Role of wild type p53 and double suicide genes in interventional therapy of liver cancer in rabbits[1]

Papel do p53 selvagem e do duplo genes suicidas na terapia intervencionista do câncer de fígado em coelhos

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ABSTRACT

PURPOSE: To investigate the feasibility of interventional lipiodol embolism and multigene therapy in combination with focal chemotherapy in the treatment of VX2 liver cancer in rabbits.

METHODS: Forty five rabbits with cancer larger than 2cm in diameter were randomly divided into five groups (n=9 per group). In Group 1, animals were treated with 0.9% sodium chloride. In Group 2, animals received lipiodol embolism. In Group 3, animals received lipiodol embolism and p53 gene therapy. In Group 4, animals received lipiodol embolism and TK/CD gene therapy. In Group 5, animals received lipiodol embolism and p53 and TK/CD gene therapy. Ultrasonography and CT were performed before and at ten days after interventional therapy.

RESULTS: The VX2 model of liver cancer was successfully established in rabbits and interventional therapy smoothly performed. At ten days after interventional therapy, significant difference in the tumor volume was noted among five groups ($p<0.05$) and different treatments could inhibit the cancer growth. The inhibition of cancer growth was the most evident in the Group 5. Factorial analysis revealed gene therapy with p53 or TK/CD and lipiodol embolism independently exert significantly inhibitory effect on cancer growth. In addition, the suppression on tumor growth rate was the most obvious in the Group 5.

CONCLUSIONS: Combination of gene therapy with lipiodol embolism can effectively inhibit the cancer growth and prolong the survival time. These findings demonstrate the effectiveness of multigene therapy in combination with lipiodol embolism in the treatment of liver cancer.

Key words: Liver Neoplasms. Genes, p53. Gene Therapy. Rabbits.

RESUMO

OBJETIVO: Investigar a possibilidade de terapia multigênica e intervenção por embolização com lipiodol em combinação com quimioterapia focal no tratamento de câncer de fígado VX2 em coelhos.

MÉTODOS: Quarenta e cinco coelhos com câncer maior do que 2cm de diâmetro foram distribuídos, aleatoriamente, em cinco grupos (n=9 por grupo). Grupo 1: animais foram tratados com cloreto de sódio 0,9% e no grupo 2 os animais receberam embolização com lipidol. Grupo 3: animais receberam embolização com lipiodol e terapia do gene p53 e grupo 4 animais receberam embolização com lipiodol e terapia do gene TK/CD. Grupo 5: animais receberam embolização com lipiodol e terapia do gene p53 e do gene TK/CD. Ultrassonografia e tomografia computadorizada foram realizadas antes e dez dias após a intervenção terapêutica.

RESULTADOS: O modelo VX2 de câncer de fígado foi estabelecido com sucesso em coelhos e a terapia intervencionista foi bem executada. Dez dias após a intervenção terapêutica, uma diferença significativa no volume do tumor foi observada entre os cinco grupos

(p<0,05) e diferentes tratamentos poderiam inibir o crescimento do câncer. A inibição do crescimento do cancer foi mais evidente no grupo 5. Análise fatorial revelou que a terapia com gene p53 ou TK/CD e embolia por lipiodol independentemente exerce um efeito inibidor significativo sobre o crescimento do câncer. Além disso, a supressão da taxa de crescimento do tumor foi mais evidente no Grupo 5.

CONCLUSÕES: A combinação de terapia gênica com embolização com lipiodol pode inibir efetivamente o crescimento do câncer e prolongar o tempo de sobrevida. Estes resultados demonstram a eficácia da terapia multigênica em combinação com embolização com lipidol no tratamento de câncer hepático.

Descritores: Neoplasias Hepáticas. Genes p53. Terapia de genes. Coelhos.

