Treatment of Cancer with a Novel Dual-Targeted Conditionally Replicative Adenovirus Armed with *mda-7/IL-24* Gene

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Abstract **Purpose:** Recent studies showed that oncolytic adenoviruses not only have capacity for destruction of tumors but also can be used as potential vectors to express therapeutic genes for therapy of cancer. However, better specificity and mode of transgene expression are required to improve the efficacy and safety if this vector is applied for clinical application.

Experimental Design: In this study, we have created adenoviral replication-based transgene expression system by replacement of 6.7K/gp19K of E3 genes with *EGFP* and *IL-24* genes so that expression of transgenes should be controlled by adenoviral E3 promoter. Transgene expression, viral replication capacity, and cytotoxicity have been studied in tumor and normal cells. Antitumor efficacy was evaluated in animal model with established tumor.

Results: Our data showed that expression of IL-24 could be detected at 6 h and reached the maximal level at 48 h after infection in tumor cells. The expression level was 14 times higher than that induced by cytomegalovirus promoter. Low level of IL-24 could be detected in normal cells only until 72 h after infection. The substitution of 6.7K/gp19K of E3 genes with transgenes did not affect viral replication in tumor cells. Strong cytotoxicity was observed only in tumor cells after infection with AdCN205-IL-24. Treatment of the established tumors induced high level of local expression of IL-24 in tumor cells and resulted in tumor regression.

Conclusions: Our data showed that AdCN205-IL-24 can provide potent and safe vector for the therapy of cancer.

Cancer gene therapy by delivery of genetic material into tumor cells with therapeutic benefit provides great promise against cancer. Replication-defective viral vectors have been used for different applications for gene therapy of cancers. However, because of limitations involving vector delivery and relatively low levels of gene transfer, such vector systems have proven relatively inefficient for the treatment of large solid tumors (1–3). Conditionally replicative adenoviruses exert intrinsic antitumor activity through selective replication in lysis cancer cells. In addition, the release of conditionally replicative adenovirus progeny by infected tumor cells provides a potential to amplify the oncolytic effect by lateral spread through solid tumors (4–6). The clinical data showed that oncolytic adenovirus exhibited some degree of antitumor activity alone or combined with chemotherapy or radiotherapy (7–10). The oncolytic viruses not only have capacity for destruction of tumors but also can be used as potential vectors to express therapeutic genes in tumor cells (11). Genes coding for tumor necrosis factor–related apoptosis-inducing ligand, second mitochondria-derived activator of caspases (Smac), Fas ligand, granulocyte macrophage colony-stimulating factor, and mda-7/IL-24 have been incorporated into oncolytic adenovirus. The tumor regression was observed in animal models with established tumor after treatment with these vectors (12–19). However, in most of these constructs, therapeutic genes were controlled by exogenous constitutive promoters. Therefore, expression of therapeutic genes in normal tissue may induce undesired effect, and even oncolytic adenovirus does not replicate in it. On the other hand, inappropriate expression of therapeutic genes with cytotoxicity during viral replication cycle may impair viral replication efficacy (20, 21). The viral replication may turn off cellular or therapeutic gene expression because adenoviral replication has been attributed to a block in cytoplasmic accumulation of cellular mRNAs and preferential translation of viral mRNA (22, 23). Therefore, transgenes controlled by exogenous promoter may lead to unpredictable expression of the foreign genes over the course of the viral infection.

In contrast to the use of exogenous promoter for controlling therapeutic genes in oncolytic adenoviruses, recent studies have explored whether adenoviral endogenous promoters could be used for controlling therapeutic gene expression (24–28). Because both E3 genes or fiber genes are only expressed during replication cycle after infection of adenovirus (29, 30), it would

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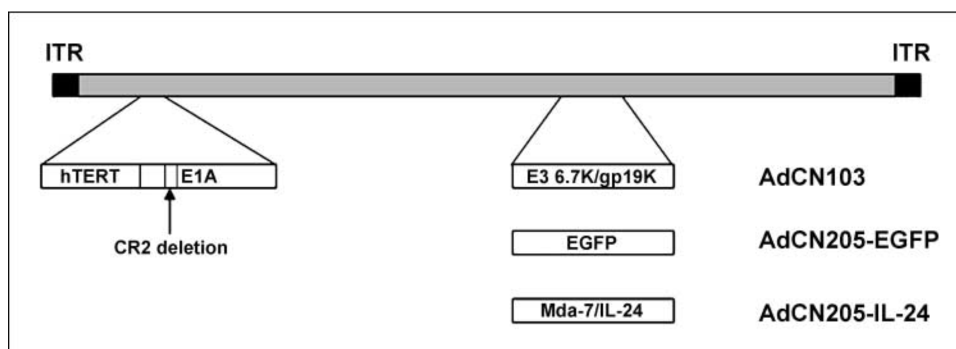

