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p53 Anti-tumor Research in Bel-7402 by Using Human-derived Vector*

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Abstract In order to study the tumor suppression effect of p53 with CMV enhancer and hTERT promoter mediated by human-derived vector pHrn in liver cancer cell Bel-7402, report plasmid pchEGFP, tumor suppressor plasmids pchp53$_{Arg}$ and pchp53$_{Pro}$ were constructed by inserting expression cassette CMVe+hTERTp+EGFP, CMVe+hTERTp+p53$_{Arg}$ and CMVe+hTERTp+p53$_{Pro}$ into pHrn respectively. 24 h after cell transfection by lipofectamine 2000, GFP expression pattern was analyzed through fluorescence microscope and flow cytometry; RT-PCR and Western blot were taken to study the p53 expression pattern. The cell apoptosis by Hoechst 33258 and Annexin V-FITC/PI staining was also studied. Results show that the expression of GFP and p53 protein in Bel-7402 were detected, but apparent cell apoptosis could not be found. The recombinant p53 mediated by human-derived vector could express in Bel-7402, but no significant tumor suppression effect was detected, which might result from the down regulation effect of the wild type p53 on hTERT promoter.

Key words human-derived vector, human telomeras reverse transcriptase (hTERT), p53

Key of the gene therapy is the choice of the vector. In 1981, two family with extra bisatellite micro-chromosome (BM) were found when we investigated the newborns inheritance disease. The family had normal phenotype and the extra BM could pass down to the next generation stably. We thought the BM had the potential to be devised as a good vector for gene therapy. The BM was located at the short arm of the chromosome 15, homologous to the short arm of the chromosome form the D, G group (chromosome 13, 14, 15, 21, 22). Based on the BM sequence, we devised the human-derived vector pHrn. In the former gene therapy study of Hemophilia using pHrn we found pHrn could target transgene into human ribosomal DNA locus of HT-1080 successfully with an site-specific integration efficiency of 2.0×10^{-5}. The transgene could be expressed efficiently ($(32\pm5)ng\cdot10^{6}\ cell^{-1}\cdot24\ h^{-1}$). The PCR assay identified the transgene could pass down stably after 50 cell cycle[1].

hTERT (human telomerase reverse transcriptase) promoter is a tumor specific promoter, and it has the merit of activating downstream gene expression in tumor cells specially, but its deficiency is the low activation efficiency [2,3]. We recently find that the combination of CMV enhancer with hTERT promoter (CMVe+hTERTp) is a highly efficient tumor specific promoter. In the gene therapy study of liver cancer with CDUPRT, we found the activation efficiency of the CMVe+hTERTp was 13 times as strong as that of the hTERT promoter alone[4].

p53 is widely recognized as a tumor suppressor gene, mutated or lost in about 50% of all human cancer cases worldwide [5]. Mice deficient in p53 are susceptible to spontaneous tumorigenesis[6] and germ-line TP53 mutations occur in individuals with the cancer-prone Li-Fraumeni syndrome[7,8]. The wild-type p53 protein exhibits a common polymorphism at amino acid 72, resulting in either a proline residue CCC (p53$_{Pro}$) or an arginine residue CGC (p53$_{Arg}$) at this position. Researches show that there are a number of differences between the p53$_{Pro}$ and p53$_{Arg}$ in their abilities to bind components of the transcriptional

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machinery, to activate transcription, to induce apoptosis, and to repress the transformation of primary cells. And it suggests the p53 related cancer gene therapy will have different results depending on the genotype of p53 used[9].

In this study we combined $p53_{Pro}$ and $p53_{Arg}$ with CMVe +hTERTp and pHrn, constructed plasmids $pchp53_{Pro}$ and $pchp53_{Arg}$ to study their function in liver cancer cell Bel-7402.