Introduction

Gene therapy has been a promising strategy for the treatment of malignancies. Combination gene therapy has the advantages of gene therapy, elevates the therapeutic efficacy and overcomes the shortcomings of single gene therapy[1,2]. Combination gene therapy has become a direction in the development of gene therapy. In our previous animal studies[3,4], target gene was introduced to cancer cells which were then inoculated into animals for tumorigenesis. Our findings demonstrated that combination therapy with WTp53 gene and TK/GCV, CD/5-Fc could effectively treat colon cancer and prevent liver metastasis of colon cancer. However, the therapeutic strategy is not practical. In clinical practice, it is impossible to introduce target gene into all cancer cells. In the present study, combination therapy of transcatheter arterial lipiodol chemoembolization with gene therapy was performed to treat liver cancer achieving favorable effectiveness. Our findings bridge basic research and clinical application of interventional therapy and also provide a novel strategy for the clinical treatment of liver cancer and metastatic liver cancer.

Methods

The pCMV-p53 plasmids were kindly provided by the Institute of Biochemistry and Molecular Biology in Shandong University. PA317 cells and PA317/TK-CD were kindly provided by the Cancer Center of Qilu Hoapital of Shandong University. A total of 50 New Zealand rabbits aged six to eight months weighing 2.0 to 2.5kg were purchased from the Academy of Agricultural Sciences of Shandong Province (License number: SCXK20090013). Lipofectamine™ 2000 (Gibco), 5-Fluorocytosine (5-Fc, Sigma), ganciclovir (GCV; Roch Syntex), VX2 cancer cells (USA), microcatheters (3F, 1.2F), sheath of femoral artery catheter made of 10-gauge syringe needle, hepatic artery catheter with fine needle, C arm DSA system and 64-slice CT (Siemens, Germany) were used in the present study.

Preparation of pCMV-p53 plasmid-liposome complex

The pCMV-p53 plasmids had expression of WTp53 and Neo, a gene for screening. Following amplification, extraction and purification (E.Z.N.A® Plasmid Miniprep Kit), these plasmids were harvested and subjected to 0.8% agarose gel electrophoresis (8 v/cm, 60 min) followed by EB staining. The bands were observed under an ultraviolet lamp. Ultraviolet spectrophotometer was employed to measure the absorbance (A) at 260 nm and 280 nm. The DNA content was calculated and the DNA purity determined. pCMV-p53 plasmids were embedded in liposome to construct pCMV-p53 plasmid-liposome complex. Studies have shown that p53 plasmid-liposome complex at a ratio of 1:2 has the best transfection efficiency. Thus, this ratio was also used in the present study for the preparation of pCMV-p53 plasmid-liposome complex.

Preparation of concentrated supernatant of TK-CD retrovirus

PA317/TK-CD cells underwent transfection with pWZLneoTKglyCD plasmids and could stably express viral products. pWZLneoTKglyCD plasmids were retroviral expression vector with TK cDNA and CD cDNA replication defects. PA317 cells and PA317/TK-CD cells were thaw and PA317 cells were used to determine the concentration of G418 for screening. G418 at the concentration for screening was added and purified PA317/TK-CD cells were harvested and then underwent passaging. The supernatant was collected by centrifugation for 5 min and then filtered through a 0.45-μm filter. Poly-L-Lysine (PLL) was added at a final concentration of 0.005% followed by incubation at 4°C for 5 min. Centrifugation was carried out at 20000 rpm for 2h at 4°C. The supernatant was removed and the sediments were re-suspended in medium of 100 volumes.

Establishment of liver cancer model in rabbits

The cancer cells were inoculated at the site near the groin

of rabbit for tumorigenesis. Two weeks later, the tumor size was about 1 to 2 cm^3. Then, the tumor was collected by surgery and the capsule was removed. The fish-like tumor tissues at the edge of tumor were cut into 1 to 2 mm^3 pieces followed by washing for further experiments [5].

A total of 50 rabbits were used in the experiment. The animals were anesthetized and fixed. The skin of upper abdomen was prepared. An inverted V-shaped incision was made at the site below xiphoid with 1 to 2 cm in length. Laparotomy was performed and the xiphoid removed. Then, the liver was exposed. The left middle lobe was clamped to block the local blood flow and exposed out of the abdomen and then fixed. The liver capsule was punctured and a hole was drilled with 3 to 5 mm in depth. A tumor tissue was placed into the hole and the wound in the liver was dressed with a gelatin sponge followed by covering OB glue. When the glue solidified, the liver was placed back to the abdominal cavity and the abdominal wound was closed. The above procedures were performed under aseptic conditions. After surgery, animals received intramuscular injection of penicillin (800000 U) for three consecutive days.

