare the expression levels of PIG11 induced by $As_2O_3$ in these cell lines. It appears that the apoptosis rate of the cells may be related to the amount of PIG11expression.

Of note, PIG11 overexpression may simply represent an epiphenomenon not directly related to the higher sensitivity of HepG2 cells to the induction of apoptosis. The use of pharmacological inhibitors of transcription/translation (e.g., Act-D or CHX) to inhibit PIG11 overexpression in HepG2 cells can help in clarifying this important point. Act-D binds to DNA at the transcription initiation complex and inhibits RNA synthesis, while CHX is an inhibitor of protein synthesis, which blocks translational elongation on ribosomes. Our results showed that Act-D reduced constitutive PIG11 levels in HepG2 cells by twofold. $As_2O_3$ increased PIG11 levels in HepG2 cells by 1.9-fold and in L-02 cells by 1.4-fold. CHX inhibited the constitutive expression of PIG11 in HepG2 cells by more than 60%. In a similar manner, the $As_2O_3$-mediated induction of PIG11 levels in HepG2 cells was higher than that obtained in L-02 cells with CHX. Thus, these results further demonstrate that the $As_2O_3$-mediated induction of apoptosis requires new protein synthesis, and an increase of PIG11 expression is involved in the cell apoptosis induced by $As_2O_3$.

To further confirm the important role of PIG11 in carcinogenesis and $As_2O_3$-induced apoptosis, we tested the effects of PIG11 siRNA on PIG11 expression. Our results clearly showed that the inhibition of PIG11 by siRNA greatly decreased the PIG11 expression induced by $As_2O_3$ and partially prevented $As_2O_3$-induced apoptosis. As shown in Fig. 6c, we found that the knockdown of the PIG11 gene did not appear to have affected the basal cell growth without $As_2O_3$ treatment. This result agrees with the above results for Act-D and CHX, suggesting the participation of many proteins through different signaling pathways in the process of apoptosis induced by $As_2O_3$. Our results indicate that the adaptive response of PIG11 proteins, not the constitutive level of PIG11 in cells, may be an important factor in higher cell sensitivity to $As_2O_3$-induced apoptosis. The $As_2O_3$-mediated induction of apoptosis requires new protein synthesis. This finding suggests that the tumor suppressor gene *PIG11* plays a role only when cells are exposed to apoptotic stimuli, including $As_2O_3$. Further studies need to elucidate the role of $As_2O_3$ in the adaptive response of PIG11 proteins and the interaction of PIG11 and related proteins.

