The Growth inhibitory Effects by Transfection of Anti-Oncogene P16 on Human Hepatocarcinoma Cell Line*

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ABSTRACT Objective: To investigate the inhibitory effect of tumor suppressor gene p16 on the growth of hepatocarcinoma cell. Methods: p16 cDNA was subcloned into pcDNA3.1 eucaryotic expressing vector; then the recombinant pcDNA3.1-p16 plasmid was transfected into human hepatocarcinoma cell line SMMC-7721 with liposome. MTT method and Western blot were employed to investigate cell growth. Results: The recombinant plasmid pcDNA3.1-p16 was constructed successfully; the growth of hepatocarcinoma cell line SMMC-7721 was obviously inhibited after transfection; the exogenous p16 protein was expressed, with the up-regulation of Bax as well as down-regulation of Bcl-2 and cIAP2. Conclusion: The recombinant plasmid pcDNA3.1-p16 is able to express p16 protein in human hepatocarcinoma cell line SMMC-7721 and suppress the cancer cell growth, which is associated with apoptosis.

Key words: Hepatocellular neoplasm; p16 gene; Gene therapy; Transfection; Liposome

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Introduction Liver cancer is one of the malignant cancers which seriously threaten human health. Many factors may be involved in the development of liver cancer, including inactivation of tumor-suppressor gene. Immunohistochemical results showed that in normal hepatic cytoplasm there was inactivation of tumor-suppressor gene and activation of oncogene involved in tumorigenesis [1]. Targeting at the oncogene activation, tumor-suppressor gene inactivation and apoptosis related genes change, gene therapy treatment already developed from single gene therapy to multiple genes therapy [2-3]. P16 gene is a kind of tumor-suppressor gene focused by many researches. It is also called multiple tumor suppressor 1 (MTS-1) because of its inactivation in suppressing tomor in a wide variety of tumor tissue, including liver cancer [4-5]. In this study, in order to explore the tumor-suppressing effect and mechanism of p16 genes, we successfully transferred the pcDNA3.1 - p16 eukaryotic expression plasmid to human liver cancer cell, and studied its effect on the growth of cancer cells, laying foundation for further study.

1 Materials and Methods

1.1 Main reagents

Rat anti human p 16 monoclonal antibody and Rat anti human Bax monoclonal antibody were products of eBioscience company; Rat anti human Bcl-2 monoclonal antibody and rabbit anti human cIAP2 polyclonal antibody were products from Santcruze company. Plasmid extraction kits were bought from Promega. G418 and liposomes were bought from Gibcol. ECL kits were bought from Merk.

1.2 Tissue samples and cells

Cells were cultured with 1640 with 10% FCS at 37℃, 5% CO_2. Human cancer tissue and its adjacent tissue were collected from liver adenocarcinoma surgery patients, and normal liver tissue were collected from glioma patients (autopsy specimens). Human liver tumor SMMC -7721 cells were preserved by our laboratory, and were cultured with 1640/10% FCS, 37℃, 5% CO2 and conventional passage.

1.3 Plasmid, strain and primer

The recombinant pUC57 plasmids vector containing cDNA sequence of p16 genes and pcDNA3.1 eukaryotic plasmid vector were bought from the Invitrogen company. The E.Coli DH5α strains were preserved by our laboratory. Primers were 5'-GGCT-GTACACTTGCAT CCTT-3', 5'-TGAGGTAAGCTAG-CACTCAG-3'.

1.4 Construction, identification and extraction of the P16 genes recombinant eukaryotic plasmid

Taking the recombinant pUC57 plasmids which includs p16 gene cDNA sequence as the template, we amplified the p16 genes encoding sequence through PCR method. Then the agarose gel electrophoresis were used to identify it. After purification and linkage, the recombinant plasmids bearing the target gene were transferred into E.Coli. DH5a and cultured at 37℃ for 12-16h. Then the colony growth could be observed. Single colonies were selected to be cultured and amplified. Plasmids were extracted out according to the method discribed by Promega company. After the plasmid extraction were identified by enzyme digestion and sequence analysis, we then extracted a large quantity of the plasmid for preparation.