Grouping and catheterization

Two weeks after surgery, further experiment started. According to the findings in ultrasonography and CT, 45 rabbits with tumors of about 2 cm in diameter were randomly assigned into five groups (n=9 per group). In Group 1, rabbits were treated with normal saline (control group); In Group 2, animals received lipiodol embolism; In Group 3, animals received lipiodol embolism and gene therapy with p53; In Group 4, animals received lipiodol embolism and gene therapy with TK/CD; In Group 5, animals received lipiodol embolism and gene therapy with both p53 and TK/CD.

Catheterization was performed through the femoral artery to the hepatic artery[6-8]. In brief, rabbits were anesthetized and fixed. The skin of groin was prepared and a longitudinal incision was made at the groin along the femoral artery. The sheath of femoral artery was opened and femoral artery with 1 to 2 cm was separated. A suture was used to wrap the distal femoral artery and then lifted to block the blood flow. A v-shaped incision was made in the anterior wall of femoral artery and the guiding wire was inserted. Then, the catheter sheath was inserted into femoral artery along the guiding wire with 4 cm catheter sheath in the artery. Then, the artery was ligated to fix the sheath. A 3F microcatheter was inserted along the sheath into the abdominal arteries following by angiography to find the proper hepatic artery. Super-selective catheterization was performed into the proper hepatic artery followed by angiography to confirm the liver parenchyma. Further angiography was carried out to confirm the right or left hepatic artery.

Treatments

Following catheterization, angiography was performed with Ultravist 300 as contrast in a digital subtraction device to confirm the target blood vessel. A 1.2 F microcatheter was inserted into the feeding artery of cancers (Figure 1).

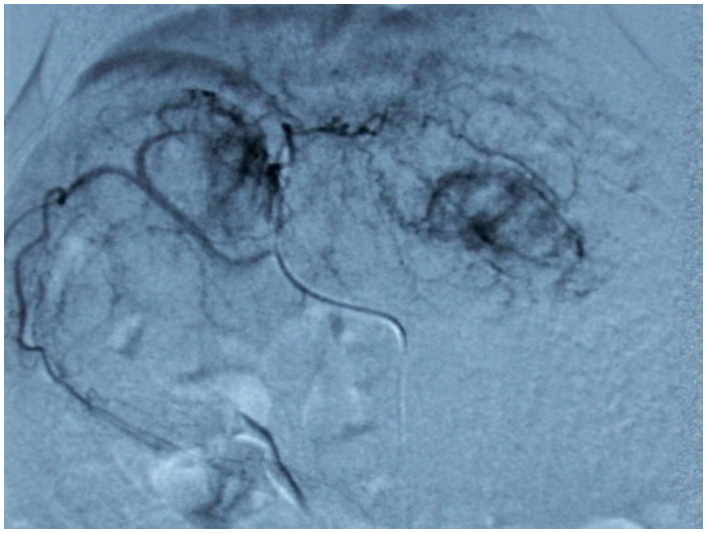

FIGURE 1 - DSA: tumor presented ball-like.

Injection of drugs was performed under radiography.

In Group 1 (control group), normal saline (1 ml per animal) was injected through the feeding artery. In Group 2, ultra-liquefied lipiodol (1 ml per animal) was injected through the feeding artery. In Group 3, ultra-liquefied lipiodol (1 ml per animal) and plasmids carrying p53 (0.1 ml per animal; 20 μg) were injected through the feeding artery. Studies have shown that the amount of p53 in a certain range was positively related to the transfection efficiency. When the amount of p53 was higher than 10 μg, the transfection efficiency remained stable. Thus, 20 μg of p53 was used in the present study. In Group 4, ultra-liquefied lipiodol (1 ml per animal) and concentrated supernatant from TK/CD (0.1 ml per animal; 1×10^9PFU) were injected through the feeding artery. In Group 5, ultra-liquefied lipiodol (1 ml per animal), concentrated supernatant from TK/CD (0.1 ml per animal; 1×10^9PFU) and plasmids carrying p53 (0.1 ml per animal; 20 μg) were injected through the feeding artery.

