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Research Paper

A comparative study of gene vaccines encoding different extracellular domains of the vascular endothelial growth factor receptor 2 in the mouse model of colon adenocarcinoma CT-26

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Key words: comparative study, VEGFR2, extracellular domain, oral, gene vaccine, immunogenicity, antitumor, colorectal cancer

Aims: **To compare the immunogenicity and anti-tumor effects of gene vaccines encoding domains 1–4 with full length of extracellular region of VEGFR2.**

Results: **Both DNA vaccines decreased VEGF levels and rose specific antibodies; the lymphocyte subsets of vaccinated mice maintained high; tumor latency period and survival time of immunized mice were prolonged; tumor size, weight, MVD and liver metastases were significantly less than the control groups.**

Methods: **Mouse model of CT-26 adenocarcinoma of colon were treated with orally immunized gene vaccine encoding extracellular1–4 and full length of VEGFR2 respectively. The effect of anti-tumor was evaluated by detecting the tumor volume, mice survival time, intratumoral microvessel density (MVD) and liver metastases. To explore the reasonable mechanism of the oral gene vaccines, the levels of VEGF and anti-VEGFR2 antibody in serum were detected by ELISA, $CD4^+$ and $CD8^+$ T cells in peripheral blood and subcutaneous tumors were analyzed by flow cytometer and immunohischemistry respectively.**

Conclusion: **Compared with full length of extracellular domain of VEGFR2, extracellular regions 1–4 of VEGFR2 has been sufficient to decrease the serum VEGF level and to inhibit tumor growth and metastasis specifically.**

Introduction

Vascular endothelial growth factor receptor 2 (VEGFR2, also known as flk-1) is the main receptor though which VEGF could modulate tumor growth and metastasis.[1-4] It is suggested that interaction with VEGF is a critical requirement to induce biological response, which include cell proliferation, migration, increase of vascular permeability, and maintenance of vascular integrity.[5-7] Several approaches have been used to block VEGFR2, including dominant-negative receptor mutants, germ-line disruption of VEGFR genes, monoclonal antibodies, dendritic cell vaccine and a series of synthetic RTK inhibitors.[8,9] Their final purpose is to block VEGF/VEGFR2 signal pathways and inhibit angiogenesis, but they all have some common defects, such as requiring constant drug injection, only blocking VEGF pathways at very high cost.

DNA vaccine against tumor has widely been used in different preventic and therapeutic strategies. Niethammer, et al[10] demonstrated that a potent antitumor response could be achieved via immunization of mice with DNA vaccine encoding full-length VEGFR2. Recently, Dong, et al[11] mentioned an antitumor effect induced by two peptides isolated from mouse VEGFR2. Evidence shows that VEGFR2 possess a characteristic structure consisting of seven extracellular immunoglobulin-like domains, a single transmembrane domain, and a tyrosine kinase domain. Deletion mutant analysis demonstrates that VEGFR2 extracellular immunoglobulin-like domains are sufficient for high affinity binding of VEGF. Detailed analysis of the interaction between VEGF and various VEGFR2 immunoglobulin-like domain deletion mutants suggest that the first Ig-like domain may regulate the ligand binding, and domain 2–4 might be important for VEGF association.[12]

We hypothesized that critical extracellular domain of VEGFR2 has sufficient immunogenicity to induce an antitumor response. For the purpose, extracellular domain 1–4 of VEGFR2 was designed to develop DNA vaccine to inhibit tumor growth and metastasis, and DNA vaccine encoding full length of extracellular domain of VEGFR2 was developed for comparative study. Attenuated Salmonella typhimurium SL3261,[13,14] an aromatic amino acid auxotrophic mutant strain, was employed to carry DNA vaccine to the host. The feasibility of the oral gene vaccine encoding extracellular domain 1–4 of VEGFR2 against tumor-associated angiogenesis in the subcutaneous and liver metastasis tumor mouse model was examined.

Results

Construction of pcDNA3.1 + $VEGFR2_{(n1\text{-}4)/(n1\text{-}7)}$. 1248 bp and 2246 bp genes of interest were cloned from BALB/c mouse embryo tissue using RT-PCR technique respectively. DNA sequencing showed that the two cloned genes completely matched the DNA sequence

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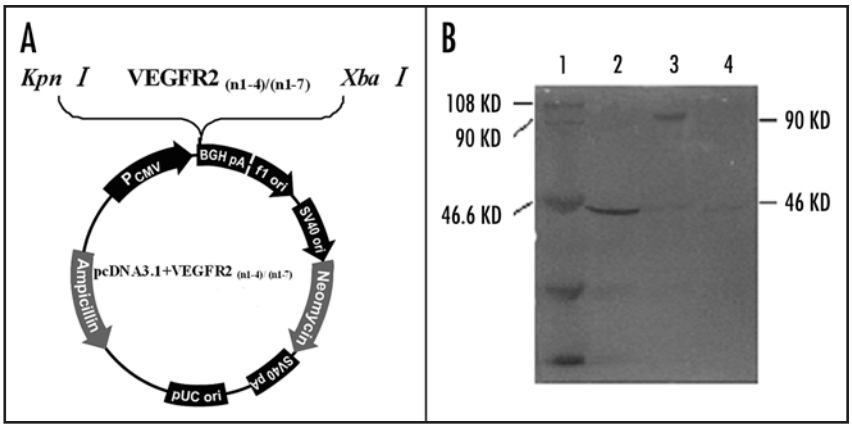

Figure 1. Construction and functionality of expression vector. (A) The DNA encoding extracellular domain 1–4 and 1–7 region were inserted into the pcDNA3.1 vector between the restriction sites *Kpn I* (5') and *Xba I*. (B) Western blot analysis of expressed product in transfected COS-7 cells cultural supernatants. Lane 1: protein marker; lane 2: COS-7 cells transfected with pcDNA3.1 + VEGFR2$_{(n1-4)}$; lane 3: COS-7 cells transfected with pcDNA3.1 + VEGFR2$_{(n1-7)}$; lane 4: blank control.

listed in GeneBank. Two constructs produced protein of the expected molecular mass, with pcDNA3.1 + VEGFR2$_{(n1-4)}$ being expressed in its active form at 46 kDa and pcDNA3.1 + VEGFR2$_{(n1-7)}$ as a 90 kDa protein (Fig. 1).

