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ORIGINAL PAPER

PIG11 is Involved in Hepatocellular Carcinogenesis and Its Over-expression Promotes Hepg2 Cell Apoptosis

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Abstract PIG11 (p53-induced gene 11) is a p53 target gene and candidate tumour suppressor gene. In this study, the expression of PIG11 protein was detected in human hepatocellular carcinoma (HCC) and normal liver tissues with an immunohistochemical method. Compared with expression in human normal liver tissues, the expression of PIG11 protein was significantly down-regulated in human HCC tissues. In addition, a recombinant pLXSN-PIG11 retroviral vector was constructed and transfected into HepG2 cells (human hepatocellular carcinoma cell line) and the role of PIG11 in apoptosis was analyzed. The percentage (18.60%) of apoptotic cells transfected with pLXSN-PIG11 was higher than that in cells transfected with pLXSN only (6.03%) or the vehicle control (3.81%) ($P<0.01$). DNA gel electrophoresis showed a clear DNA ladder in pLXSN-PIG11-infected HepG2 cells. Our results suggested that the PIG11 gene is involved in carcinogenesis and development of hepatocarcinoma. Therefore, PIG11 is considered to be a new candidate liver tumour suppressor gene, and may play an important role in tumour suppression through promotion of cell apoptosis.

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Introduction

PIG11 (p53-induced gene 11), also referred as TP53I11 (tumour protein p53 inducible protein 11), is a tumour suppressor gene belonging to the p53-induced protein gene (PIG) family. First reported and named by Polyak et al [1], these were early transcriptional targets of p53, detected by the SAGE technique after over-expression of p53. PIG11 was significantly induced by wild-type p53 both in H1299 cells [2] and in ECV-304 cells [3]. Recent investigations suggested that PIG11 was up-regulated during the induction of apoptosis or during cell growth inhibition by multiple chemo-preventive agents. PIG11 was also one of the apoptotic genes induced by curcumin in human breast cancer MCF-7 cells [4]. Chiba et al found that PIG11 exhibited substantial induction following trichostatin A-induced cell growth inhibition in multiple hepatoma cell lines [5]. Our previous studies demonstrated that PIG11 was markedly up-regulated during arsenic trioxide-induced apoptosis of MGC-803 cells [6]. PIG11 was also found to bind to dsDNA in a sequence-independent manner *in vitro* [7].

The PIG11 gene has been localized to human 11p11.2 and the protein itself consists of 121 amino acids, has a molecular weight 12.904 kDa and a PI of 8.24. Analysis for sequence homology of PIG11 by BLAST revealed no similar sequences, nor could any domains, repeats, motifs or features be predicted with confidence using SMART analysis [8]. These findings suggested that PIG11 may be a member of a novel gene family involved in the regulation of apoptosis.

Several candidate liver tumour suppressor genes from the human 11p11.2 region, including PIG11, have been identified using a functional model of tumour suppression The transcript for PIG11 appears to have been lost or decreased significantly in some human hepatocellular carcinomas (HepG2 and Hep3B) [9]. All of these observations suggest that PIG11 may be a potential candidate as a liver tumour suppressor gene.

Recently, we succeeded in expressing the PIG11 protein in E.coli by genetic engineering technology, and prepared a polyclonal rabbit anti-human antibody to PIG11. In the current study, we have detected the expression of the PIG11 protein in HCC tissues and in normal liver tissues employing immunohistochemical methods. In addition, a recombinant pLXSN-PIG11 retroviral vector was constructed and transfected into HepG2 cells to allow analysis of apoptosis in the transformed cells.

Materials and Methods

Sample Collection

The study adhered to the laws of China and the guidelines approved by the ethics committee of China. Ten human normal liver tissues and 22 HCC tissues were obtained from Department of Pathology, The First Affiliated Hospital, University of South China. All samples were formalin-fixed and paraffin-embedded for advanced pathologic examination and immunohistochemical study. Normal liver tissue samples were obtained from surgical operations that included one liver rupture, three chronic hepatitis and six hepatolithiasis surgeries. All HCC samples were from surgical operations performed on HCC patients, diagnosed on the basis of histopathological examination.

