Effects of p16 gene on biological behaviours in hepatocellular carcinoma cells

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Received: 2002-06-11 Accepted: 2002-07-12

Abstract

AIM: To investigate the effects of p16 gene on biological behaviours in hepatocellular carcinoma cells.

METHODS: HCC cell lines SNU-449 and HepG2.2.15 were infected respectively by a replication defective, recombinant retrovirus capable of producing a high level of p16 protein expression (pCLXSN-p16). G418 resistant stable p16 protein expression cell lines were selected. And the biological behaviours of the p16 gene transfected HCC cells were observed.

RESULTS: Initial in vitro experiments in HCC cell line SNU-449 with loss of p16 protein expression demonstrated the pCLXSN-p16 treatment significantly inhibited cell growth. But there was no treatment effect when the pCLXSN-p16 was used in another HCC cell line HepG2.2.15 which has positive p16 protein expression. Subsequent study in a nude mouse model demonstrated that the p16 gene transfected SNU-449 had a lower succeeding rate in the first time establishment of tumors and grew more slowly in the nude mice when compared with non-transfected SNU-449. Moreover, the nude mice inoculated with transfected SNU-449 had a longer surviving time than those inoculated with non-transfected SNU-449.

CONCLUSION: Our results show that the p16/INK4a gene transfer can inhibit the proliferation and reduce the invasion ability of hepatocellular carcinoma.

Huang JZ, Xia SS, Ye QF, Jiang HY, Chen ZH. Effects of p16 gene on biological behaviours in hepatocellular carcinoma cells. World J Gastroenterol 2003; 9(1): 84-88

http://www.wjgnet.com/v1007-9327/9/84.htm

INTRODUCTION

Hepatocellular carcinoma (HCC) is a common malignant tumor with a increasing incidence[1], and the treatment has been extremely difficult. HCCs are generally highly resistant to chemotherapeutic agents and radiotherapy. Despite a variety of treatment options including surgical resection, chemoembolization, percutaneous injection of ethanol or acetic acid, and liver transplantation, the prognosis of HCC patients is poor[2]. Thus, new treatment strategies are necessary to improve the survival rate. It is believed that molecular changes that occur during carcinogenesis, such as overexpression of the multidrug resistant gene and the loss of tumor suppressor genes may allow many kinds of tumor cell populations to become resistant to most therapeutic approaches. Gene therapy may offer certain advantages in the treatment of HCC.

P16 gene encoded for a protein that was initially identified as a specific inhibitor of the CDKs, CDK4 and CDK6. By binding to and inhibiting CDK4 and CDK6, p16 prevents both pRb phosphorylation and subsequent progression into the S phase of the cell cycle. P16 gene alterations were found in many kinds of cancers[3-13]. Our previously study has showed that the frequency of P16 gene inactivation was high (36 %)[14] in HCC. Other’s Reports have also showed frequent P16 gene inactivations[15-21]. Thus, P16 gene may be an ideal candidate for the treatment of HCC. In this study, we constructed the retrovirus p16 expression vector and then investigated whether it could control the proliferation of HCC cell lines.

MATERIALS AND METHODS

P16 cDNA subcloning and construction of pCLXSN-p16

The original P16 cDNA was amplified from the total RNA of normal human lymphocytes by reverse transcription PCR. The PCR product was subcloned into pCLXSN expression vector, a deficient retrovirus vector which derived from pLXSN (IMAGENEX). Then the recombined p16 expression vector was cotransfected with the pCL-Ampho packaging construct into 293 cells by using a modification of the HEPES-buffered saline calcium phosphate method[22]. Two days after infection the 293 cells were placed under G418 selection (800 µg/ml) and grew for 10 days. Then the G418 resistant 293 cells were amplified and the supernatant which contained the pCLXSN-p16 pseudo retrovirus were collected and filtered through a 0.45 µm pore size filter. The pseudo retroviruses were titred through infecting NIH 3T3 cells[22]. We also packaged a reporter gene expression vector pCLMFG-LacZ as a control. The viral titer was 1.6×10⁶CFU/ml.