Body distribution of recombinant vaccine strain SL3261. Our experiments are based on the working hypothesis that orally administered Attenuated Salmonella typhimurium, carrying expression vectors encoding extracellular domain 1–4 of VEGFR2 to the host. Body distribution of recombinant vaccine strain SL3261 in each group was detected by TEM. We have observed that, aside from the blank control group (group D), recombinant vaccine strain SL3261 could be found in group A, B and C in the tissues such as the small intestine, colon, liver and spleen, which proved that our DNA vaccines could be efficiently carried to the host by the attenuated S. typhimurium SL3261 (Fig. 2).

Characterization of gene integration. Since all the eukaryotic expression vectors used in this research were constructed with CMV promoter, PCR was performed to amplify a portion of the CMV promoter as the proof of integration. As had been expected, the PCR products of genomic DNA of the small intestine, colon, liver and spleen of the experimental groups were all 141 bp segments. No corresponding band was detected in group D. This indicated that the exogenous gene have been integrated into the genome of BALB/c mouse (Fig. 3).

Declination of the serum level of VEGF. The serum levels of VEGF were detected by ELISA after 2 weeks of the inoculation of CT-26 cells, and the results showed that the serum levels of VEGF in group A and B were lower than that of group C and D ($p < 0.05$), there is no statistical significance in group A compared with group B. This indicated that VEGF in the serum in experimental groups had been combined by the extracellular fragment (VEGFR2$_{(n1-4)}$) or the total length (VEGFR2$_{(n1-7)}$) of VEGFR2 (Table 1). And the declination of VEGF achieves the purpose of blocking the VEGF/VEGFR2 pathway and inducing anti-angiogenesis effect around the solid tumor.

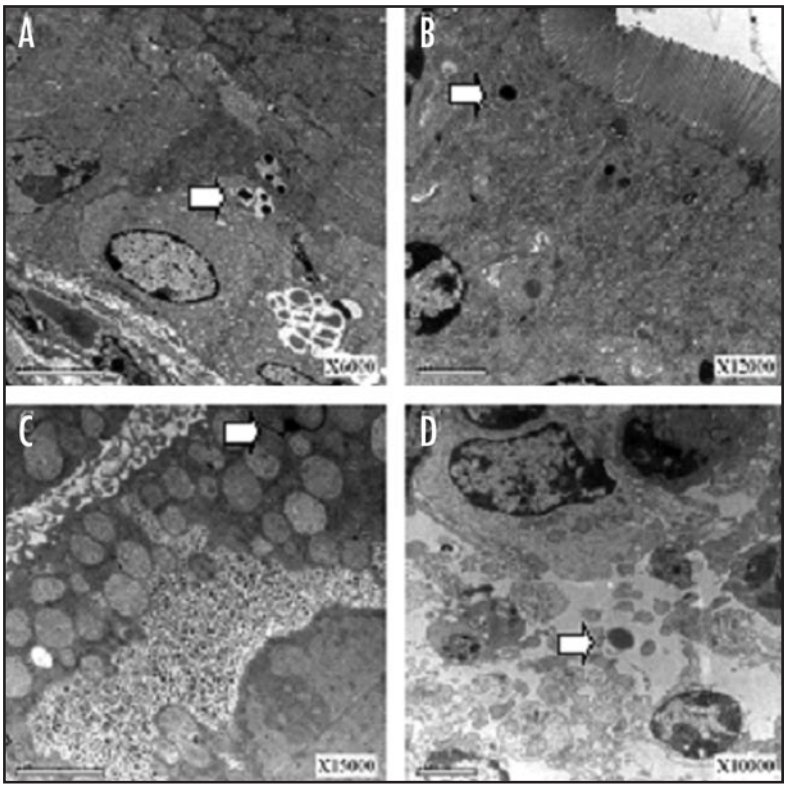

Figure 2. Body distribution of attenuated Salmonella typhimurium SL3261. (A) recombinant attenuated Salmonella typhimurium SL3261 in small intestine (X6000); (B) recombinant attenuated Salmonella typhimurium SL3261 in colon (X12000); (C) recombinant attenuated Salmonella typhimurium SL3261 in liver (X15000); (D) recombinant attenuated Salmonella typhimurium SL3261 in spleen (X10000).

Induction of specific anti-VEGFR2$_{(n1-4)}$-IgG and anti-VEGFR2$_{(n1-7)}$-IgG. We observed that, after the last immunization, mice in group A produced high levels of specific anti-VEGFR2$_{(n1-4)}$-IgG, and mice in group B produced high levels of specific anti-VEGFR2$_{(n1-7)}$-IgG. In group C and D, there was no such specific anti-VEGFR2$_{(n1-4)}$-IgG or anti-VEGFR2$_{(n1-7)}$-IgG raised (Fig. 4). This indicated that our DNA vaccines can induce immune responses against endothelial cell.

Analysis of lymphocyte subset in subcutaneous tumor model group. There was no significant difference in CD4$^+$ T cells in the 4 groups before and after vaccination, but after inoculation of CT-26 cells, we observed a obvious declination of CD4$^+$ T cells in C and D group while high levels were maintained in A and B group ($p < 0.05$, Table 2), no statistical significance was observed between group A and B ($p > 0.05$). There was no statistical significance in CD8$^+$ T cells in the 4 groups before vaccination ($p > 0.05$), but after vaccination and 3 wk later after the inoculation of CT-26 cells, CD8$^+$ T cells were obviously higher in A and B group than that of C and D group ($p < 0.05$, Table 3).

A higher lymphocyte infiltration in tumors from animals treated with DNA vaccines was observed. Immunohistochemical analysis of tumor samples revealed an enhanced accumulation of CD8$^+$

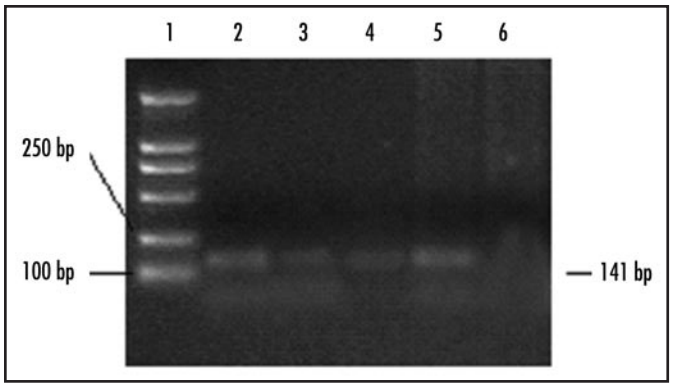

Figure 3. PCR products of genomic DNA. Lane 1: DL2000 molecular marker; lane 2: small intestinal genomic DNA; lane 3: colon genomic DNA; lane 4: liver genomic DNA; lane 5: spleen genomic DNA; lane 6: blank control.