Immunohistochemical Determinations

Expression of PIG11 protein in human normal liver tissues and in HCC tissues was determined using a standard streptavidin-horseradish peroxidase (S-P) technique (UltraSensitive^{TM} S-P kit, Fuzhou Maixin Biotechnology Development Co., Ltd., Fushou City, China), according to the manufacturer's protocol. PBS (phosphate-buffered saline) substituting for PIG11 was used as negative control. Rabbit polyclonal anti-PIG11 1:800 was diluted with PBS and incubated at room temperature for 1 h.

Semi-quantitative analysis was performed as follows: Images (magnification×400) were obtained on a PIPS-2020 tall acuity colour pathology image analysis system (Chongqinghaitian Medical Equipment Co., Ltd.). Each section was photographed in 2~3 random fields. Optical densities (O.D.) of immunohistochemistry images were also estimated by this system. The O.D. of mesenchyma (M_{OD}, at least five random points) and that of parenchyma ($P_{OD,}$ at least ten random points) were detected for each picture. Relative O.D. (R_{OD}) = M_{OD}-P_{OD}.

Establishment of a Stable HepG2 Cell Line Expressing PIG11

PA317 packaging cells, NIH3T3 cells and HepG2 cells were cultured in DMEM (Gibco,USA) medium with 10% fetal calf serum (Hangzhou Sijiqing Biological Engineering Materials Co, Ltd, Hangzhou City, China) in a humidified atmosphere of 95% air and 5% CO_2 at a temperature of 37^{o}C. PCR primers were designed and synthesized according to human PIG11 cDNA sequences (GenBank) including EcoR I and BamH I restriction sites with the following sequences:

Forward 5'-GCGAATTCCAACACCGATGCACACA-3'
Reverse 5'-CGCGGATCCTAGGCAGCTCTTTAGG-3'.

The PIG11 gene was amplified from the plasmid expression vector pcDNA3.1/NT-GFP-PIG11 [8] by PCR. Both the amplified PIG11 gene products and plasmid vector pLXSN were digested with the restriction endonucleases, EcoR I and BamH I (MBI, Lithuania), and linked with T4DNA ligase (Takara, Dalian, China). The new recombinant plasmid vectors were transferred into competent DH5α bacteria to get a recombinant pLXSN-PIG11 retroviral vector and sub-cloned PCR fragments were confirmed by DNA sequencing analysis (Shanghai Sangon Bio CO., Ltd, China).

PA317 packaging cells were used to package the recombinant retrovirus. Cells were seeded onto a 24-well plate at 1.5×10^5 cells per well and incubated for 24 h. The cells were allowed to grow to 95% confluence and were then transfected with recombinant retroviral vector pLXSN-PIG11 or pLXSN using LipofectamineTM2000 (Invitrogen, USA). Transfected PA317 cells were further cultured with G418 (500 μg/ml) (BBI, Canada) selecting medium. Viral medium was filtered through a 0.45 μm filter and then stored at −80°C. Viral titres were estimated indirectly by infecting NIH3T3 cells with different volumes of retroviral-containing supernatant.

HepG2 cells were seeded onto a 6-well plate and infected by viral medium of high titre. One millilitre of retroviral medium containing 8 μg/ml polybrene (Chemicon, USA) was added to each well. The mixtures were incubated for 6 h, then 3 ml fresh medium was added. On the day following infection, cells were passaged at a 1:2 ratio into selective medium containing G418 (600 μg/ml). When cloned HepG2 cells were marked, the concentration of drug for screening was lowered and the HepG2 cells were transferred into a new culture bottle for cell growth.

Detection of pLXSN-PIG11 Expression

PIG11 protein expression was detected by western blot analysis. Protein extraction was carried out using standard methods from the literature [7] and proteins were loaded onto 15% SDS-polyacrylamide gels. The primary antibody against PIG11 was added at a final dilution of 1:200 in TBS-5% milk powder-1% Tween 20. Blots were re-probed with β-actin (Santa Cruz,USA). The secondary anti-rabbit horseradish peroxidase-labelled antibody (1:5,000) was visualized by enhanced chemiluminescence.