Drug solution was administered with a microinjector. Following injection, the catheter was flushed with 1 ml of normal

saline with heparin. The catheter and its sheath were removed and the distal femoral artery was ligated followed by wound close. Animals were intramuscularly treated with penicillin (800000 U) for infection prophylaxis. Animals were put back to cages and housed in a specific pathogen free environment. On the second day of TK/CD treatment, intraperitoneal injection of GCV at 100 mg/(kg·d) and 5-Fc at 500mg/(kg·d) was performed for consecutive 10 days.

Observations

Before and at ten days after interventional therapy, ultrasonography and CT were performed and the maximal (a) and minimal (b) diameters were measured followed by calculation of tumor volume ($V=ab^2/2$) and tumor growth rate (volume after treatment / volume before treatment). At 8 weeks after interventional therapy, rabbits were sacrificed by cervical dislocation. Rabbits naturally died were also processed for observations. Pathological examination was carried out and the survival time was determined.

Statistical analysis

Statistical analysis was performed with SPSS version 11.5. Quantitative data were expressed as means ± standard deviation ($\bar{x} \pm s$). Comparisons between two groups were performed with Student t test and those among multiple groups with analysis of variance. Factorial analysis (2×2) was done to analyze the synergistic interaction between WTp53 gene therapy and TK/CD gene therapy. Kaplan-meier method was employed to delineate survival curve and log rank test for comparisons of survival time. α was defined as 0.05.

Results

Preparation of pCMV-p53 plasmid-liposome complex

The amplification, extraction and purification of pCMV-p53 plasmids were smooth. Agarose gel electrophoresis revealed a 4.7-kb band. Spectrophotometry showed the A260 and A280 were 1.28 and 0.70, respectively. The DNA content was 3.2 μg/μl and its purity was 1.83. Then, pCMV-p53 plasmids were embedded in Lipofectamine™ 2000 to prepare the pCMV-p53 plasmid – liposome complex, the concentration of which was 200 μg/ml.

Preparation of concentrated supernatant of TK-CD retrovirus

PA317 cells and PA317/TK-CD cells were successfully resuscitated. The concentration of G418 for screening PA317/TK-CD cells was 400 μg/ml. At two weeks after screening with G418, purified PA317/TK-CD cells were harvested followed by culture, passaging and frozen storage. A total of 20 ml of virus supernatant was collected and 2 ml of concentrated supernatant obtained. The titer of concentrated virus supernatant was 10^{10}PFU/ml.

Tumor volume and tumor growth rate

There was no marked difference in tumor volume among five groups ($p>0.05$). At ten days after interventional therapy, the tumor volume was compared among five groups with analysis of variance and Least Significant Difference (LSD) test. Results showed significant difference in tumor volume ($p<0.05$) among five groups. These findings suggest different treatment can inhibit the tumor growth, which was the most obvious in the Group 5(Table 1).

TABLE 1 - Tumor volume before and at ten days after therapy and tumor growth rate at ten days after therapy in five groups.

Group	Tumor volume (cm^3)		Tumor growth rate
	Before therapy	After therapy	
1	2.447±0.208	3.719±0.205	1.548±0.101
2	2.550±0.163	2.277±0.247	0.885±0.089
3	2.505±0.321	1.774±0.169	0.676±0.084
4	2.616±0.222	1.673±0.323	0.581±0.153
5	2.715±0.294	1.207±0.143	0.321±0.059

Factorial analysis was performed to analyze the synergistic interaction between two gene therapies. Results showed gene therapy with p53 and TK/CD alone in combination with lipiodol embolism could significantly inhibit the tumor growth but no synergistic interaction between p53 gene therapy and TK/CD gene therapy, which was not consistent with our previous study. This may be attributed to small sample size.

Analysis of tumor growth rate revealed significant difference in the tumor growth rate between different treatment groups and control group. The suppression on tumor growth rate was the most obvious in the Group 5. This suggests lipiodol

embolism in combination with multigene therapy is effective for the treatment of liver cancer.

Morphology

Macroscopically, the tumor was grey, nodular and round or oval, and had clear boundary with surrounding tissues. The tumor section revealed the tumor was fish-like and colliquative necrotic foci and cystic cavity were present at the center of tumor (Figure 2).

