# TREATMENT OF RAT HEPATOMA BY LOCALLY INJECTION OF MURINE IL-12 RETROVIRUS PACKAGING CELL

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## ABSTRACT

**Objective: To investigate the therapeutic effects of the murine IL-12 (mIL-12) retrovirus packaging cell line on hepatoma injected locally. Methods: The retrovirus vector encoding mIL-12 gene was constructed and transfected into packaging cell line PA317. The cells were then used to treat the rats with experimental orthotopic hepatoma at different time. The therapeutic effects, immune functions of the hosts, pathological and toxicological responses were documented. Results: the results showed that the mIL-12 retrovirus packaging cell line could significantly inhibit the growth of the hepatoma cells injected locally to the hepatoma. The early treatment made the rats survive long, while the medium or late stage treatment could prolong the life time of the rats compared with the bland control group or bland vector control group, though the rats did not survive. The number of NK cells and T cells increased significantly in the treatment group. The effects of the early treatment were superior to those of the medium and late stage treatment. Moreover, the transfection of IL-12 gene locally in the hepatoma tissue could make the hepatoma disappear from other liver lobe. This phenomenon demonstrated that IL-12 could activate the immune cells of the host to kill the untransfected tumor cells. This is very important for IL-12 to be used in gene therapy clinically. Meanwhile, the hepatoma would not recur in the rats that had survived more than 2 months from the early treatment after being re-challenged with tumor cells. Conclusion: the results showed that IL-12 gene injected locally in the hepatoma tissue could enhance the anti-tumor immunity of the host.**

**Key words: Interleukin-12, Hepatoma, Intra-hepatic injection, Gene therapy**

Interleukin-12 (IL-12) has some kinds of anti-tumor activities and is regarded as most effective cell factor that intervenes anti-neoplastic activity. In our previous work, we treated experimental rat hepatoma with murine interleukin-12 (mIL-12) by intraabdominal local injection. The result showed that the gene therapy with mIL-12 for rat liver cancer was of a significant therapeutic effect. In present study, using model of rat orthotopic hepatoma ($CBRH_3$), the retrovirus vector encoding mIL-12 gene, which was constructed and transferred into packaging cell line PA317, was injected into rat liver cancer locally in order to study rat survival and immune functions of host.

## MATERIALS AND METHODS

### Cellular Strain and Plasmid

Monophilic and bi-ophilic retrovirus vector packaging cell line PE501 and PA317 and NIH3T3 (determining virus titre) were supplied by our laboratory. Multi-gene expression of retroviral vector GCXEPN contained encephalomyocarditis virus (ECMV RIES) and poliovirus (Polio IRES), which ensured multi-gene high expression simultaneously, was established in our laboratory. The retroviral vector packed cell strain PA317-LacZ contained report gene LacZ, which titre was $3.5\times10^6$cpu/ml, was established in our laboratory. Plasmic P35 contained rat IL-12 and plasmid P40 PGCP35 IRESP40SN was friendly supplied by Dr. Liu Xinyuan (Shanghai Institute of Biochemistry, Chinese Academy of sciences).

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### The Model of Hepatoma

Rat hepatoma cell strain $CBRH_3$ was friendly supplied by Dr. Hong Xie (Animal Center, Chinese academy of Sciences). Wistar rat, male, 200-250g wt was purchased from Animal Center, Chinese Academy of Sciences. $CBRH_3$ was injected into abdominal cavity of rat. Tumor was formed and after 7-9 day. Rats were sacrificed and tumor was taken out from cavity. The pieces of tumor were cut to 0.05×0.1×0.1cm. One tissue of tumor piece in syringe was inoculated (injected) into rat liver for one or more points. The success of inoculation was 100%.

### Rat IL-12 Retroviral Vector GCMEXPN-IL-12

The GCMEXPN-IL-12 was expanded in PGCP35IRESP40 by PCR. The primers of fragments of mIL-12 P35 and P40 were P35 5'-primer CGGGCGGGCCGCACGTCAA-TCACGCTACCTC 3'–prime ACTGCGGCCGCTCAGGCGGAGCTCA-GATAGCCGA. Both of them had site-Not I-restrict digest. P40 5'-primer GCGGTCGACACCATGGG-TCCTCCCAG-AAGCCTAACC 3'-primer CGGTCG-ACCTAGGATCGGACGGTGCAGGGAAC. Both of them had site-Sal I-restrict digest. The products of PCR were restricted by Not I and Sal I. This fragment entry GCXEXPN vector was identified the positive and negative direction by PCR. The technique of restricted, join and distill-plasmid has previously been described.