1 Materials and methods

1.1 Materials

Human liver cancer cell Bel-7402 (China Center for Type Culture Collection); Commercial vector pEGFP-N1 (BD); pGEM-T (Promega); Lipofectamine 2000 (Invitrogen); RT-PCR kits (Promega); Mouse-anti-human P53 (BD); Mouse-Anti-huma β-actin (Sigma); horseradish peroxidase (goat anti mouse; PIERCE); ECL kit (Amersham); Annexin V-fitc apoptosis detection kit (Biovision); pHrn was constructed by our laboratory.

1.2 Plasmids construct

Fragments hTERTp (5′ ctgcagcgcgggggtggcc-ggggc 3′; 5′ aagcttggattcgcgggcacagac 3′) and CMVe (5′ ctcgagaagtcccgttgattttggtg 3′; 5′ gctagccggggtc-attagttcatagcc 3′) were amplified by PCR using human gDNA and pEGFP-N1 as template. Fragments hTERTp, CMVe, $p53_{Pro}$ and $p53_{Arg}$ were inserted into commercialization vector PEGFP-N1 orderly; obtaining intermediate vectors pchEGFP-N1, $pchp53_{Arg}$-N1 and $pchp53_{Pro}$-N1. They were cut with restriction enzymes *Nhe* Ⅰ and *Afl* Ⅱ; fragments cut down were then inserted into pHrn. Finally, the report vector pchEGFP, as well as the tumor suppressor vector $pchp53_{Arg}$ and $pchp53_{Pro}$ were constructed.

1.3 Cell culture and transfection

Cells were maintained in RPMI-1640-Medium (Sigma) with 10% FBS (37℃, 5% CO_2). 1 day before transfection, cells were seeded into 6 well cell culture microplate with the cell density of 3.0×10^5 per well. Transient transfections of human liver cancer cells Bel-7402 were performed with the lipofectamine 2000 system according to the manufacturer's instructions when the cell confluence was approximately 80%～90%.

1.4 GFP expression assay

24 h after transfection, cells were washed 3 times with PBS, followed by digesting with 0.05% tripsin-EDTA. Took appropriate cell digestion suspension (about 2×10^5 cells) into 1.5 ml EP tube; centrifuged at 300 *g* for 10 min, discarded the supernatant; resuspended cells with PBS; centrifuged again at 300 *g* for 10 min, discarded the supernatant and repeated the above procedure one more time; then added 300 μl PBS into the EP tube, mixed gently. The sample was analysed by FCM (flow cytometry) within 30 min. Cells for fluorescence microscope detection needed no other treatment; 24 h after transfection, they were detected and photographed by fluorescence microscope.

1.5 RT-PCR assay

24 h after transfection, cells were lysed by 1 ml Trizol reagent (Gibco); total RNA was extracted as the protocol of Trizol reagent. RNA was quantitated with a spectrophotometer (Beckman DU640); 1 μg RNA was used for reverse transcription (Promega). β-actin (5′ aatctggcaccacaccttct 3′; 5′ agcacagcctggatagcaac 3′) and p53 (5′ gagctgaatgaggccttgga 3′; 5′ ctgagtcagg-ccttctgtctt 3′) were amplified using the same amount RT-PCR product as template. PCR product was determined by 2% agarose gel.

1.6 Western-blot assay

Western-blotting was performed according to the Molecular Cloning (3rd ed). Briefly, cell extracts were boiled at 100℃ for 10 min and centrifuged for 10 min at 13 000 r/min, 4℃ (Eppendorf centrifuge). The supernatants were resolved by 10% SDS-PAGE. The resolved proteins were transferred onto PVDF membranes and incubated overnight at 4℃ in blocking solution (PBS, 5% powdered milk, 3% BSA). Membranes were exposed to a combination of anti-p53 monoclonal antibodies (1∶500) with anti-β-actin monoclonal antibodies (1∶2 000) in blocking solution for 1 h at room temperature, followed by two washes for 10 min in PBS with 0.1% Triton and one wash for 10 min in blocking solution. Membranes were then incubated with diluted anti-mouse antibody HRP (1∶5 000) for 1 h at room temperature, followed by three washes of 15 min in PBS with 0.1% Triton. The presence of antibody was detected with an enhanced chemiluminescence (ECL) kit (Amersham Life Science).