Cell lines

Human HCC cell lines SNU-449 (with loss of p16 expression) and HepG2.2.15 (with p16 expression) were kindly provided by Dr. Zhang Mingyi (Tumor Hospital of Sichuan Province, Chengdu, China,) and Dr. Gao Yong (the Union Hospital, Tong Ji Medical University, Wu han, China) respectively. They were grown in RPMI 1640 containing 10 % fetal bovine serum.

Infection of retroviral vectors and selection of stable expression cell lines

SNU-449 and HepG2.2.15 cell lines were seeded and cultured in two 6 cm plates at a density of 2×10⁴ cells for 24hs respectively. Immediately before infection, the culture medium was replaced and 1 ml viral supernatant of pCLXSN-p16 or pCLMFG-LacZ (control) was added. Then the polybrene was added to the medium in 8 µg/ml. The infected cells were grown for 24hs and splited into 10 cm plates at 1:10 dilution, then grown in the medium containing G418 in 800 µg/ml. The
medium was replaced every 3-4 days and the G418 resistant cells were selected and named SNU-449/pCLXSN-p16, HepG2.2.15/pCLXSN-p16, SNU-449/pCLMFG-LacZ and HepG2.2.15/pCLMFG-LacZ, respectively.

**Immunohistochemical analysis and western blotting**

SNU-449 cells were collected 24hs after infected by pCLXSN-p16. The stable G418 resistant SNU-449/pCLXSN-p16 cells were also collected. Both of them were examined for p16 expression immunohistochemically by the labeled streptavidin-biotin method. The SNU-449/pCLXSN cells were also screened for p16 expression by Western blotting. The cells for immunohchemical analysis were grown on the glass slides.  

Immunohistochemical detection procedure was carried out according to the manufacture's recommendations. Western blotting was performed as previously described[23].

**Growth curves**

The effect of p16 expression on cell growth was examined in all six cell lines, including SNU-449, SNU-449/pCLXSN-p16, SNU-449/pCLMFG-LacZ, HepG2.2.15, HepG2.2.15/pCLXSN-p16, and HepG2.2.15/pCLMFG-LacZ. Triplicate samples of log phase cells were seeded at a density of 1×10^4 in 6 holes culture plates. Cells were harvested for counting by digestion with trypsin/EDTA for 3 min, followed by the addition of fresh media to inhibit further digestion. Cells were then pelleted by centrifugation at 1000rpm for 5 min, followed by resuspension in fresh media. Triplicate sets of cells were counted at 1, 3 and 5 days after seeded. The mean cell number for each harvesting was calculated, and cell growth curves were determined.

**Cell cycle and apoptosis analysis**

The effect of pCLXSN-p16 expression on cell cycle dynamics and cell apoptosis was examined in both the SNU-449/pCLXSN-p16 and HepG2.2.15/pCLXSN-p16 cell lines using FACs analysis. In the meanwhile, the cell cycle distribution and apoptosis analysis of SNU-449, SNU-449/pCLMFG-LacZ and HepG2.2.15 cells were also examined for controls. Briefly, 1×10^6 cells of each cell line were collected by centrifugation at 1000 rpm for 5 min, followed by two washes in ice-cold PBS, Cells were then fixed in 2.0 ml of 70 % ethanol and stored at 4°C for a minimum of 1 h. Prior to FACs analysis, cells were washed twice with ice-cold PBS, and the cell pellet was resuspended in 10 μg/ml of propidium iodide (Sigma Chemical Co.). Add 100 μg/ml of Rnase (Sigma Chemical Cop.) and incubated at 37°C for 30 min. Then the FACs analysis was performed.

**In vivo studies**

Nude mice were used at the weight of 23-27 grams. Log phase SNU-449/pCLXSN-p16 and SNU-449 cells were harvested and centrifuged at 1000 rpm for 5 min, then resuspended in 0.9 % salt solution at 1×10^6, respectively. Cells were immediately implanted subcutaneously into the right flank of nude mice (1×10^6 cells for each animal). Tumor formation and subsequent growth were monitored. The tumor volume at 23 days after implantation and the survival rate of the mice at 52 days after implantation were also observed. Statistical significance was assessed by Mann-Whitney statistical analysis (Statmost for windows).