Table 1 **Mice VEGF levels of each group (×10^{-2}, mean ± SD)**

Group	*n*	A_{450}		
		Before immunization	2 weeks after last immunization	2 weeks after tumor challenge
A	8	58.24 ± 6.42[a]	122.88 ± 18.07[b]	2.66 ± 1.91[c]
B	8	62.13 ± 5.68[d]	114.20 ± 14.21[e]	16.43 ± 3.42[f]
C	8	60.45 ± 6.20	172.44 ± 12.43	85.65 ± 10.36
D	8	59.86 ± 5.77	161.18 ± 6.72	92.86 ± 7.28

2 weeks after last immunization, the differences of VEGF level in the serum following treatment with the our DNA vaccines were statistically significant compared to control groups (A/C, bp = 0.000; A/D, bp = 0.000; B/C, ep = 0.000; B/D, ep = 0.000; A/B, p = 0.100); 2 weeks after tumor challenge the VEGF level in the serum of experimental groups declined significantly with respect to control groups (A/C, cp=0.000; A/D, cp = 0.000; B/C, fp = 0.000; B/D, fp = 0.000; A/B, p = 0.135).

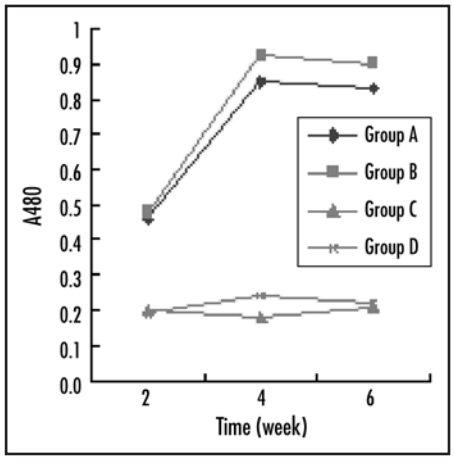

Figure 4. Levels of specific anti-VEGFR2$_{(n1-4)}$-IgG and anti-VEGFR2$_{(n1-7)}$-IgG in serum of immunized mice. (A) SL3261-pcDNA3.1 + VEGFR2$_{(n1-4)}$ group; (B) SL3261-pcDNA3.1 + VEGFR2$_{(n1-7)}$ group; (C) empty vector group; (D) $NaHCO_3$ group.

cytotoxic T lymphocytes, as well as an increase of CD4$^+$ cells in the tumors of animals treated with the two gene vaccines compared to tumors from control group mice (Fig. 5).

In vivo evaluation of tumor growth of pcDNA3.1 (+)/VEGFR2$_{(n1-4)/(n1-7)}$ against CT-26. We test our hypothesis by demonstrating that an effective antitumor response was induced against subcutaneous tumors by our vaccines. Marked inhibition of subcutanetous (s.c.) tumor growth was observed in BALB/c mice challenged two weeks after the third vaccination with SL3261-pcDNA3.1 + VEGFR2$_{(n1-4)}$ or SL3261-pcDNA3.1 + VEGFR2$_{(n1-7)}$ by s.c. injection of CT-26 cells (Fig. 6). In contrast, animals in control groups revealed uniformly rapid s.c. tumor growth. And we also noted that tumor latency period and survival time were significantly longer in group A and B than that in group C and D ($p < 0.05$, Fig. 7); the tumor size, weight were significantly less in group A and B than that in group C and D ($p < 0.05$, Table 5, Fig. 8).

Effect of oral immunization on tumor vessel counts. Macroscopic structure showed the tumor tissue was hard and adhered to the surrounding tissue. Intratumoral microvessel density was evaluated as an indirect assessment of effects on tumor angiogenesis. Immunohistochemical staining for CD31 to detect microvessel in CT-26 tumor revealed a significant decrease in tumor-vessel counts in group A ($p < 0.05$) and group B ($p < 0.05$), compared to those in the control groups (Fig. 9).

Protective effect of pcDNA3.1 (+)-VEGFR2$_{(n1-4)/(n1-7)}$ against CT-26 In the liver metastasis tumor model group. We established that our DNA vaccines are effective in inhibiting colon cancer liver metastases. We noted a marked reduction in liver metastases in all experimental animals following threee immunization with our DNA vaccines. Mice of different groups in the liver metastases tumor model group were sacrificed 2 wk later after tumor inoculation and liver metastases were enumerated, we observed that mice in group A and B both had less liver metastases than that of group C and D ($p < 0.05$, Table 5, Figs. 10 and 11). The liver metastases of group A and B have no statistical significance compared with each other respectively.

Discussion

Tumor growth and metastasis are angiogenesis-dependent processes.[18] Angiogenesis is typically stimulated in response to tumorsecreted angiogenic factors such as VEGF, which bind to high-affinity VEGFR2 and promote endothelial cell proliferation, invasion and the formation of new capillaries.[19-22] Increased vascular endothelial growth factor (VEGF) expression is associated with both primary and metastatic tumor growth. We hypothesized that competitive binding of VEGF can inhibit the growth of carcinoma cells and decrease metastases; this antiangiogenic therapy strategy may provide a novel addition to current antineoplastic approaches.

