· Original article ·

Treatment of gastric cancer with replication-competent adenovirus carrying interleukin-12 gene

XUE Xu-Chao[1], FANG Guo-En[1], ZHANG Qi[2], XUE Hui-Bin[2], BI Jian-Wei[1], CAO Gui-Song[1], QIAN Qi-Jun[2]* (1. Department of Surgery, Changhai Hospital, Second Military Medical University, Shanghai 200433, China; 2. Viral & Gene Therapy Laboratory, Eastern Hepatobiliary Surgery Institute, Shanghai 200438)

[**ABSTRACT**] **Objective**: To investigate antitumor effects of a replication-competent adenovirus carrying mouse interleukin-12 gene on gastric cancer. **Methods**: Replication-competent adenovirus carrying mouse IL-12 gene (CNHK200-mIL-12) was constructed to transfect gastric carcinoma cell line SGC-7901. The cytopathic effect, the expression of the mouse IL-12 gene, and the replication of the virus were observed respectively by cell pathology, ELISA and virus replication assay. **Results**: The vector system, CNHK200-mIL-12, possessed the same characteristic as the replicative adenovirus ONYX-015, replicating and proliferating in the gastric carcinoma cells but not in the normal cells, thus specifically killing gastric carcinoma cells. The replication level of CNHK200-mIL-12 in the gastric carcinoma cells was more than one thousand times higher compared with that of the conventional adenovirus vector; the mIL-12 expression level increased by 100 folds compared with that of the conventional adenovirus vector carrying mIL-12 gene. **Conclusion**: CNHK200-mIL-12 can replicate specifically in gastric carcinoma cells and kill them. Meanwhile, it greatly increases the target gene expression, suggesting that CNHK200-mIL-12 may be used to treat gastric carcinoma.

[**KEY WORDS**] replication-competent adenovirus; interleukin-12; stomach neoplasms; gene therapy

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A fundamental problem for cancer gene therapy is the lack of a tumor-selective delivery system. Commonly used delivery vectors can be divided into 2 types: nonviral and viral vectors. Compared with nonviral vectors, viruses may be more efficient. Among them, replication-competent adenoviruses present a novel platform and draw much attention. Making use of the biology difference between tumors and normal tissues, recombinant replication competent adenovirus may selectively proliferate in tumor cells, resulting in oncolysis but is greatly attenuated in normal cells.

ONYX-015, an adenovirus from which the E1B 55 000 protein is deleted, replicates preferentially in p53-defecient cancer cells and may be a promising approach for cancer therapy[1-3]. However, the antitumor effect of ONYX-015 alone is insufficient and we constructed a new replicating adenovirus containing mouse interleukin-12 (mIL-12) gene, expecting such a recombinant adenovirus would take advantage of both virotherapy and genetherpay, leading to a synergistic antitumor activity. We tested it in gastric carcinoma cells, hoping to provide a new strategy for gastric cancer treatment.

1 MATERIALS AND METHODS

1.1 Cells and cell culture The human gastric adenocarcinoma cell line SGC-7901 and human normal liver cell line L02 were purchased from Institute of Biology, Chinese Academy of Science, Shanghai, China. SGC-7901 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL) supplemented with 10% neonatal calf serum (Hyclone) and L02 in RPMI 1640 with 10% fetal bovine serum. 293 cells, a human embryonic kidney cell line transformed with the left end arm of the adenoviral genome and containing the adenovirus E1A and E1B genes (Microbix Biosystem Ltd., Canada) was maintained in DMEM supplemented with 10% FBS. All the media were supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin and maintained in incubator at 37 C with 5% CO_2.