**RESULTS**

**Detection of p16 protein expression**

SNU-449 cells exhibited a loss of p16 protein expression (Figure 1), and HepG2.2.15 cells showed positive p16 staining. About 20 % SNU-449 cells showed positive p16 staining 24 hours after infected by pCLXSN-p16 pseudo virus. All the G418 resistant SNU-449/pCLXSN-p16 cells exhibited p16 protein expression (Figure 2). Western blot analysis also confirmed that SNU-449 cells showed loss of p16 protein expression and SNU-449/pCLXSN-p16 cells could express p16 protein (Figure 3).

**Figure 1** SNU-449 cells show negative p16 staining (original magnification ×400).

**Figure 2** SNU-449/pCLXSN-p16 cells show positive p16 staining (original magnification ×200).

**Figure 3** Western blot analysis. Lanes 1 and 3: SNU-449/pCLXSN-p16 cells; Lane 2: SNU-449 cells.

**Figure 4** In vitro growth curves of SNU-449/pCLXSN-p16, SNU-449/pCLMFG-LacZ and SNU-449 cell lines.
Cell volume in nude mice injected with SNU-449/pCLXSN-p16 cell line, a selected stable p16 protein expression cell line, were significantly inhibited when compared with cell lines SNU-449 and SNU-449/pCLMFG-LacZ in which p16 protein expression was lost. Nearly equal growth rates were observed in both HepG 2.2.15 and HepG 2.2.15/pCLXSN-p16 cell lines.

Cell cycle distribution and cell apoptosis status

The underlying mechanism of in vitro growth inhibition seen in the pCLXSN-p16 treated cell line SNU-449/pCLXSN-p16 was investigated by cell cycle analysis using flow cytometry. As shown in table 1, SNU-449/pCLXSN-p16 cell line exhibited significant increase in the percentage of cells in G<sub>0</sub>-G<sub>1</sub> phase (68 %), consistent with a cell cycle arrest at the G<sub>1</sub> transition when compared with cell lines SNU-449 and SNU-449/pCLMFG-LacZ. However there was no obvious cell cycle difference between cell lines HepG2.2.15/pCLXSN-p16 and HepG2.2.15.

Table 1 Cell cycle analysis

| Cell type                  | G<sub>0</sub>-G<sub>1</sub> | G<sub>2</sub>-M | S  |
|----------------------------|---------------------------|---------------|----|
| SNU-449                    | 37                        | 36            | 27 |
| SNU-449/pCLXSN-p16         | 68                        | 21            | 11 |
| SNU-449/pCLMFG-LacZ        | 39                        | 38            | 23 |
| HepG2.2.15                 | 29                        | 42            | 29 |
| HepG2.2.15/pCLXSN-p16      | 32                        | 43            | 25 |

Cell apoptosis analysis was performed in cell lines SNU-449, SNU-449/pCLXSN-p16, HepG2.2.15 and HepG2.2.15/pCLXSN-p16 and showed no difference between these cell lines.

Effects of pCLXSN-p16 treatment on HCC tumor growth in vivo

The therapeutic potential of pCLXSN-p16 to treat HCC tumors in vivo was studied in nude mice. Tumors were created by injecting 1×10<sup>6</sup> SNU-449 or SNU-449/pCLXSN-p16 cells in 100 µl 0.9 % salt solution into the right flank of nude mice as described above. The succeed rate of tumor establishment at the first time injection of tumor cells SNU-449/pCLXSN-p16 in nude mice was 70 % (7/10). At the same time only 40 % (4/10) nude mice succeeded to establish tumors at the first time injection of SNU-449 cells. Animals who were succeeded to establish tumors were observed over a period of 52 days from the day of injecting tumor cells. During the observing period animals injected with SNU-449/pCLXSN-p16 cell line survived significantly longer than those injected with SNU-449 (P<0.05). The mean survival time of animals injected with SNU-449/pCLXSN-p16 and SNU-449 were 42.1±9 days and 32.7±6.7 days, respectively (Table 2). The survival rate of nude mice injected with SNU-449/pCLXSN-p16 cell line was higher than that of those injected with SNU-449. Figure 5 was the survival curves of nude mice injected with cell lines SNU-449/pCLXSN-p16 and SNU-449, respectively.