1.7 Hoechst 33258 staining

Cell transfection was performed as described in Cell Culture and Transfection. 24 h after transfection, cells were washed with PBS for 3 times, followed by maintaining in Hoechst 33258 solution (0.1 mg/L) for 30 min in the dark. Cell apoptosis was detected with

fluorescence microscope.

1.8 Annexin V-FITC/PI staining

Annexin V-FITC/PI double staining was performed with the corresponding kits (Biovision) according to the manufacturer's instructions. The cell suspension was performed with FCM (flow cytometer) assay to detect the cell apoptosis.

2 Results

2.1 Plasmid construct and sequencing

Plasmids pchEGFP, pchp53$_{Pro}$ and pchp53$_{Arg}$ were successfully constructed. The plasmids digested with *Nhe* Ⅰ and *Afl* Ⅱ were resolved by 1% agarose gel (Figure1). Figure 2 was the ideograph of pchp53$_{Arg}$. Figure 3 was the sequencing results of p53$_{Arg}$ and p53$_{Pro}$.

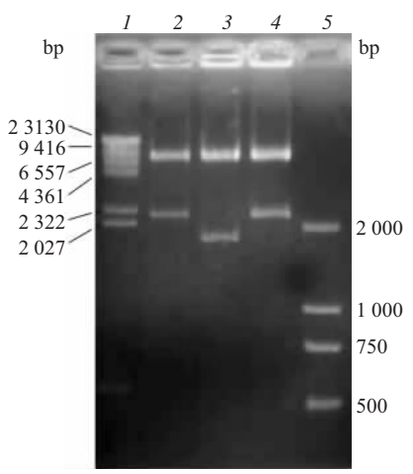


Fig. 1　Confirmation of target fragments insertion into human-derived vector pHrn

Nhe Ⅰ and *Afl* Ⅱ cut pchp53$_{Pro}$ and pchp53$_{Arg}$ into 8 608 bp and 2 216 bp two fragments, cut pchEGFP into 8 608 bp and 1 692 bp fragments. *1*: λDNA/*Hind*Ⅲ; *2*: pchp53$_{Arg}$; *3*: pchEGFP; *4*: pchp53$_{Pro}$; *5*: Marker DL200.

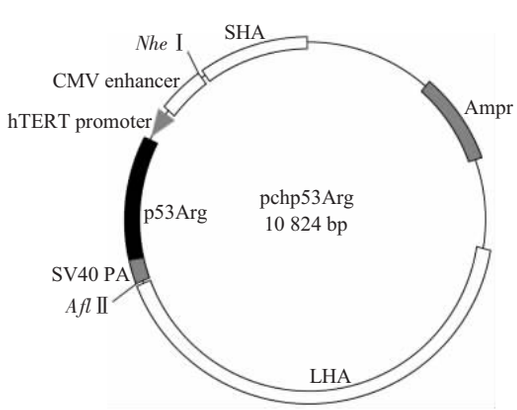


Fig. 2　Ideograph of pchp53$_{Arg}$

SHA: Short homologous arm; LHA: Long homologous arm. Both derived from human ribosomal gene region.

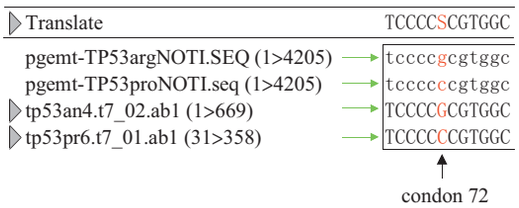


Fig. 3　p53 sequencing results

For p53$_{Arg}$ the 72 codon is CGC; for p53Pro the 72 codon is CCC.