1.5 pcDNA3.1−p16 plasmid transfection and SMMC−7721

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identification

The Lipofectamine mediated DNA transfection were processed according to the kit instruction of Gibcol company, and positive cells were screened out by G418 and cloned. Western blot method was used to detect the expression of cell p16 protein. We prepared three groups: (1)saline groups; (2) pcDNA3.1 wild type plasmid group, i.e. vector group, (3) pcDNA3.1-p16 genes treatment group, namely p16 group. Each group was loaded into six holes in parellel. The saline groups and pcDNA3.1 vector group were set up as control.

1.6 Detecting cell growth by MTT

MTT detection and analysis were carried out according to the literatures [6]: cells were inoculated into and cultured in a 6-orifice plate of 5 x 10^4/cm^2 density, with nutrient solution exchanged every two days. Living cells were counted. MTT test were repeated for 3 times, the result were expressed as Mean + Standard Deviation.

1.7 Detection of expression pattern of apoptosis related protein

According to methods in literature [7], cell proteins were extracted processed with conventional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and PVDF membrane. Changes of apoptosis related proteins were detected, such as Bax molecule, which promotes cell apoptosis and apoptosis inhibiting protein Bcl-2 and cIAP2. An antioxidant (Bax, p16, Bcl-2, cIAP2) was diluted to a concentration of 1:200-500, room-temperature incubated for 90 min; HRP-conjuncted second antibody (goats anti mouse/rabbit IgG - HRP 1:2000) room-temperature incubation 60 min. ECL show color 3-5 min rush chips. After enhancement darkroom.

1.8 Statistical treatment

SPSS10.0 statistical software was applied to conduct single factor analysis of variance among three sets of data. Differences with a statistical significance were presented by $P < 0.05$.

2 Results

2.1 p16 expressions in Human liver cancer, paracancerous tissue, normal liver tissue

p16 was strongly positive expressed in Normal liver tissue , deispayed as yellow particles (figure 1c); while it was weakly positive in the paracancerous tissue, (FIG.1b); But it was negative in liver cancer cells (figure 1a). It could be inferred from the figures that p16 protein expression was significantly lower in cancer tissues than in paracancerous tissue group and normal group.

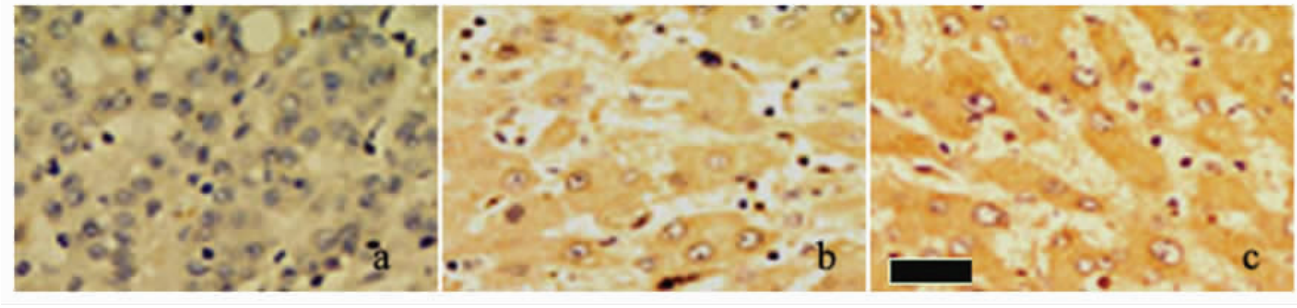

Fig. 1 The expression of p16 protein in liver cancer(1a), paracancerous tissue(1b) and normal liver tissue(1c)

2.2 amplification of p16 genes and construction and identification of pcDNA3.1−p16 recombinant eukaryotic plasmid

Taking the recombinant pUC57 plasmids which includes the full length of p16 gene cDNA sequence as the template, we amplified the whole p16 gene encoding sequence through PCR method (figure 2a). After the wild type pcDNA3.1 plasmid were digested with EcoRI and XbaI we constructed the pcDNA3.1-p16 recombinant eukaryotic plasmid by linking the target segment and the pcDNA3.1. The pcDNA3.1-p16 recombinant eukaryotic plasmid were also double digested with EcoR I and Xba I, and then the target segment was 960bp, as expected (figure 2b). Results from ABI Prism377 sequencing determination showed that the cDNA sequence of the cloned nucleotides p16 genes is consistent , with the right direction, no mutation, insert or lost.