Our strategy was to construct extracellular region of VEGFR2 based DNA vaccine to inhibit angiogenesis by decreasing VEGF level of serum, blocking VEGF/VEGFR2 pathway and destructing endothelial cells that express VEGFR2 by immune responses. Recent advances in the understanding of VEGFR2 have shown that extracellular domain 1–4 of VEGFR2 is essential for tight binding with VEGF.[12] It is suggested that the extracellular domain 1–4 of VEGFR2 is a suitable target to develop DNA vaccine for the suppression of tumor growth and metastases. We cloned the domains 1–4 of extracellular region of VEGFR2, developed DNA vaccines SL3261-pcDNA3.1 + VEGFR2$_{(n1-4)}$. Additionally, DNA vaccine SL3261-pcDNA3.1 + VEGFR2$_{(n1-7)}$ encoding full length of extracellular region was constructed for comparative study on antitumor effect. The antitumor effects of both gene vaccines were

Table 2 **CD4+T cells of each group (mean ± SD)**

Group	*n*	Before immunization	after immunization	2 weeks after tumor challenge
A	8	62.34 ± 6.63[a]	65.82 ± 7.25[b]	62.12 ± 6.44[c]
B	8	59.36 ± 4.54[d]	74.12 ± 3.46[e]	73.13 ± 3.27[f]
C	8	64.15 ± 7.65	67.86 ± 8.24	44.79 ± 5.16
D	8	62.78 ± 6.54	65.89 ± 7.94	43.76 ± 6.12

2 weeks after tumor challenge, flow cytometer results showed that CD4+T cells of experimental groups still maintained high compared to the control groups (A/C, cp = 0.000; A/D, cp = 0.000; B/C, fp = 0.000; B/D, fp = 0.000; A/B, p = 0.355).

Table 3 **CD8+T cells of each group (mean ± SD)**

Group	*n*	Before immunization	after immunization	2 weeks after tumor challenge
A	8	10.16 ± 0.84[a]	15.64 ± 1.46[b]	11.92 ± 2.60[c]
B	8	9.65 ± 1.01[d]	16.22 ± 2.01[e]	13.45 ± 2.76[f]
C	8	9.78 ± 0.59	9.58 ± 1.24	7.54 ± 2.97
D	8	10.72 ± 1.54	9.49 ± 1. 39	5.53 ± 2.40

After immunization, flow cytometer results showed that CD8+ T cells of experimental groups were higher than the control groups (A/C, bp = 0.000; A/D, bp = 0.000; B/C, ep = 0.000; B/D, ep = 0.000; A/B, p = 0.224); and 2 weeks after tumor challenge, CD8+ T cells of experimental groups were higher than the control groups (A/C, cp = 0.000; A/D, cp = 0.000; B/C, fp = 0.000; B/D, fp = 0.000; A/B, p = 0.370).

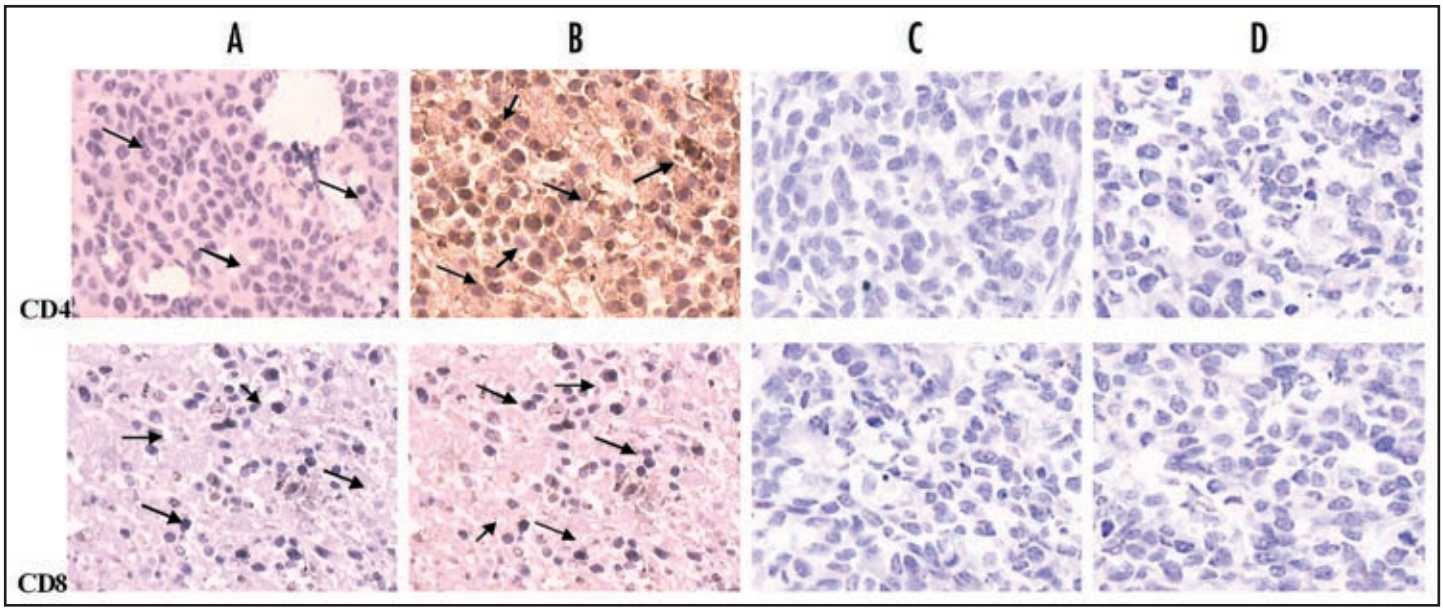

Figure 5. Immunohistochemistry for CD4+ and CD8+ T cells. Up shows Immunohistochemistry result of CD4+ T cells in each group. Photographs taken at 400 xs of representative areas within distinct primary tumors. Black arrows indicate infiltrating cells with characterisitc brown membrane staining. Down shows Immunohistochemistry result of CD8+ T cells in each group. Photographs taken at 400 xs of representative areas within distinct primary tumors. Black arrows indicate infiltrating cells with characterisitc brown membrane staining. (A) SL3261-pcDNA3.1 + $VEGFR2_{(n1-4)}$ group; (B) SL3261-pcDNA3.1 + $VEGFR2_{(n1-7)}$ group; (C) empty vector group; (D) $NaHCO_3$ group.

evaluated through detecting the tumor volume, mouse survival time, intratumoral microvessel density (MVD) and liver metastases. The results showed that vaccinated mice in two vaccine groups (group A and B) had longer survival time, lower weight of tumors and liver metastases. Furthermore, MVD of immunized mice (group A and B) were lower than that of control groups (group C and D). We concluded that the oral vaccine encoding 1–4 extracellular domain of VEGFR2 could induce an anti-tumor effect as the vaccine encoding 1–7 extracellular domains of VEGFR2.