2.2 GFP expression and FCM assay

24 h after transfection, cell transfected with pchEGFP was studied with fluorescence microscope and flow cytometry. GFP was expressed in Bel-7402 cells (Figure 4). The FCM assay showed GFP expression efficiency was 17% (Figure 5).

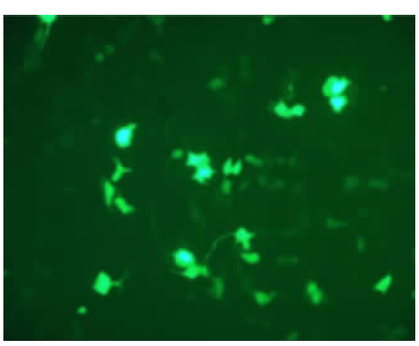
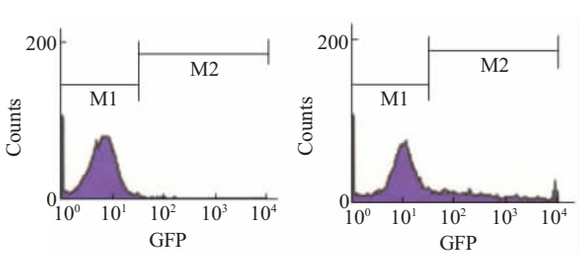


Fig. 4　GFP expression in Bel-7402 cells 24 h after transfection with pchEGFP



	M1	M2	M1+M2	M2/(M1+M2)×100%
001(control)	9 981	19	10 000	0.19%
002(pchEGFP-phrn)	8 251	1 756	10 000	17.56%

Fig. 5　GFP transfection efficiency in Bel-7402 detected by FCM

24 h after transfection with pchEGFP.

2.3 RT-PCR assay

Figure 6 showed the electrophoresis results of RT-PCR products. p53 mRNA expression could be detected in all lanes in Figure 6a. The β-actin acted as an endogenous control for semi-quantitating p53 expression.

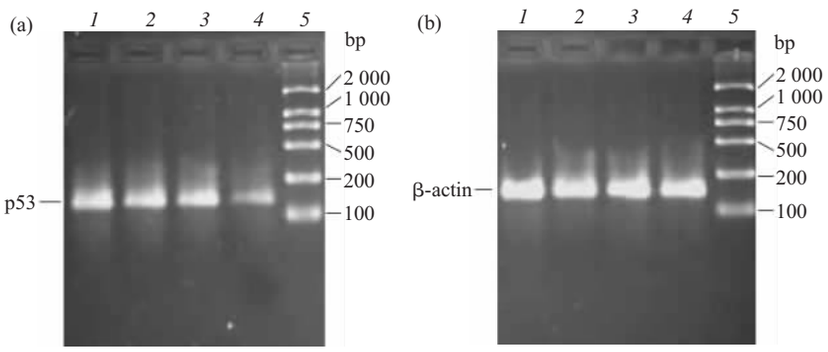


Fig. 6 p53 mRNA (a) and β-actin in (b) expression in Bel-7402 cells

24 h after transfection with pcDNA3.1-p53$_{Arg}$, pchp53$_{Pro}$, pchp53$_{Arg}$ and pHrn. *1*: pcDNA3.1-p53$_{Arg}$; *2*: pchp53$_{Arg}$; *3*: pchp53$_{Pro}$; *4*: pHrn; *5*: Marker DL2000.

2.4 Western-blot assay

Figure 7 showed Western-blot results. p53 molecular mass is 53 ku. The β-actin molecular mass is 43 ku. The β-actin acted as an endogenous control for semi-quantitating p53 expression.