Fig. 1. Schematic structure of AdCN103, AdCN205-EGFP, and AdCN205-IL-24. In AdCN103, the E1A promoter was replaced by hTERT promoter and deletion of the adenoviral genome 923 to 946 nucleotides, which enables viral replication within malignant cells with abnormal retinoblastoma functions. In AdCN205-EGFP and AdCN205-IL-24, E3 6.7K/gp19K genes were substituted by *EGFP* reporter gene and *IL-24* therapeutic gene, respectively.

be expected that the transgene can be only expressed in tumor cells if oncolytic adenovirus is applied. To fully evaluate therapeutic potential of oncolytic adenoviral vector carrying this novel expression system, we have constructed novel double-controlled oncolytic adenovirus, in which 6.7K/gp19K of E3 genes was replaced with potent antitumor gene *mda-7/IL-24*. Our previous and other studies have shown that mda-7/IL-24 exerted strong antitumor activity by induction of tumor cell apoptosis, enhancement of immune responses, and inhibition of tumor angiogenesis (31–34).

Data presented here showed that expression of IL-24 could be detected at 6 h and became maximal expression at 48 h after

Fig. 2. Expression of transgene in cells after infection with oncolytic adenoviruses. Tumor cells (SW620 and BEL7404) and normal cells (QSG7701 and MRC5) were infected with AdCN205-IL-24, Ad-IL-24, or AdCN205-EGFP at a multiplicity of infection of 10. At different time points, the supernatant was collected for determination of IL-24 levels by ELISA. *A,* IL-24 level in cells infected with AdCN205-IL-24. *B,* IL-24 level in cells infected with Ad-IL-24. Columns, mean of three independent experiments; bars, SD. *C,* representative photomicrographs were obtained from BEL7404 and MRC5 infected with AdCN205-EGFP. Original magnification, ×200.

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infection in tumor cells. Treatment of the established tumors resulted in tumor regression. Our data showed that this novel AdCN205-IL-24 can provide potent and safe vector for the therapy of cancer.

Materials and Methods

Cell culture. Human hepatocellular carcinoma cell line (Hep3B), human colorectal carcinoma cell line (SW620), and human normal lung fibroblast cell lines (NHLF and MRC5) were obtained from the American Type Culture Collection. Human hepatocellular carcinoma cell lines (BEL7404 and SMMC7721) and human normal liver cell lines (QSG7701 and L-02) were purchased from the Shanghai Cell Collection. HEK293 was obtained from Microbix Biosystems, Inc. Cells were maintained in humidified 37°C atmosphere containing 5% CO_2 and cultured in DMEM (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), 4 mmol/L glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin.

Virus construction and production. The constructs including pCN205-EGFP and pCN205-IL-24 were generated according to the standard molecular cloning protocol. The homologous recombination between pCN205-EGFP and pCN205-IL-24 plasmids and pCN103 plasmid carrying oncolytic adenoviral backbone was done in BJ5183 to create pAdCN205-EGFP and pAdCN205-IL-24, respectively. Viral particles were produced in HEK293 cells by transfection with *Pac*I-digested pAdCN205-EGFP and pAdCN205-IL-24 to obtain recombinant AdCN205-EGFP and AdCN205-IL-24. All viruses were propagated and purified on a CsCl gradient using standard methods. The viruses were titered for viral particles using standard methods based on spectrophotometry at 260 nm. Functional titer (plaque-forming units) was determined with plaque assay on HEK293 cells according to the validated method developed by Quantum Biotechnology.

Viral progeny assay. To determine viral replication capacity, tumor or normal cells were infected with AdCN205-EGFP, AdCN205-IL-24, Ad-IL-24, AdCN103, or Ad-Wt at a multiplicity of infection of 5. Adenoviruses were removed after 6 h of infection. The cells were washed twice with PBS and incubated at 37°C for 48 h. The cellular pellets were lysed with three cycles of freeze thawing and the supernatant was collected. Viral titers were determined by plaque assay on HEK293 cells.

Cytotoxicity assay. Cells were plated in 96-well plates and treated with various adenoviruses. At the indicated times, medium was removed and fresh medium containing 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to each well. Cells were incubated at 37°C for 4 h, the medium was removed, and 100 μL DMSO was added and mixed thoroughly for 30 min. Absorbance was read on Tecan DNA Expert at 590 nm.