It is well known that a lower molecule weight protein is benefit, especially utilization of an attenuated bacterial carrier, to stable express foreign protein in host cell, which result in effective tumor-protective immunity. In comparison with gene vaccine developed with the full-length of extracellular domain of VEGFR2, the protein expression of SL3261-pcDNA3.1 + $VEGFR2_{(n1-4)}$ is a difference of approximate 44 kDa. Based on evaluating the antivasculature and antitumor effects against CT-26 tumor cells, we infer that the smaller fragment ($VEGFR2_{(n1-4)}$) is sufficient for VEGF binding and inducing immune response, and the DNA vaccine encoding extracellular domain 1–4 of VEGFR2 is stable and safe for the immunocompromised host. As shown in animal studies, DNA vaccine encoding extracellular domain 1–4 of VEGFR2 can efficiently induce long-term immune responses. Other research groups show that VEGFR2 based DNA vaccines could inhibit tumor growth and metastasis though immune response and cell-mediated immune response. In our study, we observed CD4+ and CD8+ T cells in peripheral blood and subcutaneous tumors in immunized mice were obviously higher than that of the control groups, and specific anti-VEGFR antibodies was induced after administration by oral DNA vaccine. However, we also found that serum level of VEGF significantly decreased in immunized mice. The results presumed that competitive binding of VEGF by extracellular domain 1–4 of VEGFR2 may also contribute to block the VEGF/VEGFR2 pathway and induce antiangiogenesis effect around the solid tumor.

Taken together, our studies demonstrate that the extracellular domain 1–4 of VEGFR2 is a suitable target for the suppression of tumor growth and metastases against CT-26 colon cancer cells. Our results confirm the study that the first four extracellular region of

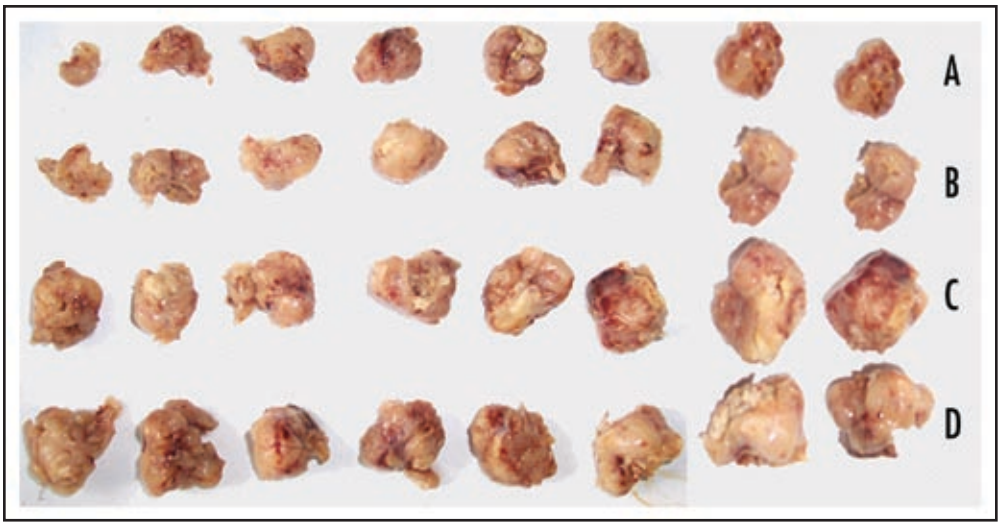

Figure 6. Representative colon carcinoma specimens of mice challenged with CT-26 cells 4 weeks after removal of the subcutaneous primary tumors. (A) SL3261-pcDNA3.1 + VEGFR2$_{(n1-4)}$ group; (B) SL3261-pcDNA3.1 + VEGFR2$_{(n1-7)}$ group; (C) empty vector group; (D) $NaHCO_3$ group.

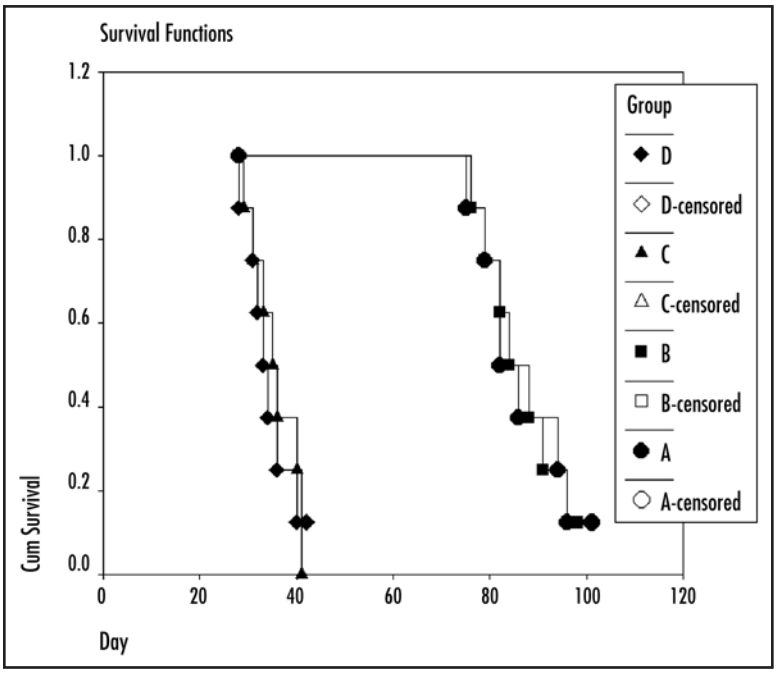

Figure 7. Lifespan of BALB/c mice after *s.c* challenge with CT-26 colon carcinoma cells. The lifespan of groups of mice ($n = 8$) are shown following tumor cell challenge after repeated vaccination. Death occurred in control groups 28 days after tumor challenge, and in group A and B, death occurred 75 days after tumor challenge. ●, group A; ■, group B; ▲, group C; ♦, group D.

VEGFR2 have been shown to be required for ligand binding.[23,24] It is anticipated that this multifunctional DNA vaccine may aid in the rational design of such vaccines for the immunotherapy of colorectal cancer. Further research is needed in this direction.

Materials and Methods

Materials. Bacterial strains. Attenuated Salmonella typhimurium SL3261 (aromatic amino acid auxotrophic mutant) was kindly presented by SJ Dunstan (British empery science medical college) and JF Bian (Lab of Molecular biology, School of Medicine, Shandong university). Bacteria were routinely grown at 37°C in LB broth or agar, supplemented with 100 μg/ml of Ampicillin when required.

Cell line. CT-26 cell line and COS-7 cell were purchased from ATCC and GIBCO respectively.