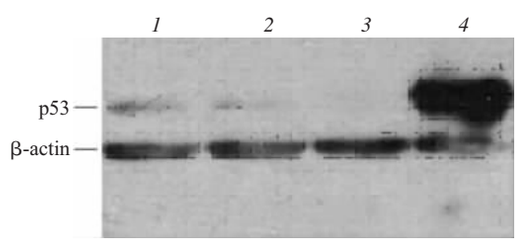


Fig. 7 Western blot analysis of p53 and β-actin expression levels in Bel-7402 cells

24 h after transfection with pchp53$_{Arg}$, pchp53$_{Pro}$, pHrn and pchp53$_{Arg}$. *1*: pchp53$_{Arg}$; *2*: pchp53$_{Pro}$; *3*: pHrn; *4*: pcDNA3.1-p53$_{Arg}$.

2.5 Hoechst 33258 staining

Figure 8 showed cell staining results with Hoechst 33258. There were not much difference between the control, pchp53$_{Arg}$ and pchp53$_{Pro}$. Compared with the control, the other two seemed have more cell nuclear stained deeply, but it was not apparent.

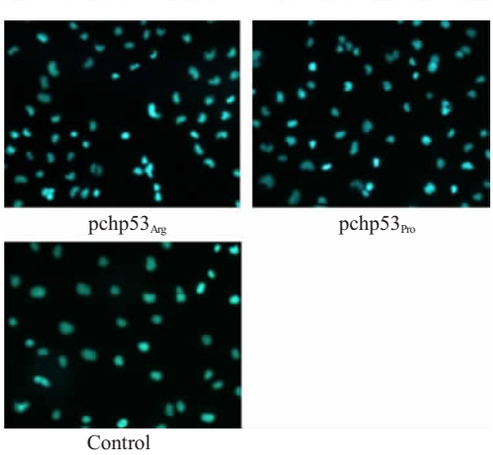


Fig. 8 Bel-7402 cells apoptosis results, detected by Hoechst 33258 stainning

24 h after transfection with pchp53$_{Arg}$, pchp53$_{Pro}$, and control (pHrn)

2.6 Annexin V-FITC/PI staining

Figure 9 was the results of Annexin V-FITC/PI staining assay. From the left to the right the cell apoptosis (FITC+/PI) rate was 2.82%, 3.06%, 3.08% and 3.47%(the mean). The control was transfected not plasmid but lipofectamine.

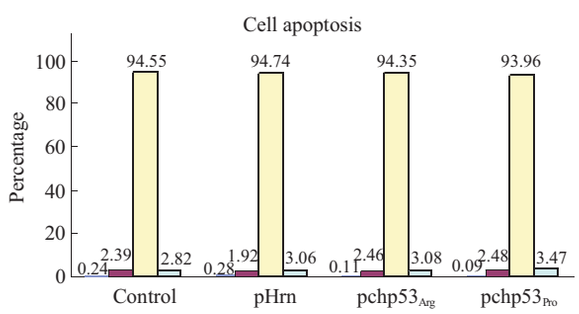


Fig. 9 Bel-7402 cells apoptosis FCM results

24 h after transfection with control (PBS), pHrn, pchp53$_{Arg}$, and pchp53$_{Pro}$, cells were stained by Annexin-V-FITC/PI. ■: FITC−/PI+; ■: FITC+/PI+; □: FITC−/PI−; □: FITC+/PI−.

3 Discussion

The plasmid combination pHrn with CMVe+hTERTp could express well in Bel-7402. 24 h after Bel-7402 was tansfected with pchEGFP, the GFP expression rate reached 17%. RT-PCR results showed there was endogenous p53 expression in Bel-7402. Western blot results indicated that p53 expression amount in pcDNA3.1-p53$_{Arg}$ was much more than that in pchp53$_{Arg}$ and pchp53$_{Pro}$; The p53 expression could not be detected in pHrn due to the low expression

amount. Compared with control, apparent tumor suppression effect of pchp53$_{Arg}$ and pchp53$_{Pro}$ could not be found. Totally, the plasmids can express effectively in Bel-7402, but the expression amount was too low to have apparent tumor suppression effect.