Detection of IL-24 by ELISA. The two-antibody sandwich ELISA was developed for detection of human mda-7/IL-24. The antibodies used are monoclonal mouse anti-human IL-24 antibody (R&D Systems), goat anti-human IL-24 antibody (R&D Systems), and peroxidase-conjugated rabbit anti-goat IgG (H&L). The absorbance was read at a 450-nm wavelength. The concentration of IL-24 was evaluated with standard curve.

Tumor xenograft in nude mice. Female BALB/c nude mice at 3 to 4 wk of age were obtained from Charles River Laboratories, Inc. and maintained at pathogen-free conditions. All procedures were done according to institutional guidelines and conformed to the NIH guidelines on the ethical use of animals. BEL7404 cells (2×10^6) were injected s.c. into flanks of mice. When the tumors had grown to 100 to 150 mm^3, the animals were randomized into five groups. Each group was treated with either PBS or 2×10^8 plaque-forming units of vectors by intratumor injection every other day for 5 d. Tumors were measured twice weekly and tumor volume was calculated according to the following equation: V (mm^3) = width^2 × length / 2. Animal death was documented. Animals were sacrificed when the diameter of the tumor reached 17 mm or when mean diameter is >15 mm.

Immunohistochemistry. Deparaffinized tumor sections were treated with goat monoclonal anti-IL-24 antibody (diluted 1:100; Santa Cruz Biotechnology). After incubation with anti-rabbit secondary antibody, IL-24 expression was detected with 3,3′-diaminobenzidine (Sigma) by enhancement with an avidin-biotin reaction ABC kit (Vector Laboratories). The slides were then counterstained with hematoxylin.

Statistical analysis. Statistical analyses were done with Student's t test to determine the significance. Pearson's χ^2 test was also used to test the hypothesis of no association of columns and rows in tabular data. $P < 0.05$ was considered significant.

Results

Constructions of oncolytic adenoviral vectors. Previously, we developed double-controlled oncolytic adenovirus AdCN103, in which hTERT promoter was used to control expression of mutant E1A without CR2 region (35). This vector allows a selective adenoviral replication in tumor cells harboring over-expression of hTERT and dysfunction of retinoblastoma. In the present study, we constructed a system, which allowed to substitute 6.7K/gp19K of E3 genes for the *EGFP* reporter gene (AdCN205-EGFP) and *mda-7/IL-24* gene (AdCN205-IL-24), respectively, so that expression of transgenes was driven by the endogenous E3 promoter. The structures of AdCN205-EGFP and AdCN205-IL-24 were shown in Fig. 1.

Expression of transgene in cells after infection with oncolytic adenoviral vectors. To show kinetics of IL-24 expression in the cells after infection with AdCN205-IL-24, we have infected tumor (SW620 and BEL7404) and normal (QSG7701 and MRC5) cells with AdCN205-IL-24. Our data showed that IL-24 could be detected at 6 h in tumor cells after infection. There was a steady increase of IL-24 expression up to 48 h,

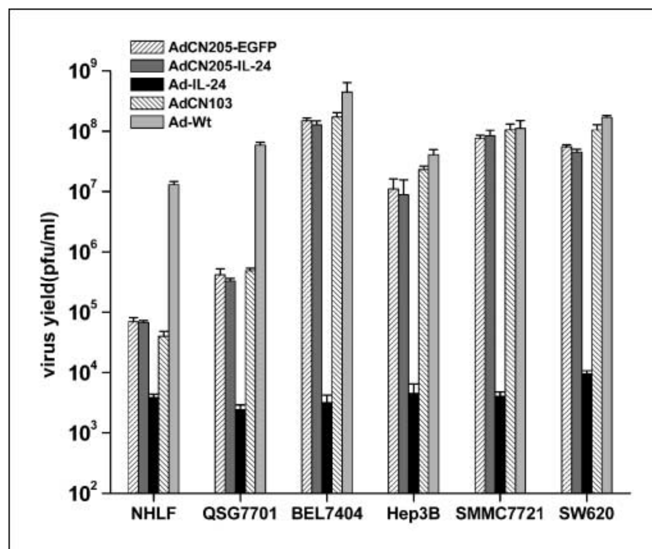

Fig. 3. Selective replication of oncolytic adenoviral vector *in vitro*. Tumor cells (BEL7404, Hep3B, SMMC7721, and SW620) and normal cells (NHLF and QSG7701) were infected with AdCN205-EGFP, AdCN205-IL-24, Ad-IL-24, AdCN103, and Ad-Wt at a multiplicity of infection of 10. At 48 h after viral infection, cells and medium were harvested, and viral particles were released by three cycles of freezing and thawing. The viral titers were measured by plaque assay on HEK293 cells. Columns, mean of three independent experiments; bars, SD. pfu, plaque-forming unit.