Apoptosis Assays

The cells (HepG2, pLXSN-HepG2 and pLXSN-PIG11-HepG2) were harvested in cold PBS, fixed in 700 mL/L ethanol, and stored at 4°C for subsequent analysis of cell apoptosis. Fixed cells were washed twice with PBS and 15 μl RNaseA (10 g/L) was added. After 3–5 min, the cells were resuspended in 400 μl PI staining reagent (50 mg/L). Samples were incubated in the dark for 30 min before apoptosis analysis by FACS (Coulter EPICS XL flow cytometry.USA).

For investigation of DNA laddering, low molecular size DNA was isolated using a DNA ladder extraction kit (Beijing BioDev-Tech. Scientific & Technical Co. Ltd., Beijing City.China) and total DNA was analyzed by 1.5% agarose gel electrophoresis. The gels were stained with ethidium bromide and the DNA fragments visualized under ultraviolet light were photographed.

Statistics Results were analyzed by the SPSS11.5 statistical software package. All data were expressed as means ± S.D. (standard deviation). Comparisons between different groups were made by one-way ANOVA or the Student's t-test. $P<0.05$ was taken as being statistically significant.

Results

Expression of PIG11 Protein in Human Normal Liver and Hepatocellular Carcinoma (HCC) Tissues

Positive results were shown as brown-stained particles in the cells, using the S-P immunohistochemical method. PIG11 protein was primarily located in the cytoplasm. The expression of PIG11 protein was clearly shown in normal liver tissues and was significantly down-regulated in HCC tissues (Fig. 1). Semi-quantitative analysis using a pathology image analysis system indicated that expression of PIG11 protein in HCC tissues was significantly lower than that in normal liver tissues ($p<0.01$, Table 1)

Identification of Stable Expression of PIG11 in HepG2 Cells

In order to look for a biological role for PIG11, a fusion gene was constructed consisting of pLXSN linked to the PIG11 gene coding sequence. The recombinant retroviral vector was identified by its DNA sequence and HepG2 cells were infected with a pLXSN-PIG11 vector or a pLXSN vector. After selection culture, a HepG2 cell clone containing pLXSN-PIG11 was obtained. Before infection, the viral titre was determined by infecting NIH3T3 cells with retrovirus; the results showed that the titre of PA317 packaged recombinant retrovirus pLXSN-PIG11 was 1×10^5 cfu/ml.

The expression of PIG11 protein was detected by western blotting and was found to be much higher in HepG2 cells transfected with pLXSN-PIG11 than in cells transfected with pLXSN only. No change was noted in the levels of β-actin (an abundant protein used as a control) (Fig. 2a and b).

Over-expression of PIG11 Protein Induced Apoptosis in HepG2 Cells

Apoptotic cells were quantified by FACS as the proportion of cells that had a DNA content of less than 2N (sub-G1DNA content) (Fig. 3a). FACS analysis indicated a significantly higher ($P<0.01$) percentage of apoptotic cells in pLXSN-PIG11-transfected cells (18.60%) than in pLXSN-only (6.03%) or vehicle control (3.81%) cells. (Fig. 3b). The results of DNA gel electrophoresis showed a clear DNA ladder in the HepG2 cells transfected with pLXSN-PIG11, which was not evident in cells transfected with pLXSN only or the vehicle control (Fig. 3c). Taken together, the results indicated that over-expression of PIG11 could induce apoptosis in HepG2 cells.

Discussion

PIG11 is one of the downstream target genes of p53 and is directly transactivated by p53 [1, 10].The PIG11 gene has been localized to human 11p11.2 and displays an unusual structure, apparently containing only one exon. Recently, several candidate liver tumour suppressor genes from this region have been identified using a functional model of tumour suppression. It was reported that the transcript for PIG11 was lost, or significantly decreased, in some human hepatocellular carcinomas (e.g., HepG2 and Hep3B) [9]. This suggested PIG11 as a candidate liver tumour suppressor gene existing in this chromosome region. Chiba et al [11] found, using a cDNA microarray, that eight genes, one of which was PIG11, exhibited substantial induction (ratio>2.0) following histone deacetylase (HDAC) inhibitor, trichostatin