Animal. BALB/c mice were fed at Laboratory Animal Center of Kunming Medical College. All mice (female, specific pathogen free, with age of 6–8 wk and weight of 15–20 g) were acclimated for 1 wk and caged in groups of eight, and fed a diet of animal chow and water. Principles of laboratory animal care (NIH publication No. 85-23, revised 1985) were followed.

Main reagents. pcDNA3.1 (+) and lipofectamine 2000 Reagent were products of Invitrogen. *Kpn I*, *Xba I* and dNTP were products of TaKaRa (Japan). Protein DetectorTM TMB Western blot kit, Rabbit Anti-goat IgG Horseradish Purified Antibody, Monoclonal Antibody MOUSE CD8-Pe and CD4-FITC were obtained from KPL (U.S). Rabbit Anti Mouse CD4 monoclonal Antibody and rabbit Anti Mouse CD8 monoclonal antibody were supplied by BIOS (China). Mouse VEGF ELISA kit was purchased from JingMei (China). Mouse anti-VEGFR2 extracellular fragment antibody was provided by R&D. CD31 antibody was a product of Santa Cruz (U.S). RMPI1640, DMEM, FBS and G418 were purchased from GIBCO (U.S). Micro BCA protein assay was from Pierce (U.S).

Methods. Culture of cells. *Cell cultures.* CT-26 murine colon carcinoma cells were cultured and maintained in RPMI1640 supplemented with 150 ml/L fetal bovine serum, 2 units/ml penicillin-streptomycin, at 37°C in 50 ml/L CO_2 and 95% air. Cells were harvested from subconfluent cultures with trypsin-EDTA for 1 min, suspended in media, centrifuged at 1500 rpm for 6 min at room temperature, and then resuspended to a final concentration of 10^6 viable cells/ml PBS. Trypan blue exclusion was performed to ensure cell viability. COS-7 cell was cultured in DMEM containing 100 ml/L fetal bovine serum and 50 ml/L CO_2 at 37°C.

Vaccine preparation. *Construction of the expression vector encoding murine VEGFR2 extracellular domain 1–4/1–7.* DNA encoding murine VEGFR2 extracelluar domain 1–4 was cloned with the upstream primer: 5'-CCGGTACCATGGAGAGCAAGGCGC-3' and downstream primer: 5'-GCTCTAGATTATTCCAAGTTG-GTCTTTTCCTG-3', DNA encoding murine VEGFR2 extracelluar domain 1–7 was cloned with the upstream primers 5'-CCGG-TACCATGGAGAGCAAGGCGC-3' and downstream primer: 5'-GCTCTAGATTATTCCAAGTTGGTCTTTTCCTG- 3', then the cloned genes were inserted into the pcDNA3.1(+) vector between

Table 4 **Tumor weight, volume and microvessel density of tumor in vivo (mean ± SD)**

Group	*n*	Weight of tumors (g)	Tumor volume (mm^3)	Microvessel density
A	8	3.64 ± 1.34[a]	2.62 ± 0.54[b]	2.06 ± 1.02[c]
B	8	3.21 ± 1.22[d]	2.34 ± 1.03[e]	2.53 ± 1.33[f]
C	8	8.40 ± 0.66	6.01 ± 0.14	6.93 ± 2.34
D	8	8.26 ± 0.44	5.92 ± 0.25	7.34 ± 4.12

Tumor weight of the experimental groups were lower than that of control groups (A/C, ap = 0.000; A/D, ap =0.000; B/C, dp = 0.000; B/D, dp = 0.000; A/B, p = 0.270), tumor volume of the experimental groups were less than that of the control group (A/C, bp =0.000; A/D, bp = 0.000; B/C, ep = 0.000; B/D, ep = 0.000; A/B, p = 0.623); MVD of immunized mice in two vaccine groups were significantly less than the control groups (A/C, cp = 0.000; A/D, cp = 0.000; B/C, fp = 0.000; B/D, fp = 0.000; A/B, p = 0.728).

Table 5 **Liver metastases of each group (mean ± SD)**

Group	*n*	The number of tumor nodules
A	8	3.26 ± 1.82[a]
B	8	4.02 ± 1.32[b]
C	8	18.54 ± 3.82
D	8	22.54 ± 4.52

A marked reduction was observed in liver metastases in all experimental animals following threee immunization with our DNA vaccines (A/C, p = 0.000, A/D, p = 0.000; B/C, p = 0.000, B/D, p = 0.000; A/B, p = 0.634).

the restriction enzyme sites *Kpn I and Xba I* to generate recombinant plasmids pcDNA3.1 + $VEGFR2_{(n1-4)}$ and pcDNA3.1 + $VEGFR2_{(n1-7)}$ respectively.

Expression of the pcDNA3.1 + $VEGFR2_{(n1-4)}$ and pcDNA3.1 + $VEGFR2_{(n1-7)}$ in the COS-7 cell. Protein expression of pcDNA3.1 + $VEGFR2_{(n1-4)}$ and pcDNA3.1 + $VEGFR2_{(n1-7)}$ were demonstrated by transient transfection of each vector into COS-7 cells and by performing Western blots of the respective cell lysates (pcDNA3.1 + $VEGFR2_{(n1-4)}$ and pcDNA3.1 + $VEGFR2_{(n1-7)}$) and supernatant with anti-VEGFR2 extracellular fragment Ab, respectively.

Preparation of the oral DNA vaccines SL3261-pcDNA3.1 + $VEGFR2_{(n1-4)}$ SL3261-pcDNA31 + $VEGFR2_{(n1-7)}$. Attenuated Salmonella typhimurium SL3261 was used as vaccination vector. The recombinant plasmids pcDNA3.1 + $VEGFR2_{(n1-4)}$ and pcDNA3.1 + $VEGFR2_{(n1-7)}$ were transformed into competent SL3261 by using $CaCl_2$ transformation method[15] to develop oral DNA vaccines SL3261-pcDNA3.1+$VEGFR2_{(n1-4)}$ and SL3261-pcDNA3.1 + $VEGFR2_{(n1-7)}$ respectively.