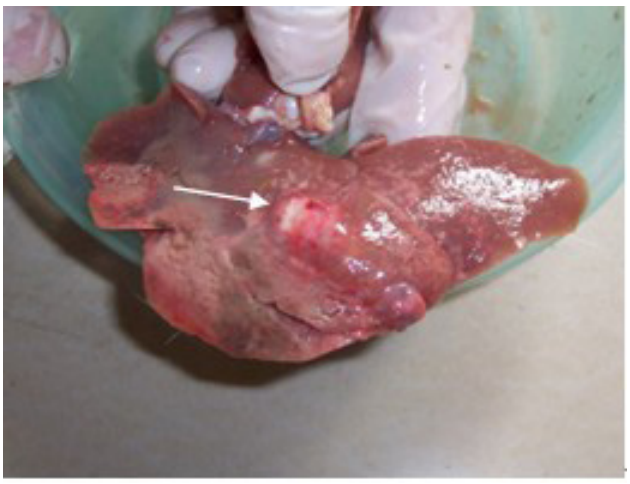

FIGURE 2 - Single, nubby cancer in the left middle lobe of the liver in the Group 5.

Under a light microscope, normal hepatic lobules were absent and the tumor presented invasive growth. Cancer cells possessed obvious atypia and arranged in nest-like or patchy manner. Karyokinesis was also noted in some cancer cells. The normal hepatic tissues surrounding the cancer nest were oppressed and formed fibrous septum (Figure 3).

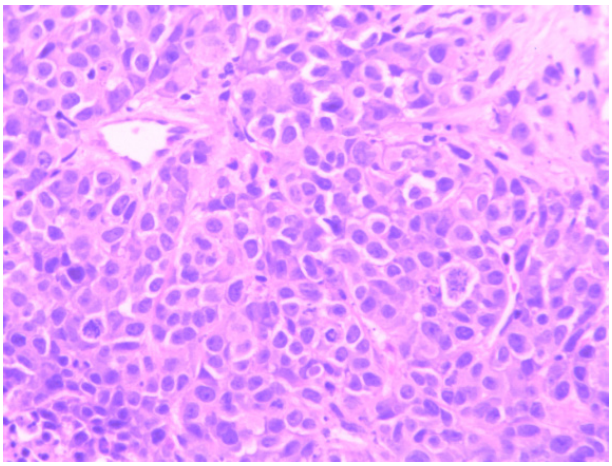

FIGURE 3 - Histopathological staining of cancer tissues (HE, 400x).

Survival time

In the control group, rabbits died consecutively at 24 to 40 days after interventional therapy. In four treatment groups, a total of 13 rabbits died in the period of study including five in Group 2, four in Group 3, three in Group 4 and one in Group 5. The survival time was analyzed with survival curve (Figure 4).

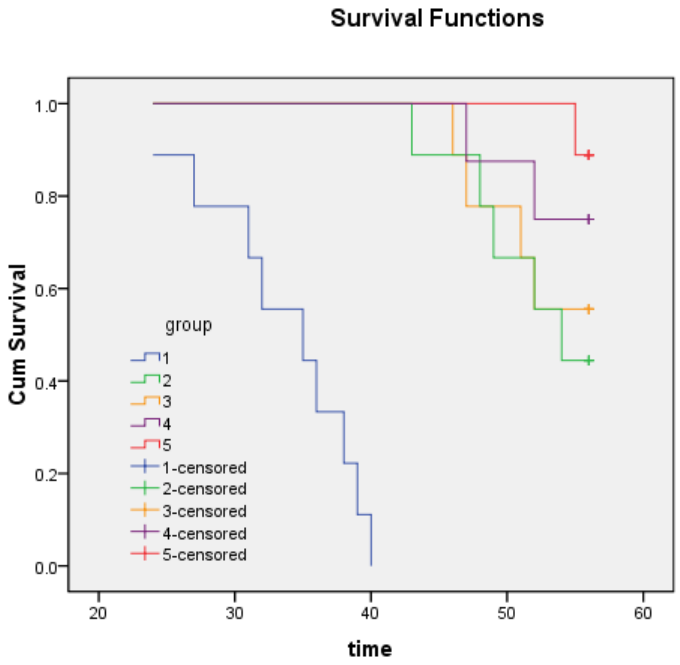

FIGURE 4 - Survival curve in different groups.