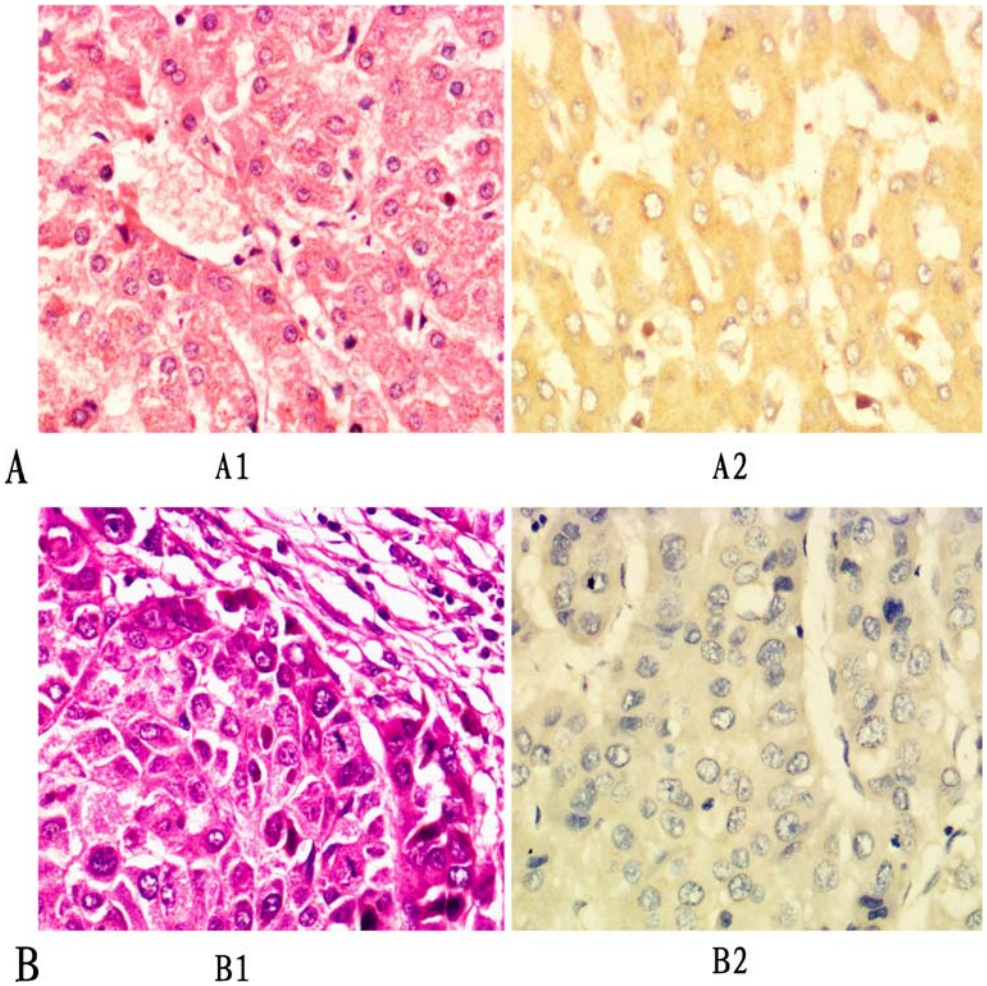

Fig. 1 Expression of PIG11 protein in normal human liver tissues and HCC tissues (original magnification×400) **a** normal liver tissues, *A1*: HE staining; *A2*: S-P immunohistochemical method. This figure shows that PIG11 protein was primarily present in the cytoplasm, showing anintense positive expression in normal liver tissues. **b** HCC tissues, *B1*: HE staining; *B2*: S-P immunohistochemical method. Staining of cells was very weak in HCC tissues

A (TSA) induced apoptosis in human hepatoma cell lines, including HuH7, Hep3B, HepG2, and PLC/PRF/5. This evidence indicated that the PIG11 gene was possibly a liver tumour suppressor gene and that its function was related to cell apoptosis.

In this study, we found that the expression of PIG11 protein was significantly down-regulated in hepatocellular carcinoma tissues using immunohistochemical methods ($p<0.01$). The results were consistent with the common observation that the protein expression of tumour suppressor genes, such as p53 and p16, are usually lost or down-regulated in malignant tumours [12, 13]. This suggested that the PIG11 gene was a candidate liver tumour suppressor gene and was involved in tumourigenesis and development of hepatocarcinoma. To identify that PIG11 was a tumour suppressor gene, its mutation frequency in tumour cells and cellular growth inhibition *in vivo* and *in vitro* were further investigated.