Oral immunization and tumor cell challenge. In the subcutaneous tumor model, thirty-two mice were equally divided into group A, B, C and D namely immunized with SL3261-pcDNA3.1 + $VEGFR2_{(n1-4)}$, SL3261-pcDNA3.1 + $VEGFR2_{(n1-7)}$, pcDNA3.1 (+) and 100 mg/L $NaHCO_3$ respectively. All mice were immunized by oral gavage 3 times at 2-wk intervals, at the concentration of 1 x 10^9 cfu/ml, and then all mice were challenged 2 wk later by s.c. injection of 1 x 10^6 CT-26 colon cell at right armpit.

In the liver metastasis tumor model, thirty-two mice were equally divided into group A, B, C and D, mice in different group were treated with 100 μl of SL3261-pcDNA3.1 + $VEGFR2_{(n1-4)}$, SL3261-pcDNA3.1 + $VEGFR2_{(n1-7)}$, empty plasmid and 100 mg/L$NaHCO_3$ respectively, at the concentration of 1 x 10^9 cfu/ml, three times every 7 d by gastrogavage, after 2 wk of the last immunization, mice in each group were anesthetized and 1 x 10^5 of CT-26 tumor cell were injected beneath the splenic capsule.

Body distribution of the recombinant vaccine strain SL3261. Our experiments are based on the working hypothesis that orally administered Attenuated Salmonella typhimurium, carrying expression vectors encoding extracellular domain 1–4 of VEGFR2 to the host. In order to confirm that our DNA vaccines could be carried to the host by the Attenuated Salmonella typhimurium SL3261, 2 days after the last

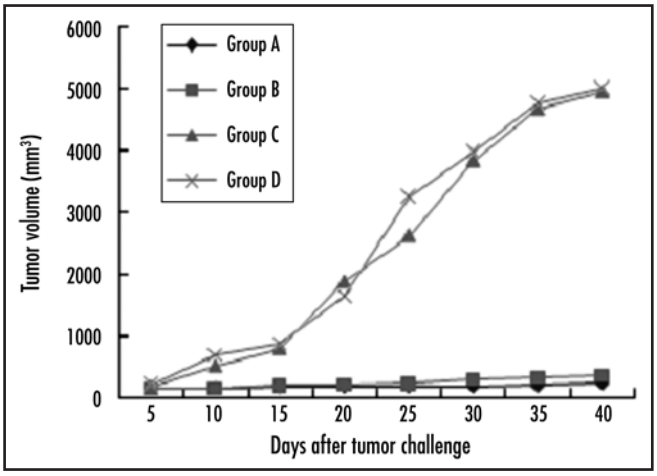

Figure 8. Curve of tumor growth in mouse model in the mouse model of colon adenocarcinoma CT-26. (A) SL3261-pcDNA3.1 + $VEGFR2_{(n1-4)}$ group; (B) SL3261-pcDNA3.1 + $VEGFR2_{(n1-7)}$ group; (C) empty vector group; (D) $NaHCO_3$ group.

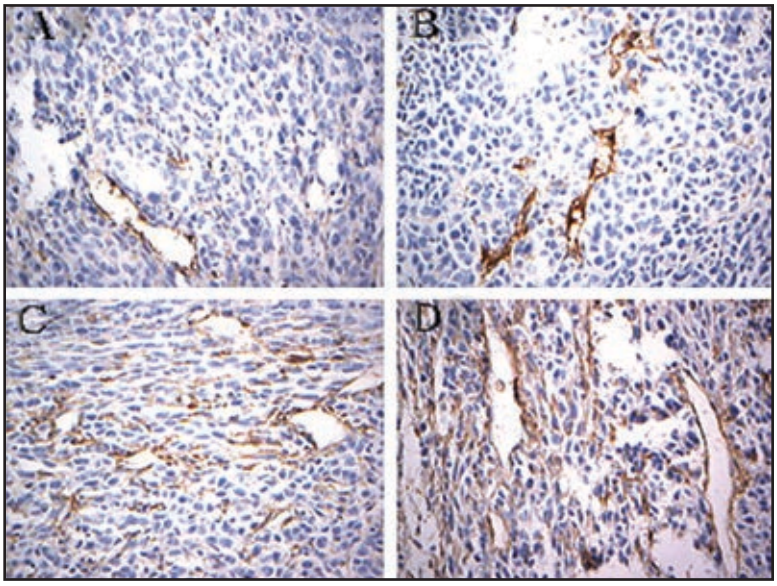

Figure 9. Immunohistochemistry staining of tumor tissue in the subcutaneous tumor model (CD31 x 400). (A) Microvessel density (MVD) in tumor tissue of SL3261-pcDNA3.1 + $VEGFR2_{(n1-4)}$ group (CD31 x 400); B: Microvessel density (MVD) in tumor tissue of SL3261-pcDNA3.1 + $VEGFR2_{(n1-7)}$ group (CD31 x 400); (C) Microvessel density (MVD) in tumor tissue of empty vector group (CD31 x 400); (D) Microvessel density (MVD) in tumor tissue of $NaHCO_3$ group (CD31 x 400).

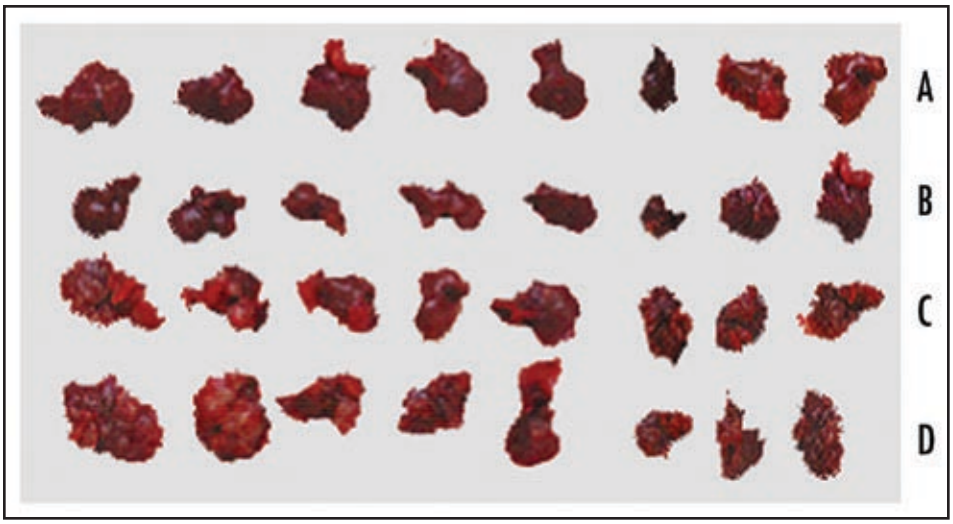

Figure 10. Liver metastases lesions in the liver metastasis tumor model. (A) SL3261-pcDNA3.1 + VEGFR2$_{(n1-4)}$ group; (B) SL3261-pcDNA3.1 + VEGFR2$_{(n1-7)}$ group; (C) empty vector group; (D) $NaHCO_3$ group.