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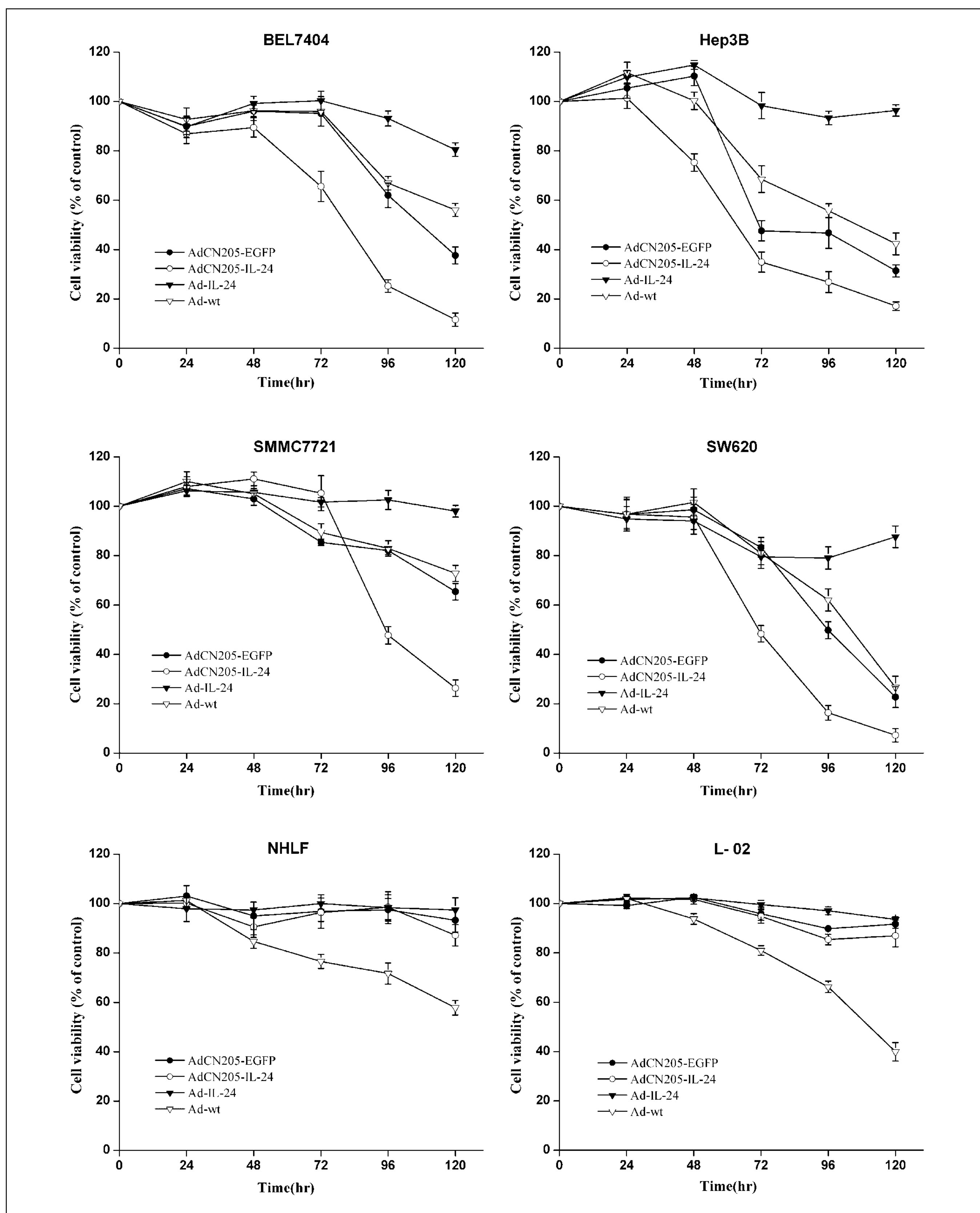

Fig. 4. Cytotoxicity of oncolytic adenoviral vectors in tumor cells *in vitro*. Tumor cells (BEL7404, Hep3B, SMMC7721, and SW620) and normal cells (NHLF and L-02) were infected with AdCN205-EGFP, AdCN205-IL-24, Ad-IL-24, and Ad-Wt at a multiplicity of infection of 10. At different time points after infection, the cell survival was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Results were expressed as percentage of untreated control. Points, mean of three independent experiments; bars, SD.