Table 1 Expression of PIG11 protein in normal human liver tissues and HCC tissues

Groups	Numbers(n)	Relative optical density values ($\bar{\chi} \pm s$)
Normal liver tissues	10	65.15±8.64
HCC tissues	22	40.69±12.92*

*$p<0.01$ vs. Normal liver tissues

Other tumour suppressor genes can inhibit cellular growth through induction of apoptosis [14]. To investigate whether over-expression of PIG11 induced cell apoptosis, a pLXSN-PIG11 retroviral vector was constructed and identified by DNA sequencing analysis. Using retrovirus mediated gene transfer and G418 selection, a HepG2 cell line stably expressing PIG11 was created. Expression of PIG11 protein in HepG2 cells transfected with pLXSN-PIG11 was confirmed by western blotting. Our results showed that the percentage of apoptotic cells was higher in cultures of pLXSN-PIG11-infected cells (18.60%) than in cells transfected with pLXSN only (6.03%) or the vehicle control (3.81%). The results of DNA gel electrophoresis showed a clear DNA ladder in HepG2 cells transfected with pLXSN-PIG11. Thus, over-expression of PIG11 could induce apoptosis in HepG2 cells.

The PIG11 gene, as a p53 downstream target gene, most likely operates in p53-mediated apoptosis through a three-

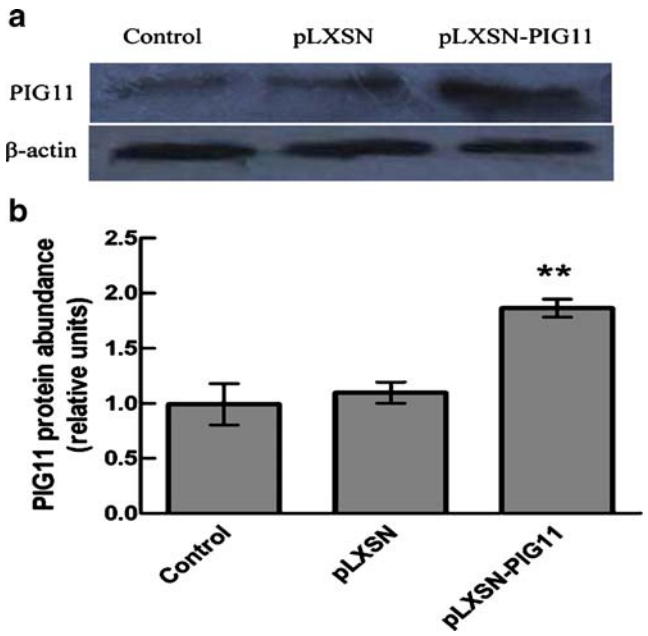

Fig. 2 Identification of stable expression of PIG11 in HepG2 cells **a** Expression of PIG11 was monitored by Western blotting. Expression of PIG11 protein in HepG2 cells transfected with pLXSN-PIG11 was much higher than that in cells transfected with pLXSN only and the vehicle control. **b** Data represent the means ± S.D (n=3). **P<0:01vs Control

step process: the transcriptional induction of redox-related genes; the formation of ROS; and the oxidative degradation of mitochondrial components, leading to cell death. In general, the PIGs are direct downstream targets induced by p53 and therefore trigger apoptosis through a p53-dependent pathway [15]. As such, they appear to encode proteins that generate or respond to oxidative stress [1, 16], so that it is possible that all of these are redox-related genes [1]. Through a p53-mediated cascade, redox-related genes such as PIG11 are transcriptionally induced, resulting in the production of ROS, which in turn leads to oxidative damage to mitochondria and apoptosis.