### Constitution of Rat IL-12 Retrovirus Vector Packed Cell Strain

Monophile retrovirus packed cell strain PE501, using electroporation, was transfected by GCXEXPN-IL-12. The supernant and 1:100 polybrene (8μg/ml) were added to PA317 next day. After 4hr, common culture medium was replaced. G418 600μg/ml was added for screening in 48hr. The clone was formed in two weeks. The screening was repeated for two weeks. Virus titre was then screened by NIH3T3, and the highest virus titre of PA317 was termed for PA317-mIL-12. Routine screening was performed.

### Determination of mIL-12 protein

It was determined by ELISA, and this Kit was purchased from Endogen (USA).

### Group of Animal

The rats had been divided into inoculation 0.05×0.1×0.1cm tissue of hepatoma in one lobe and inoculation 0.05×0.1×0.1cm tissue of hepatoma in two lobes. The former, the development of tumor treating with IL-12 in this lobe was observed. Both of these two groups was once more sub divided into blank control, virus vector control and therapeutic groups. In blank control and virus vector control the physiologic saline (0.2 ml per rat) and PA317-LacZ ($1\times10^7$ cell per rat) were injected into local cancer after inoculation of hepatoma 1 day late, respectively. In therapeutic group the PA317-mIL-12 ($1\times10^7$ cell per rat) was injected into local cancer in inoculation of hepatoma in 1,3,5 and 7 day. Ever group consisted of ten rats. The rats, that survival time after inoculation was more than two months, were termed long survival time and would be inoculated again.

### MRI and CT of Rat

The methods of MRI and CT for test of rat model of hepatoma were routine.

### Immunohistochemistry

The tissue from animal model of hepatoma would be used for rabbits immunohistochemistry ABC. The monoclonal antibody OX8 (anti-NK and cytotoxic T lymphocyte) was friendly supplied by Dr. Jinquan Wu. When rats died, the liver will be routinely fixed and pathology will be checked.

### Statistical Analysis

X2-Test was used for statistical analysis.

## RESULTS

### Construction of Retrovirus Vector encoding mIL-12 Gene Pgxexpn-mIL-12

The fragments of P35 and P40 of rat mIL-12 were expanded by amplification of PGCP35IRES40S, which were inserted into the cloned site of Not I of GCXEXPN and site of Sal I after restrict digest by Not I and Sal I. It was proved that P35 and P40 were inserted into the site because electrophoresis mapping comprised 650BP, 775bp and 1017bp, 738bp, respectively. It indicated that two sections were jointed in positive direction because expansion to 126bp by using PCR P35 5' primer, ECMV 3'primer and expansion to 1632bp by using PCR ECMV 5'primer and P35 3'primer.

### Screen and Clone of Retrovirus Vector Encolding mIL-12 Gene Packing Cell Strain PA317-mIL-12

Monophilic retrovirus packing with cell strain

PE501 was transfected with retrovirus vector encoding mIL-12 gene. The supernatant was added to bi-ophilic retroviral pack cell strain with 1:100 polybrene (8 μg/ml) next day. Supernatant was changed after 4h and performed screen with G418 600μg/ml. 10 clones were formed and seeded to 24 well plate. Then, two weeks after, using NIH3T3 to screen virus, the maximum titre of virus was 1.2cpu/ml and the mIL-12 sectet was 27ng/$10^6$/48h, detected by ELISA, which termed PA317-mIL-12.

## Comparison of Rat Survival in Local Therapy of Hepatoma

The death of one liver lobe inoculated hepatoma was 100% in control groups with physiologic saline and virus (Figure 1). The mean lifetime of control was 13.1±1.8d and 12.6±1.2d, respectively. Furthermore, the therapeutic group treated at 1,3,5, and 7 days, showed long survival rate of 100%, 100%, 30% and 10%, respectively. The mean lifetime of therapeutic group was 21.3± 3.2d and 18.2±2.9d, respectively. No case died due to acute toxicity. The mean life time of therapeutic group made the rats survival long markedly compared to control ($P$<0.01). In addition, in rats that had survived long the liver cancer did not developed after inoculation with liver cancer again in two months. Meanwhile, when liver cancer was inoculated in both liver lobes, it would completely vanished in single lobe after treating it with IL-12 in the other liver lobe. The lifetime of rats was similar to others who inoculated hepatoma in single liver lobe (Figure 2).

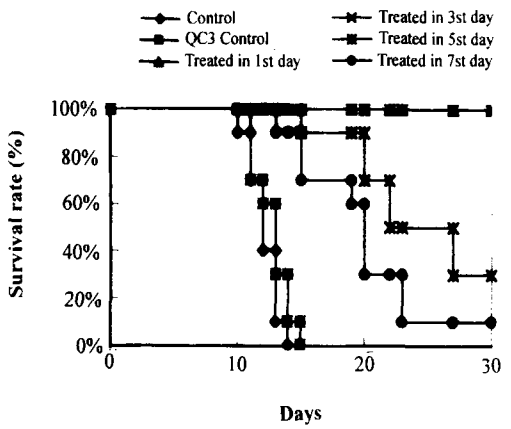


Fig. 1. The result of treatment with hepatoma vaccination in liver in different time

## The Results of CT and MRI in Therapeutic Group

The rat liver cancers were formed by inoculation of hepatoma at 7 day, which was detected by MRI. The tumors were vanished in CT image after treatment with IL-12 for one month.