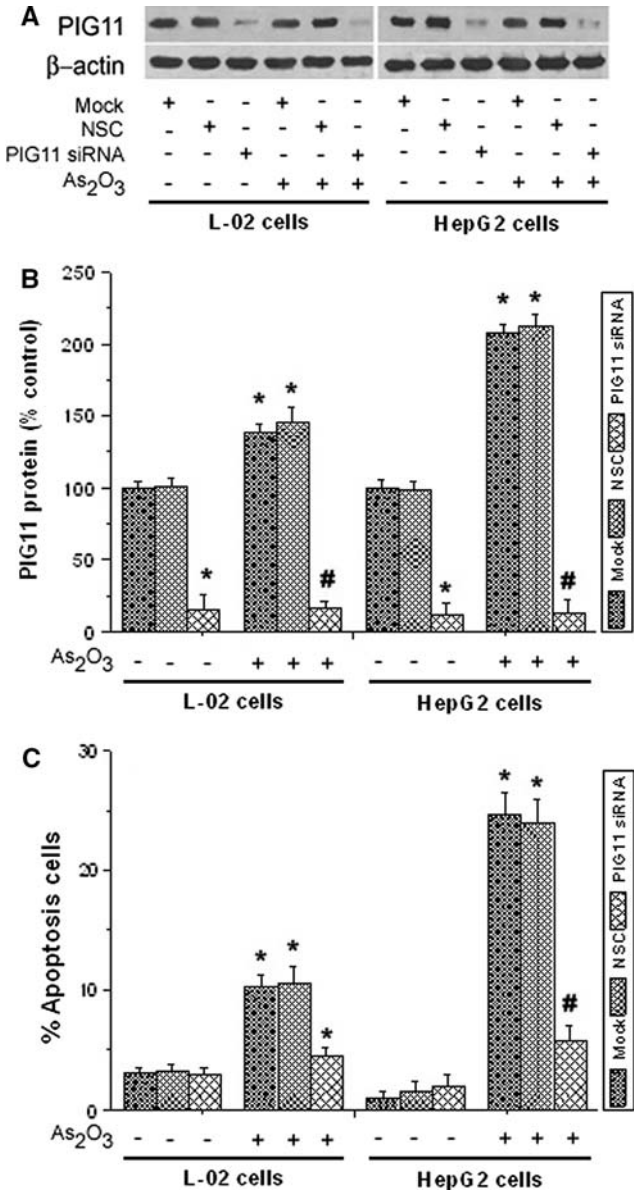


**Fig. 6** **a**, **b** Suppression of PIG11 prevents arsenate-induced PIG11 expression in cells. Cells were transfected with PIG11 siRNA, nonspecific control siRNA (*NSC*), or mock transfected. After 24 h cells were treated with 12 μM $As_2O_3$ for 48 h and harvested for determination of PIG11 protein level. **a** Suppression of PIG11 was confirmed via Western blot of the samples. **b** Cells transfected as in (**a**) were assayed for PIG11 protein level. Data values are means ± SD of three independent experiments; * $P < 0.01$, compared to mock control. **c** Cells transfected as in (**a**) were assayed for apoptosis. Data values are means ± SD of three independent experiments; * $P < 0.01$, compared to mock control

Other tumor suppressor genes could also inhibit cellular growth through induced apoptosis. We have observed that p53 protein levels increased with the $As_2O_3$ dose increase in $As_2O_3$-treated cells, and the $As_2O_3$-induced activation of p53 protein was inhibited by *N*-acetylcysteine. At the same time, the expression of PIG11 was also inhibited (unpublished data). There was a positive correlation between the expression levels of PIG11 and p53. The *PIG11* gene, as a p53 downstream target gene, is related to p53-mediated apoptosis finishing through a three-step process: the transcriptional induction of redox-related genes; the formation of ROS; and the oxidative degradation of mitochondrial components, leading to cell death. Many studies have confirmed the participation of ROS in the process of apoptosis [20–22]. Most PIGs encode proteins that generate or respond to oxidative stress, such as PIG1 (galectin-7), PIG6, PIG8, and PIG12 [1, 3–5], so that it is possible that all of them are redox-related genes [1, 23, 24]. Overexpression of PIG11 could induce cell apoptosis at low levels and enhance the apoptotic effects of $As_2O_3$; this process involves the intracellular generation of ROS [13]. Herein, we showed that PIG11 expression increased the sensitivity of $As_2O_3$-induced apoptosis by further increasing ROS generation, in turn leading to oxidative damage to mitochondria in p53-induced apoptosis. Expression of PIG11 may augment the apoptotic effects of $As_2O_3$ in tumor cells that are less sensitive to $As_2O_3$, resulting in more efficacious treatment.

## Conclusion

In the present study, we found that the expression of PIG11 protein was downregulated significantly in HCC tissues and HCC cell lines. HepG2 cells with lower PIG11 expression were more sensitive to $As_2O_3$-induced apoptosis than L-02 cells with higher PIG11 expression. The expression of PIG11 mRNA and protein was more highly upregulated in HepG2 than in L-02 cells. Knockdown of PIG11 inhibited PIG11 expression and partially prevented $As_2O_3$-induced apoptosis in both these cell lines. These findings further demonstrated that the *PIG11* gene may be involved in $As_2O_3$-induced apoptosis in HepG2 cells and suggested that the adaptive response of PIG11 expression is one of the important factors in enhancing cell sensitivity to $As_2O_3$-induced apoptosis.

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