Much research has confirmed the participation of ROS in the process of apoptosis. Generation of ROS could lead to apoptosis, mediated through mitochondrial swelling with subsequent opening of the inner mitochondrial PT pore, release of cytochrome *c* (Cytc) and other apoptosis-inducing factors (AIFs) across the inner mitochondrial membrane to the cytoplasm, up-regulation of Bax expression, activation of the caspase cascade and ultimately, apoptotic cell death. After PT pore opening, ROS, as mediators of calcium signalling, could cause calcium leakage and mitochondrial electron transport chain uncoupling, promoting further generation of ROS and leading to apoptosis [17, 18]. In addition, ROS could cause apoptosis through activation of JNK of the

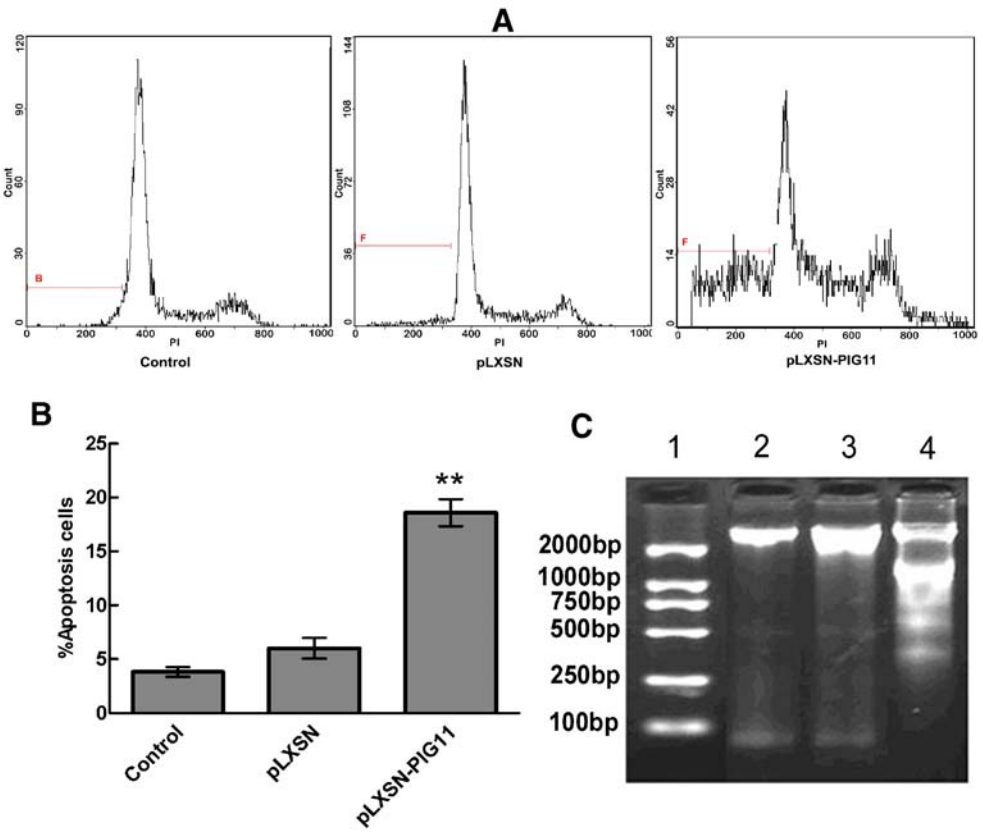

Fig. 3 Over-expression of PIG11 induces apoptosis in HepG2 cells **a** Apoptotic cells were quantified by FACS as the proportion of cells that had a DNA content of less than 2N (sub-G1DNA content). The sub-G1DNA content in HepG2 cells transfected with pLXSN-PIG11 was higher than others. **b** Data represent the means ± S.D. The percentage of apoptotic cells was obtained from three independent experiments. **P<0:01vs Control. **c** DNA ladder formation. The HepG2 cells transfected with pLXSN-PIG11 show a typical DNA ladder on agarose gel electrophoresis, *Lane 1*: marker; *lane 2*: HepG2; *lane 3*: pLXSN-HepG2; *lane 4*: pLXSN-PIG11-HepG2

Ras pathway, or p38 and Erk of the MAPK signalling pathway [19].

In conclusion, down-regulation of PIG11 protein was involved in carcinogenesis and development of hepatocarcinoma. PIG11, a new candidate liver tumour suppressor gene, may play an important role in tumour suppression through the promotion of cell apoptosis.

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