2.3 Expression of the transfected P16 genes in human cancer cell SMMC −7721

The cellular proteins were extracted respectively from the three group and were analyzed on PAGE electrophoresis. Results from Western blot analysis showed (figure 3), the protein expression level of pcDNA3.1-p16 genes treatment group was significantly higher than that of the control group, indicating that exogenous P16 genes has been successfully transfected into hepatumor cells SMMC - 7721 and effectively expressed in it.

2.4 Effects of P16 genes on the proliferation of human liver cell SMMC− 7721

The results from the MTT method showed that after the cells were culterued for 24h ~ 96h, each group showed different degree of increase in cell numbers. But the cell increase in pcDNA3. 1-p16 genes treatment group is not as much as in the control group; Compared with vector group, the cell growth-inhibiting rate [IR = (1 - p16 / vector) x 100%]in the gene treatment group in 24h, 48h,72h and 96h were respectively12.2% , 17.5% , 34.7% and 46.7% (table 1).

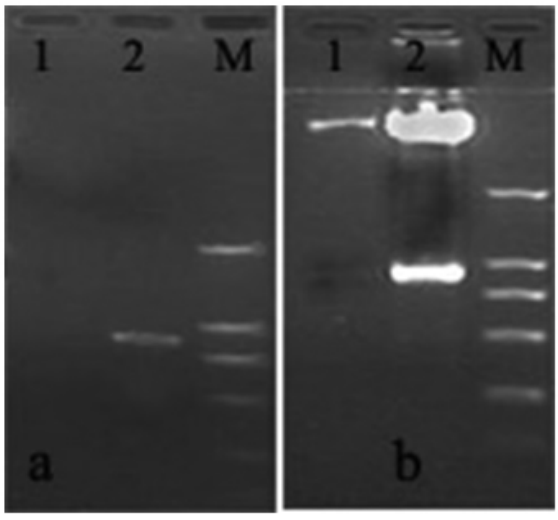

Fig.2 PCR augment cDNA of targeted P16 gene(2a) and identification of the recombinant pcDNA3.1-p16 vector(2b)
(a 1: negative control, 2: cDNA of targeted P16 gene, M:100bp DNA Markers; b 1: negative control; 2: pcDNA3.1-p16 recombinant plasmid were digested with EcoRI and XbaI ; M:100bp DNA Markers)

2.5 Effect of P16 genes expression on the apoptosis of SMMC -7721 hepatumor cells

Western blot results showed that (figure 4) : the Bax expression of p16 treatment group is significantly higher than the two control groups. The Bcl-2 and cIAP2 expression in the two control groups were obviously higher than that in p16 treatment group. These results suggested that after being transfected in SMMC -7721 hepatumor cells, the p16 genes upregulated the expression of Bax and downregulated the expression of Bcl-2 and cIAP2.

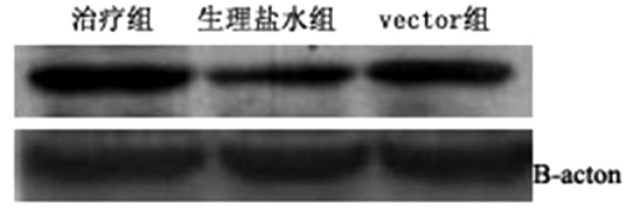

Fig.3 p16 protein expression in human liver cancer cell line SMMC-7721 after transfection

3 Discussion

At present, although the traditional treatments on liver cancer (surgery, radiotherapy, and chemotherapy) have been improved a lot, the survival rate is still very low for cancer patients. Cancer gene therapy may bring hope for patients with hepatocellular carcinoma. In 1994, Kamb etc [4] firstly discovered p16 genes; P16 protein can be combined with CDK4 or CDK6, inhibits the activity of cyclin D and CDK complex, prevents the cells transfered from G period to S period, so that can inhibit cells'proliferation and growth. A wide variety of tumor cells had p16 genes lossing or mutating, but after transfected by p16 genes, the growth of tumor cells were obviously inhibited; So p16 genes is considered to be an important tumor suppressor genes so far. Foreign scholars transfected the p16 genes into tumor cells via adenovirus carrier, and found that the growth of these cells in vivo/ vitro all were restrained; So they con sidered p16 genes as the main candidate genes for cancer gene therapy [8, 9].