Two groups of nude mice with each of 6 animals were established with tumors by injection of cell lines SNU-449/pCLXSN-p16 and SNU-449, respectively and were observed for a period of 23 days. All the animals survived during the observing period. At 23th day after injection of tumor cells they were all sacrificed for measurement of tumor volumes. The tumor volume in nude mice injected with SNU-449/pCLXSN-p16 was 234±125 mm<sup>3</sup>, significantly smaller than that in those injected with SNU-449 cell line (726±513 mm<sup>3</sup>).”}

DISCUSSION

The hypothesis that the inactivation or loss of certain genes, specially tumor suppressor genes, leads to both tumor growth and progression is now well established and provides a unique opportunity in cancer gene therapy. The decision to use p16 gene as a therapeutic agent against human hepatocellular carcinoma was based on our earlier work where we had found p16 gene was frequently inactivated in HCCs[14]. In the fact, P16 gene was widely used in the study of tumor gene therapy and showed obvious tumor suppressing effects in many kinds of tumors[22-31]. In the present study we cloned human p16 cDNA and constructed a replication-defective, recombinant retrovirus that express high levels of the tumor suppressor protein p16.

The pCLXSN vector was modified from pLXSN vector. Its more safe and can produce higher titers of retrovirus when cotransfected a report gene LacZ expression vector pLMFG-LacZ and packaging constructs pCL-Ampho into 293 cells. The transfecting efficiency was determined and only 1 % (data not shown), much lower than reported 30 %[9]. Considering the lower transfecting efficiency we selected a stable virus-producing cell lines after the p16 protein expression vector pCLXSN-p16 was cotransfected with pCL-Ampho into 293 cells. The titer of pCLXSN-p16 retrovirus produced by the stable virus-producing cell line was determined to be 1.6x10<sup>6</sup>CFU/ml. It is much lower than reported 5x10<sup>6</sup>-2x10<sup>7</sup>[22]. Only 20 % SNU-449 cells showed positive p16 protein expression 24hs after infected by the pCLXSN-p16 pseudo retroviruses. We then determined to select a SNU-449 cell line which stably expresses p16 protein after infected by the pCLXSN-p16 and use it to study the treatment effect of p16 gene transfer to the HCC cells.

Initial in vitro experiments demonstrated that the reintroduction of p16 gene significantly inhibited cell growth
by inducing G1-S cell cycle arrest in the SNU-449 HCC cell line in which p16 gene was originally inactivated. In contrast, no inhibition of cell growth was found when wild type p16 gene was transfected into another HCC cell line HepG2.2.15 in which p16 protein was normally expressed. This suggested that inactivation of p16 gene and its downstream gene may contribute to the carcinogenesis of HCC. Our results were similar to others’ reports about treatment study of some tumors using p16 gene transfer. No changes of tumor cell apoptosis were found when wild type p16 gene was introduced to cell lines SNU-449 or HepG2.2.15. This suggested that introduction of p16 gene may not cause apoptosis of HCC cells.

Subsequent in vivo studies showed that the first time establishment of tumors in nude mice was more difficult using p16 gene treated SNU-449 cells (SNU-449;pCLXSN-p16) than using the cells without treatment. This suggested that reintroduction of p16 gene could reduce the invasion ability of HCC tumor cells. Further observation demonstrated that reintroduction of p16 gene into tumor cells significantly inhibited tumor growth rate in tumor-established animals and it will offer a much useful addition to present therapeutic regiments. PCLXSN-p16 may potentially introduce p16 gene could reduce the invasion ability of HCC tumor cells. Further observation demonstrated that reintroduction of p16 gene into tumor cells significantly inhibited tumor growth rate in tumor-established animals and it will offer a much useful addition to present therapeutic regiments. PCLXSN-p16 may potentially

Our results support the use of retrovirus-mediated p16 gene treatment for HCC and provide an important foundation from which to build future preclinical studies and initiate future human clinical trials. If pCLXSN-p16 is proved to be effective in the clinical setting, it will offer a much useful addition to present therapeutic regiments. PCLXSN-p16 may potentially develop into an important adjuvant therapy, expedient in present therapeutic regiments. PCLXSN-p16 may potentially

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Edited by MaJY