The reason for the low expression amount of p53 and almost no tumor suppression effect of our plasmids might be partly the relatively low transfection efficiency due to the large size of the pHrn; but according to the results of our coworkers' research which also involved with pHrn (date not shown), this could not be the main reason for the low expression of p53. Taro and Satoru studied the relationship between p53 and hTERT. They introduced wild-type p53 into SiHa cells via a recombinant adenoviral vector. 36 hours after infection, down-regulation of human telomerase catalytic subunit mRNA expression was observed, whereas no change in telomerase activity was observed in the cells infected with control vector which had no p53 [10]. Xu *et al* [11]. cotransfected luciferase report vector p330 (driven by the hTERT proximal promoter) with wild-type p53 vectors into HeLa cells. hTERT promoter activities were suppressed by up to 90% compared to that in the same cells cotransfected with p330 and the empty vector. And they found wild-type p53 forms a complex with Sp1 and inhibits Sp1 binding to the hTERT proximal promoter. The following researchers investigating the p53 and hTERTp have reached the same recognition: wild-type p53 can downregulate hTERT mRNA expression through the interaction with hTERTp[12～16].

Figure 10 showed the hTERTp used in this research. The whole sequence was 292 bp long, containing 5 Sp1 binding sites. In the plasmids we constructed, the coding sequence of wild-type p53 was just followed the hTERTp sequence. This might explain why the p53 expression amount was very low and why there was almost no effect of the plasmids on suppression. On the other hand, our results further verified wild-type p53 can downregulation hTERTp.

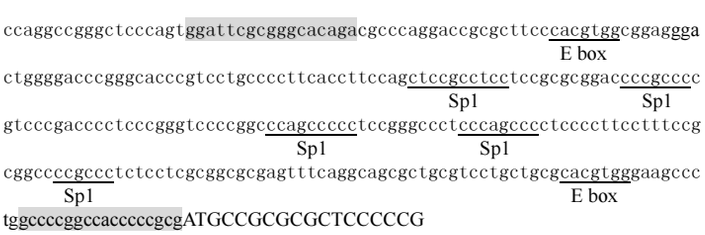


Fig. 10 hTERT promoter sequence analysis

The underlined region was core binding site of transcription factors. The shadowed region was the location of the primers used to amplify hTERT promoter.

Both the p53$_{Arg}$ and p53$_{Pro}$ could express well in Bel-7402. And the p53$_{Arg}$ expressed relatively more p53 than p53$_{Pro}$ did. Due to low expression amount, we could not compare their tumor suppression effect. This might be for some information for the future p53 related gene therapy.

Cancer has become one of the most deadly disease in the twentieth century. So far no really effective method has been found to cure cancer. With the knowledge of molecular mechanisms governing cancer formation expanding and the modern molecular technology fast improving, using gene therapy to cure cancer brings us hope.

References

1 Liu X H, Liu M J, She H, *et al*. Expression of reconstructive hFⅧ in the hrDNA by using hrDNA targeting vector. Chin Sci Bull, 2005, **50** (19): 2187～2192

2 Wirth T, Zender L, Schulte B. A Telomerase-dependent conditionally replicating adenovirus for selective treatment of cancer. Cancer Research, 2003, **63**: 3181

3 Jacob D, Bahra M, Schumacher G, *et al*. Gene therapy in colon cancer cells with a fiber-modified adenovector expressing the TRAIL gene driven by the hTERT promoter. Anticancer Research, 2004, **24** (5A): 3075～3079

4 Wang L, Xue Z G, Li Z, *et al*. Investigation of hrDNA targeting vector-mediated tumor-specific suicide gene therapy for hepatocellular carcinoma. Chin Sci Bull, 2006, **51** (19): 2342～2350

5 Hollstein M, Sidransky D, Vogelstein B, *et al*. p53 mutations in human cancers. Science, 1991, **253**: 49～53

6 Donehower L A, Michele H, Betty L, *et al*. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature, 1992, **356** (6366): 215～221