Table 1 Effect of p16 gene on proliferation of human liver cancer cell line SMMC-7721(n=6)

Groups	OD ($\bar{X}\pm$ S)			
	24h	48h	72h	96h
Sodium Chloride group	0.621± 0.041	0.768± 0.044	0.807± 0.039	0.822± 0.045
vector group	0.604± 0.043	0.702± 0.045	0.721± 0.030	0.752± 0.036
p16 group	0.531± 0.043	0.578± 0.038	0.449± 0.042	0.401± 0.039
IR	12.2%	17.5%	34.7%	46.7%

In this study, the overall length of cDNA of p16 genes were cloned into pcDNA3.1, and the eukaryotic plasmid vector pcDNA3.1 - p16 were successfully constructed. Mediated by the lipofectamine, eukaryotic plasmid vector pcDNA3.1 - p16 were transfected into hepatumor cells SMMC-7721; then the grouth of SMMC-7721 cells were found restrained obviously with an inhibition rate up to 46.7%. at the mean while, results of western blot methods demonstrated the SMMC -7721 cells have expression of heterologous protein p16, which is obviously higher than that of the two control groups. That is to say, P16 gene has already successfully imported and played a role in inhibiting the growth of tumor cell. Further Western blot results showed that the high expression of p16 upregulated the expression of Bax and down regulated the expression of cIAP2. These results showed that the inhibiting

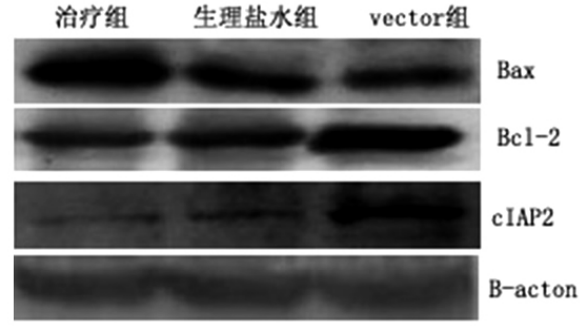

Fig.4 The influence on apoptosis in SMMC-7721 hepatumor cell line by transfection of p16 gene

mechanism of p16 genes was closely related with its induction on cell apoptosis. Our results were similar to the research reported by other scholars[10,11], results from these researches suggested that p16 genes inhibit the growth of hepatumor cells SMMC-7721 after being transfected into cells. This inhibiting effect was highly concerned with its high expression which induced tumor cell apoptosis, but the exact inhibiting mechanism needs further clarification.

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抑癌基因 p16 对人肝癌细胞生长抑制的机制研究 *

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摘要 **目的**:探讨抑癌基因 p16 对肝癌细胞生长的抑制作用及其机制。**方法**:将 p16 cDNA 亚克隆至 pcDNA3.1 真核表达载体上,并经脂质体介导转染至人肝癌细胞株 SMMC-7721。用 MTT 法和 Western blot 分析转染细胞的生长情况。**结果**:成功构建重组表达质粒 pcDNA3.1-p16,转染 pcDNA3.1-p16 的 SMMC-7721 细胞生长速度受到明显抑制,转染后有外源 p16 蛋白的表达,且伴随 Bax 上调,Bcl-2 和 cIAP2 的下调。**结论**:重组 pcDNA3.1-p16 质粒能在人肝癌细胞 SMMC-7721 内表达,且能抑制 SMMC-7721 的生长,其机理与诱导肿瘤细胞凋亡相关。

关键词:肝肿瘤;p16 基因;基因治疗;转染;脂质体

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