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[**Biography**] XUE Xu-Chao(1963-),male(Han nationality),MD. E-mail:qianqj@hkucc.hku.hk

1.2 Construction of plasmids and recombinant viruses Ad-mIL-12, an E1 region deficient adenovirus carrying the single chain gene of mIL-12, was preserved in our laboratory. ONYX-015, an adenovirus lacking the E1B 55 000 protein was kindly provided by professor Berk AJ. Adenovirus vectors pXC1, pCA13 and pBGHE3 were purchased from Microbix Biosystems Ltd. Canada. pCA13-mIL-12 (an adenovirus variant with E1 region deleted carrying single chain mIL-12 gene), was constructed in our lab. The vector pXC1 was digested with *Bgl* Ⅱ at the site of 3329, then partially digested by *Hind* Ⅲ. The retrieved DNA fragment of 9 372 bp was a new vector backbone in which the fragment from 2 809-3 329 bp was deficient compared with pXC1. The fragment was ligated by a synthetic linker, sequenced by E1B upstream primer (5′-CTG GCC AAT ACC AAC CTT A-3′) and E1B downstream primer (5′-ATA TGA GCT CAC AAT GCT TC-3′) and designated as pXC-delE1b. The vector pCA13-mIL-12 was digested by *Bgl* Ⅱ and a fragment containing CMV promoter + mIL-12 gene + SV40 polyA was released and then inserted into pXC-delE1b to produce pXC-delE1b-mIL-12. The obtained adenoviral vector plasmids pXC-delE1b and pXC-delE1b-mIL-12 were respectively co-transfected with pBHGE3, a plasmid of type 5 adenoviral right arm, into HEK293 cells using Lipofectamine Transfection Reagent (QIAGEN Inc.) to generate recombinant adenovirus. About 9-14 d after transfection, recombinant adenovirus was isolated from a single plague, purified 3 times and extracted with QIAamp DNA Blood Mini Kit (QIAGEN Inc.). After PCR identification under the following conditions: denaturation at 94℃ for 5 min; 94℃ 30 s; annealing at 50℃ for 30 s; and extension at 72℃ for 45 s(30 cycles); 72℃ 10 min, the recombinant adenoviruses were designated as CNHK200 and CNHK200-mIL-12 respectively.

1.3 Amplification and purification of recombinant adenoviruses CNHK200 and CNHK200-mIL-12 were amplified in HEK293 cells and purified by ultra-centrifugation on cesium chloride (CsCl) gradients. The titre was measured with the Tissue Culture Infection Dose (TCID50) method established by Qbiogene Inc. (IIIKich, France), and showed as plague-forming units per milliliter.

1.4 *In vitro* viral replication assay Monolayer SGC7-901 cell cultures in 6-well dishes (1×10^5 cells/well) were infected with ONYX-015, CNHK200-mIL-12 and Ad-IL-12 at an MOI of 10 pfu per well. Virus inocula were removed after 2 h. The cells were then washed twice with PBS and incubated at 37℃ for various time(0, 6, 12, 24, 48, 96 h). Lysates were prepared with 3 cycles of freezing and thawing. Serial dilutions of the lysates were titred on HEK193 cells with TCID50 method.

1.5 ELISA determination of mouse IL-12 expression SGC7901 cells were seeded in 24-well plates at a density of 1×10^5 cells/well and cultured overnight, followed by infection with Ad-mIL-12 and CNHK200-mIL-12 respectively at an MOI of 10. On day 1, 3, 5, 7 post infection, the supernatants of cell cultures were harvested, and assayed for mouse IL-12 gene expression using Mouse IL-12 ELISA Kit (Endogen Inc., USA) according to the manufacture's instructions.

1.6 Cytopathic effect assays Cells were seeded at a density of 5×10^5/well in 6-well plates 24 h before infection and then infected with adenoviruses CNHK200-mIL-12, ONYX-015 or Ad-mIL-12 at the indicated MOI (1 000, 100, 10, 1, 0.1, 0). The plates were fixed in 10% formaldehyde for 10 min at room temperature, stained with 1% crystal violet for 5 min on day 7 after infection, and then photomicrographs were taken.

1.7 *In vitro* cell viability assay MTT assays were performed to determine cell viability at various MOIs. SGC-7901 cells were seeded at a density of 1×10^4 in 96-well plates (Falcon) and 24 h later, the cells were infected with ONYX-015, CNHK200-mIL-12 or Ad-mIL-12 at an MOI of 10. Virus inocula were removed after 2 h of infection. On day 1, 3, 5, 7, cell viability was measured by the MTT assay using the non-radioactive cell proliferation Kit (Roche Molecular Biochemicals) according to the Kit protocol. The percentage of cell viability was calculated using the following formu-

la: cell killing rate = (the amount of dead cells/ the amount of total cells) ×100%.