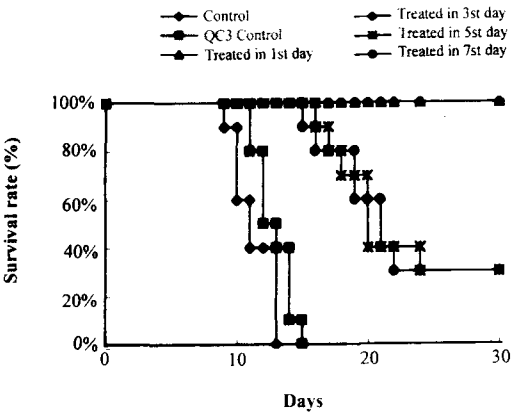


Fig. 2 The result of treatment of single lobar hepatoma vaccination in both lobar in different time.

## Expression of OX8 in Hepatoma Tissue

Compared to controls with physiologic saline or blank vector, a number of peripheral lymphocyte was found around tumor with injected retrovirus vector encoding mIL-12 gene packing cell strain into local hepatoma. Increase of positive rate of OX8 indicated that a number of NK cells, and cytotoxic T lymphocyte increased markedly in therapeutic group by rabbits immunehistochemistry.

## DISCUSSION

We studied the gene therapy for experimental hepatoma model of rat abdominal cavity and screened the aim gene including IL-2, IL-12, B7 (co-stimulated tumor gene), thymidine kinase gene of herpes simplex virus and apoptosis gene (ICE) etc. The effectiveness of IL-12 was similar to the report by Rakhmilevich in 1997. Meanwhile, some reports showed that IL-12 had significant effect to inhibit proliferation of hepato-B-virus. In present work the IL-12 was chosen as main candidate gene for liver cancer gene therapy in pre-clinical research.

To mimic human liver cancer, the model of rat orthotopic liver cancer from Dr. Hong Xie was used which was induced by diethylnitrosamine in Wistar rat. The success rat of orthotopic hepatoma inoculation was 100% and stable. Mean survival time was 13d. In present study long survival time of rat was observed after administrating IL-12 for gene therapy of liver othotopic cancer at day 1th and day 3th. Moreover, the lifetime only was prolonged by treating at day 5th and day 7th. This indicated that the effect of early gene therapy is superior to those of

medium and late stage treatment. Treating single liver lobe with IL-12 for inoculating liver cancer in both lobes was shown that other side of liver cancer was disappeared. This implied that IL-12 could activate immune system to kill beyond liver cancer. Increase of OX8 lymphocyte was positive by rabbit immune-histochemistry, which was NK cell and cytotoxic T lymphocyte. It is important for clinic gene therapy, which can not transfect the gene into all of tumor cell. Our data also showed that gene therapy of IL-12 prevented growth of liver cancer and protected liver cell.

IL-12 gene therapy is intensive studied recently. Transfecting retrovirus vector encoding mIL-12 to fibroblast has been used at II stage clinic test for tumor gene therapy in America. In China the adenovirus vector of mIL-12 has been constructed by the group of Dr. Xuetao Cao. We also constructed highly reduced toxic vaccinia virus vector (MVA) encoding mIL-12 gene. Compared to that of adenovirus vector, the expression of vaccinia virus vector was superior secret. We believe, followed improving of vector system, the gene therapy of mIL-12 would be much effective for liver cancer.

## REFERENCES

[1] Lamont AG, Adorini L. IL-12, a key cytokine in immune regulation. Immunol Today 1996; 17: 214.

[2] Yang Jiahe, Qian Qi-jun, Wu Meng-chao, et al. Cell immune and antitumor effects of interleukin-12. Bulletin biotech 1998; 9: 219.

[3] Rekhmilevich AL, Janssen K, Turner J, et al. Cytokine gene therapy of cancer using gene gum technology: superior antitumor activity of interleukin-12. Human Gene Ther 1997; 8: 1303.

[4] Cavanaugh VJ, Guidohi LG, Chisari FV, et al. Interleukin-12 inhibits hepatitis B virus replication in transgenic mice. J Virol 1997; 71:3236.

[5] Zhang Wei-ping, Cao Xue-tao. The construction and application of adenovirus vector coexpressing the heterodimer of human IL-12. Chin Med J 1998; 78: 33.