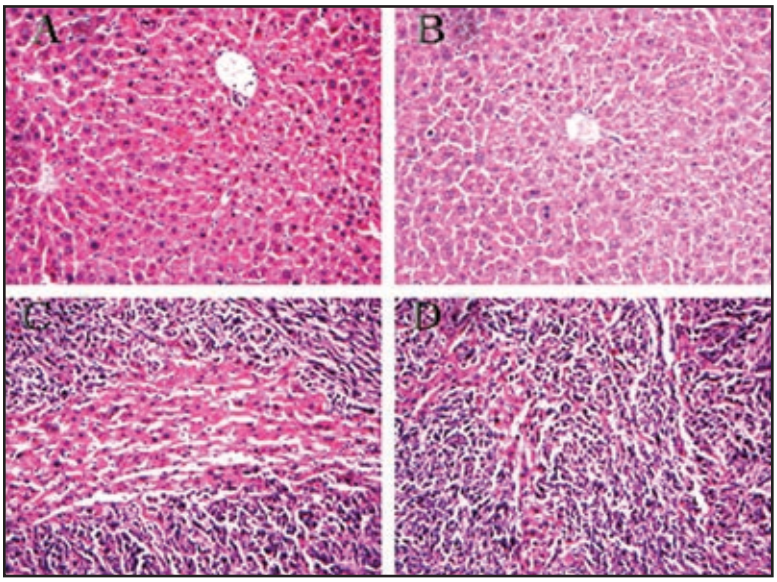

Figure 11. HE staining of liver (x200). (A) liver of SL3261-pcDNA3.1+VEGFR2$_{(n1-4)}$ group mouse (HE x 200); (B) liver of SL3261-pcDNA3.1+VEGFR2$_{(n1-7)}$ group mouse (HE x 200); (C) liver of empty vector group mouse (HE x 200); (D) liver of $NaHCO_3$ group mouse (HE x 200).

immunization, mouse in subcutaneous model group were killed randomly (1 mouse/group) to harvest the small intestine, colon, liver and spleen under aseptic conditions, the ultrastructure of the specimen were observed individually under Transmission electron microscopy (TEM) to detect the body distribution of the recombinant vaccine strain SL3261.

Detection of the eukaryotic expression vector in the genome. After the oral DNA vaccines were carried to the host, attenuated bacteria die due to their inability to synthesize aromatic amino acids, releasing a large number of plasmids that are subsequently transferred into the cytosol and nucleus of the infected cells. To confirm that the exogenous gene has been integrated into the genome of the BALB/c mice, the DNA of the small intestine, colon, liver and spleen of the mice were extracted and PCR was used to detect the eukaryotic promoter CMV of the recombinant plasmids, the upstream primer was: 5'-CCCAGTACATGACCTTATGGG-3', and downstream primer was 5'-GGAGACTTGGAAATCCCCGT-3'.[16] PCR was performed for 30 cycles as follows: denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s, and then extension at 72°C for another 10 min. Then the cloned gene was detected by 5 g/L gel electrophoresis.

Analysis of the serum levels of VEGF. We hypothesized that the downregulation of VEGF was attributed to antitumor response, and the serum levels of VEGF in mice of different group were assayed to prove that the serum VEGF was bonded by our vaccines and VEGF expression. The serum of each mouse was collected and kept at -20°C, and the levels of VEGF were assayed by ELISA as manufactures' instruction and Measure absorption value at 450 nm and calculate values of the samples.

Analysis of specific anti-VEGFR2$_{(n1-4)}$-IgG and anti-VEGFR2$_{(n1-7)}$-IgG. To confirm that the immune response induced by our DNA vaccines, the serum level specific anti-VEGFR2$_{(n1-4)}$-IgG or anti-VEGFR2$_{(n1-7)}$-IgG of BALB/c mouse in each group was assayed by ELISA as directed by the manufactures' instructions.

Anti-tumor growth effect of SL3261-pcDNA3.1 + VEGFR2$_{(n1-4)/(n1-7)}$ against CT-26. 1 wk after challenge of the CT-26 cells, tumor was formed in all mice in subcutaneous tumor model group. The tumor weight and diameter were recorded every two day and the survival time of each mouse was also recorded. Observation was kept until deaths of the animals.

Analysis of lymphocyte subset by flow cytometry and immunohistochemistry. We tested our hypothesis that $CD4^+$ and $CD8^+$ T cells are also responsible for the antitumor response. Fresh anti-coagulating blood (100 ul) of each mouse in the subcutaneous tumor model group was taken after 14 d, 28 d and 42 d of the last immunization, and then the $CD4^+$, $CD8^+$ T cells were analyze by the FCM.

After the mice were dead, tumor tissues were taken from vaccinated mice and non-vaccinated mice as a control. In brief, tumor tissues were fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin. Specimens were stained with hematoxylin/eosin and assessed immunohistochemically for CD4, CD8 using the corresponding monoclonal antibodies.

Counting of microvessel density in CT-26 solid tumor. MVD count was determined using the Chalkley method.[17] In brief, tissue sections were immunostained with a monoclonal anti-CD31 antibody. MVD count was determined by identifying the areas of highest vascular density for each cancer slide at 100x magnification. These areas were then examined at 200x magnifications with the 25 point Chalkley eyepiece graticule oriented to permit maximum number of points to hit on blood vessels. The number of vessels to hit upon a Chalkley

point represented the Chalkley score. Two slides per cancer specimen were recorded, and their average score was used.

Anti-liver metastasis effect of SL3261-pcDNA3.1 + VEGFR2$_{(n1-4)(n1-7)}$ against CT-26. In the liver metastasis tumor group, the immunized mice were sacrificed 2 wk later after tumor inoculation and liver metastases were enumerated, and the liver was routinely fixed and embedded in paraffin for H&E staining.

Statistical analysis. Results were expressed as mean ± SD. Data were analyzed by the method of ANOVA using SPSS 10.0 software. $p < 0.05$ was considered statistically significant.

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