1.8 Statistical analysis All the data were analysed by One-way ANOVA.

2 RESULTS

2.1 Identification of CNHK200-mIL-12 and Ad-mIL-12 The results of sequencing indicated that pXC-delE1b was deleted E1b 55 000 and inserted with two terminate code TAATGA compared with his parental pXC1. Using the IL-12 gene primers (upstream: 5′-ACC ATG GGT CCT CAG AAG-3′, downstream5′-CTT TCA GGC GGA GCT CAG ATA-3′), CNHK200-mIL-12 and Ad-mIL-12 were confirmed containing mIL-12 gene by PCR(Fig 1).

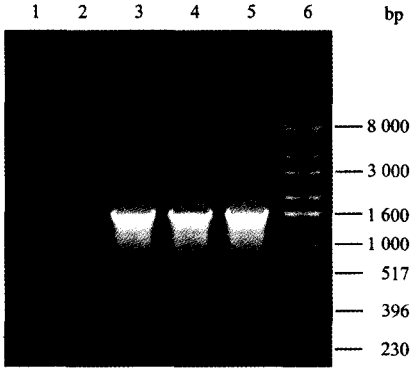

Fig 1 Recombinant adenovirus identification

1: Negative; 2: ONYX-015; 3: CNHK200-mIL-12; 4: Ad-mIL-12; 5: pXC-delE1b-mIL-12 plasmid; 6: Marker

2.2 Viral replication comparison As shown in Fig 2, ONYX-015 and CNHK200-mIL-12 replicate to multiples in SGC-7901 cells while Ad-mIL-12 replicate rather poorly. The replication capacity of replication competent adenovirus is 1 000 folds stronger than that of non-replicative adenovirus.

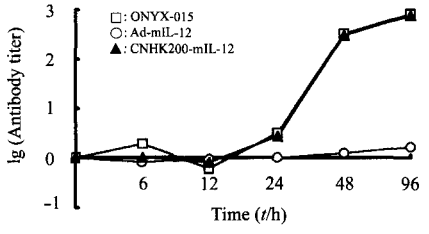

Fig 2 Virus replication in SGC-7901 cells

2.3 Mouse IL-12 gene expression in SGC-7901 cells Mouse IL-12 protein was detected in the supernatants of SGC-7901 cultures either infected with CNHK200-mIL-12 or Ad-mIL-12, but the former produced much more protein than the latter (Fig 3). The production of mouse IL-12 gene was accumulated more quickly at early stage than late stage, which is consistant with the replication of adenovirus. The expression level on day 7 of SGC-7901 cells infected with CNHK200-mIL-12 was hundreds of times of that on day 1. The expression of Ad-mIL-12 also increased a little after infection but decreased quickly along with time.

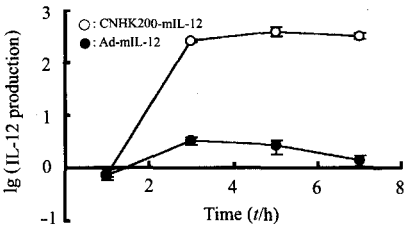

Fig 3 Determination of mIL-12 expression by ELISA

2.4 *In vitro* cytopathic effect (CPE) ONYX-015 and CNHK200-mIL-12 demonstrated strong cell killing ability: at MOI of 1 000 or 100, all SGC-7901 cells were killed; at MOI of 10, more than half cells were killed and partial at MOI of 1. On the contrast, only a slight CPE in normal cells L02 was observed at MOI of 100. Ad-mIL-12 showed rather weaker cell killing activity and no CPE was observed even at MOI as high as 500.

2.5 MTT assay When SGC-7901 cells were infected with virus at an MOI of 10, both ONYX-015 and CNHK200-mIL-12 showed greatly stronger antitumor ability *in vitro* than Ad-mIL-12 ($P<0.01$). However, there is no difference between ONYX-015 and CNHK200-mIL-12(Fig 4).