7 Malkin D, Li F P, Strong L C, *et al*. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms.

Science, 1990, **250** (4985): 1233～1238
8 Srivastava S, Zou Z Q, Pirollo K, *et al*. Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. Nature, 1990, **348** (6303): 747～749
9 Miranda T, Kalita A, Labrecque S, *et al*. Two polymorphic variants of wild-type p53 differ biochemically and biologically. Molecular and Cellular Biology, 1999, **19** (2): 1092～1100
10 Taro K, Satoru K, Katsuyuki H, *et al*. Adenoviral expression of p53 represses telomerase activity through down-regulation of human telomerase reverse transcriptase transcription. Clinical Cancer Research, 2000, **6** (4): 1239～1247
11 Xu D W, Wang Q, Gruber A, *et al*. Downregulation of telomerase reverse transcriptase mRNA expression by wild type p53 in human tumor cells. Oncogene, 2000, **19** (45): 5123～5133
12 Stampfer M R, Garbe J, Mizumoto K, *et al*. Loss of p53 function accelerates acquisition of telomerase activity in indefinite lifespan human mammary epithelial cell lines. Oncogene, 2003, **22** (34): 5238～5251
13 Boldrini L, Faviana P, Gisfredi S, *et al*. Regulation of telomerase and its hTERT messenger in colorectal cancer. Oncology Reports, 2004, **11** (2): 395～400
14 Rahman R, Latonen L, Wiman K G. hTERT antagonizes p53-induced apoptosis independently of telomerase activity. Oncogene, 2005, **24** (8): 1320～1327
15 Yan B Z, Wang J, Zhang B, *et al*. Inhibitory effect of p53 gene on telomerase activity and proliferative activity in salivary adenoid cystic carcinoma cells. Chin J Stomatol, 2006, **41** (1): 45～48
16 Tang F, Gu D H, Wang H, *et al*. Expression and significance of hTERT mRNA in breast carcinoma and its relation to p53. Chin J Oncol, 2006, **28** (3): 192～195

人源基因载体 pHrn 介导 p53 基因在 Bel-7402 中的抗癌研究 *

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摘要 利用人源基因载体结合人巨细胞病毒 (CMV) 增强子，人端粒酶逆转录酶 (human telomerase reverse transcriptase，hTERT)启动子及肿瘤抑制基因 p53 构建肿瘤抑制载体，研究它们在肝癌细胞株 Bel-7402 中的功能. 将 CMV 增强子(CMVe)与肿瘤特异性启动子 hTERTp 组合，分别结合 GFP 及精氨酸型和脯氨酸型肿瘤抑制基因 p53，构建 GFP 表达载体 pchEGFP 以及 p53 表达载体 $pchp53_{Arg}$ 和 $pchp53_{Pro}$；脂质体 2000 转染肝癌细胞株 Bel-7402；24 h 后利用荧光显微镜以及流式细胞仪检测 GFP 的表达；RT-PCR、蛋白质印迹检测 p53 基因的表达；利用 Hoechst 33258、 Annexin V/PI 染色，检测 p53 促使肿瘤细胞的促凋亡情况. 结果表明：GFP 荧光表达载体在 Bel-7402 细胞中能有效表达；RT-PCR 及蛋白质印迹检测到精氨酸型和脯氨酸型 p53 基因在 Bel-7402 细胞内表达；Hoechst 33258 染色及 Annexin V/PI 染色检测细胞凋亡实验没有得到明显的细胞凋亡结果. 结果表明：实验所构建的肿瘤抑制载体在肝癌细胞 Bel-7402 能有表达，并能形成外源性 p53 蛋白，但没有检测到明显的 p53 促使肝癌细胞调亡的结果. 这可能与 p53 对 hTERT 的负调控作用有关.

关键词 人源基因载体，人端粒酶逆转录酶(hTERT)，p53
学科分类号 Q78

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