Log rank test showed significant difference in survival time among five groups ($p<0.001$). In addition, the survival time in control group was dramatically different from that in four treatment groups ($p<0.01$). These findings suggest different treatments can prolong the survival time, which was the most evident in the Group 5.

Discussion

The p53 includes wild type and mutant type. In more than 50% of liver cancers, mutant p53 is noted[9]. The physiological function of wild type p53 is to involve in the DNA repair, induce apoptosis and reduce the P-glycoprotein expression which then elevates the sensitivity of cancer cells to chemotherapeutics. Mutant p53 losses the anti-tumor activity and mutant p53 gene transforms from tumor suppressor gene into oncogene promoting the malignant transformation[10]. Thus, loss or deficiency in wild type p53 activity may result in resistance to chemotherapeutics

and reduce the efficacy of chemotherapy. There is evidence showing that cancer cells with mutation of p53 gene can combat with the anti-tumor effect of suicide genes which may compromise the efficacy of suicide gene therapy. Thus, to reverse mutant p53 gene to wild type p53 gene has been a promising strategy in the treatment of cancers.

Therapy with both suicide gene and prodrugs has been a hot topic in the treatment of cancers. In the double suicide gene therapy, two suicide genes were fused and then introduced into cancer cells through vectors. The cancer cells can stably generate the products of fusion gene which possess the activities of both suicide genes promoting the sensitivity of cancer cells to chemotherapeutics[11]. Then, prodrugs are administered for treatment achieving the synergistic effect of both suicide genes. Among numerous suicide genes, TK gene and CD gene have been extensively studied and gene therapy with them has definite effectiveness. Double gene therapy with both TK and CD fusion gene has been confirmed to be more efficient, broad-spectral and safe, cancer cells are not susceptible to generate drug resistance and this therapy can reduce the required dose of pro-drugs[12]. The major characteristic of suicide gene therapy is its bystander effect which overcomes the shortcomings in low transduction efficiency and expands the anti-tumor effect of suicide genes. Studies have shown that double gene therapy may confer more potent bystander effect when compared with single gene therapy. Moreover, the bystander effect may expand the anti-tumor effect of wild type p53 on cancer cells to surrounding cells exhibiting more potent anti-tumor effect.

The gene therapy requires the favorable expression efficiency and the target gene is locally expressed in target cells which may maximize the anti-tumor effect but has less influence on normal cells[13]. Thus, targeted gene transfer has been a key in the gene therapy aiming to enhance the transfection efficiency. In previous studies, it has been demonstrated the transfection and expressions of p53 gene and double suicide genes (TK and CD gene) in cancer cells[14,15], which were not described herein. Currently, intratumor injection and intravenous administration are two important ways for gene therapy.

Zhu *et al.*[16] reported injection of p53 gene through hepatic artery could achieve protein expression of p53 in cancer cells demonstrated by western blot assay and immunohistochemistry. This suggests interventional gene therapy is feasible and effective. In the present study, target genes were administered into liver cancer through hepatic artery which solves the problem of targeted gene transfer. Interventional gene therapy may also reduce the influence on normal hepatocytes and the side effects. In addition, lipiodol embolism may aggravate the hypoxia in the cancer and can exert long-term effect on cancer with the anti-tumor effect of suicide genes. Thus, we speculate that interventional gene therapy may be an ideal strategy for the gene therapy of malignancies. In the present study, on the second day of interventional therapy, pro-drugs (GCV and 5-Fc) were administered through the portal venous system for consecutive ten days. Intraperitoneal administration of prodrugs may achieve stable, sustained and high concentration of prodrugs in the abdominal cavity, portal vein and liver.

Conclusions

The favorable combination of gene therapy and administration of prodrugs in time promotes the direct effect of gene therapy and makes the gene therapy more specific and targeted. Our findings demonstrate double gene therapy with WRp53 and TK/CD in combination with focal chemotherapy with GCV and 5-Fc can effectively inhibit the growth of liver cancer.

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