3 DISCUSSION

Tumor gene therapy aims to deliver therapeutic genetic material in a safe and efficient manner to tumor tissues where it can accumulate. Poor transduction efficiency and low therapeutic gene expression are 2 major hurdles for successful gene therapy up to now. Conventional viral vectors are replication defective in which the expression of trans gene is far away from that needed. This shortfall

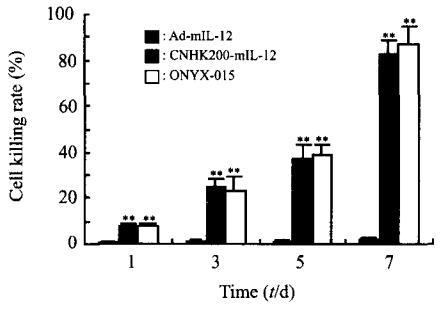

Fig 4　Cell killing rate comparison in SGC-7901 cells

** $P<0.01$ *vs* Ad-mIL-12 group

may be due in large part to the one-dimensional nature of these approaches, asking for a successful therapeutic outcome against a highly complex biological target like a human tumor through the activity of a single gene. Therapeutic gene delivery needs a multidimensional system if it is to be successful in treating human cancers. A natural evolution of gene therapy is its incorporation into replication-selective oncolytic viruses, combing the antitumor properties of the viral infection with the action of the therapeutic proteins.

Replicating viruses are designed to selectively replicate in tumor cells but greatly attenuated in normal cells. For example, ONYX-015 is a replication-selective Ad lacking a viral gene, E1B 55 000, whose product is required to inactivate the cellular tumor suppressor p53. This virus replicates preferentially in tumor cells that lack function of p53[4]. P53 function is abrogated in the majority of human gastric cancers. We constructed recombinant adenovirus CNHK200, an analog of ONYX-015, which can infect, replicate and lyse gastric cancer cells SGC-7901. The virus yields in SGC-7901 infected with CNHK200 or ONYX-015 increase significantly and reach hundreds of folds 96 h after infection. However, treatment with replicative adenovirus as a single agent was not sufficient and it should combine with other approaches such as chemotherapy or radiotherapy. We constructed CNHK200-mIL-12, expecting that it would take advantage of both virotherapy and genetherapy. When containing therapeutic gene mouse IL-12, CNHK200-mIL-12, CNHK100-mIL-12 replicates as well as ONYX-015, which means that inserted transgene didnot impair the replication capacity. Compared with conventional viral vectors, replicative oncolysis viruses produce more therapeutic gene protein[5]. By ELISA we observed that mouse IL-12 gene expression in SGC-7901 cells infected with CNHK200-mIL-12 is much higher than that infected with Ad-mIL-12, a non-replicative adenovirus, just as we expected. At the same time, we also observed transient gene expression not only in non-replicative but also in replicative adenoviruses due to gene-viral delivery system. The tranductivity and expression of transgene will decrease along with the division of viral infected tumor cells. The production of mouse IL-12 protein accumulated quickly at early stage after infection but slowly at late stage, which consists with the replication condition of oncolytic virus. Although transient expression is a big obstacle for gene therapy[6,7], it may provide potential benefits when taking IL-12 gene as therapeutic agent for it may cause severe inflammation or fatal liver impairment. Transient expression reduces side-effects of IL-12. From CPE and MTT assay, we observed selective cell killing effect of CNHK200-mIL-12 and ONYX-015 in SGC-7901 cells. Compared with non-replicative adenovirus, CNHK200-mIL-12 demonstrated stronger antitumor activity.

In summary, replicative-adenovirus mediated gene therapy offers more potential advantages than non-replicative adenovirus and it may be a promising approach for gastric cancer treatment.

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增殖型腺病毒载体介导 mIL-12 基因对胃癌细胞的杀伤作用

薛绪潮[1],方国恩[1],张 琪[2],薛惠斌[2],毕建成[1],曹贵松[1],钱其军[2*]

(1. 第二军医大学长海医院普通外科,上海 200433; 2. 东方肝胆外科医院病毒基因治疗实验室,上海 200438)

[**摘要**] **目的**:观察增殖型腺病毒载体介导的 mIL-12 基因对胃癌细胞的杀伤作用。**方法**:利用携带 mIL-12 基因的增殖型腺病毒转染胃癌细胞株 SGC-7901,通过病毒增殖实验、细胞病理效应、酶链免疫反应等分别观察病毒复制能力、病毒对胃癌细胞的杀伤作用及 mIL-12 表达水平。**结果**:携带 mIL-12 基因的增殖型腺病毒转染胃癌细胞株 SGC-7901 具有肿瘤增殖型腺病毒 ONYX-015 的相似作用,可在肿瘤细胞内复制、增殖并杀死肿瘤细胞,而并不能在正常细胞内复制及增殖。该病毒在肿瘤细胞内的增殖能力是传统载体的近千倍。该载体携带的 mIL-12 基因的表达量明显高于传统基因治疗的腺病毒载体体系,是传统基因治疗表达量的百倍。**结论**:CNHK200-mIL-12 能在胃癌细胞中增殖并杀死胃癌细胞,并提高目的基因的表达水平,可能为胃癌治疗提供一新途径。

[**关键词**] 增殖型腺病毒载体; 白细胞介素 12; 胃肿瘤; 基因疗法

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2.1 *疫苗运输、保管与抗体效价* 第 1 组 512 名学员使用批号 2990103-1 疫苗完成免疫程序 12 个月后抗体阳转率仅达 67.2%;第 2 组 276 名学员使用 2000310-1 疫苗接种 1 年后抗体阳转率为 84.4%,比文献[1]的 90.54%低(与本研究使用的疫苗同公司,批号 2951211-2);又比文献[2]的 67.2%高(同公司疫苗,批号 2990103-1)。在排除真、假性免疫应答低下及疫苗质量、接种方法存在问题的基础上,考虑存在疫苗失活或活性下降的问题。回顾我们为第 1 组学生接种的疫苗,因仓库通知取货延误了数天时间,可能是造成疫苗活性下降的原因,接种后抗体阳性率仅达 67.2%;第 2 组学生接种的疫苗排除了以上的时间延误问题,接种后抗体阳性率达 84.4%,阳性率增加了 17.2%。但我们的疫苗接种阳性率仍低于文献[1]的 90.54%,考虑我校每年的疫苗均系 9 月份前后运到,天气炎热,又经过长途运输,且无冷藏设备,可能是造成部分疫苗失活或活性下降的一个原因。在今后的疫苗运输、接收和保管等多项环节上应严格把关,以确保接种成功。

2.2 *进行全程免疫接种的必要性* 鉴于有些学生不能完成全程免疫接种,我们对全程和非全程接种学生进行了系统的观察,以期引起相关人员的重视,提高进行全程免疫接种的自觉性。我们的观察结果表明全程免疫接种比非全程接种抗体阳性率增加了 14.5%,但因第 2 针注射后 5 个月进行的抗体检测,存在观察时间不够的缺陷,故说服力不够强。

2.3 *加强健康教育知识的普及* 乙肝基因工程疫苗已被证实是一种安全、有效的疫苗,可用于医学生入学和实习前的免疫接种,也可用作医务人员的预防性接种,以达到最大限度控制和减少乙肝病毒感染的机会。从我校学员接种乙肝疫苗 12 个月后抗体的产生情况来看,20%～33.3%的接种者没有免疫应答,这些学生属于乙肝病毒高度易感的人群。我国是乙肝感染很严重的国家,约 10%的人为乙肝病毒携带者,做好传染病的预防工作十分重要。对未产生抗体的学生采取必要的防范措施是学员队防疫工作者的重要任务。集体生活单位是传染病的高发区域,住校学员入校应严格体检把关。我们的观察结果提示:接种过乙肝疫苗并不是万事大吉了,应该把 HBV 血清标志物阳性的学生与其他学生分开居住,以减少交叉感染的机会;利用广播、黑板报宣传乙肝的危害及其传播途径,并为抗体阴性的学员进行乙肝疫苗重复接种。

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