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and it was maintained at high level until 96 h after infection (Fig. 2A). In contrast, no IL-24 expression could be detected in normal cells until 72 h after infection because at this time point low level of IL-24 could be detected (Fig. 2A). However, similar kinetics and level of IL-24 expression were found in both tumor and normal cells infected with Ad-IL-24, in which IL-24 was controlled by cytomegalovirus promoter (Fig. 2B). The expression level of IL-24 was ~14 times higher in tumor cells infected with AdCN205-IL-24 than in tumor cells infected with Ad-IL-24. The high level of EGFP expression was only observed in tumor cells accompanied with cytopathic effect and no or low EGFP was observed in normal cells after infection with AdCN205-EGFP (Fig. 2C). The difference in kinetics and level of transgene expression may be due to viral replication induced by oncolytic adenoviral vectors in tumor cells.

***Selective replication of oncolytic adenoviral vectors* in vitro.** The viral progeny assay was used to test whether the deletion of the E3 6.7K/gp19K and insertion of the *mda-7/IL-24* transgene altered the capacity of viral replication. As presented in Fig. 3, virus production by the AdCN205-EGFP and AdCN205-IL-24 was similar to that induced by AdCN103 and Ad-Wt in cancer cell lines. The virus production by Ad-IL-24 was significantly less than that induced by oncolytic adenoviruses in cancer cell lines. In contrast, virus production by the AdCN205-EGFP, AdCN205-IL-24, and AdCN103 in NHLF and QSG7701 normal cells was higher than that induced by Ad-IL-24 but much less than that of Ad-Wt. Thus, replacement of adenoviral E3 6.7K/gp19K gene with therapeutic gene *IL-24* or reporter gene *EGFP* does not interfere with adenoviral replication and production in tumor cell lines.

***Cytotoxicity of oncolytic adenoviral vectors in tumor cells* in vitro.** To show the ability of the oncolytic adenovirus with therapeutic gene *mda-7/IL-24* to destroy tumor cells, we did cytotoxicity assay in the cells after infection with oncolytic adenoviruses. As shown in Fig. 4, replication-defective adenovirus Ad-IL-24 only induced minor cytotoxicity to the tumor cells, whereas AdCN205-EGFP or Ad-Wt induced obvious cytotoxicity. The cytotoxic effect of AdCN205-IL-24 on tumor cells was much more apparent than that induced by AdCN205-EGFP or Ad-Wt. In contrast, AdCN205-EGFP, AdCN205-IL-24, and Ad-IL-24 did not induce any cytotoxicity to normal cells. This result suggested that AdCN205-EGFP and AdCN205-IL-24 can selectively replicate in cancer cell lines and AdCN205-IL-24 displayed a powerful efficacy in killing cancer cells.

Antitumor activity of oncolytic adenoviral vectors in animal model with the established tumor. To know antitumor potency of oncolytic adenoviral vectors, we have treated the established tumors in an animal model by intratumoral injection of Ad-IL-24, AdCN205-EGFP, AdCN205-IL-24, and Ad-Wt. Our data showed that animals receiving PBS experienced progressive tumor growth. Treatment with Ad-IL-24 resulted in minor inhibition of tumor growth (Fig. 5A). Both AdCN205-EGFP and Ad-Wt exhibited strong antitumor activity at similar level. In contrast, treatment with AdCN205-IL-24 resulted in significant inhibition of tumor growth compared with that induced by AdCN205-EGFP and Ad-Wt ($P < 0.01$; Fig. 5A). Two of eight animals treated with AdCN205-IL-24 had complete tumor regression. Treatment with AdCN205-IL-24 resulted in long-term survival (Fig. 5B).

Immunocytochemical analysis has shown that expression of IL-24 was observed only in tumor sections from animals treated with Ad-IL-24 and AdCN205-IL-24. The density of IL-24 expression was much higher in tumor sections from animals treated with AdCN205-IL-24 compared with that treated with Ad-IL-24 (Fig. 6). Pathologic analysis revealed that the necrotic area could be found in tumors treated with Ad-IL-24, whereas intensive necrotic area was observed in tumors treated with AdCN205-EGFP and Ad-Wt (Fig. 6). Treatment with AdCN205-IL-24 resulted in more intensive necrosis throughout tumor sections. The detection of adenoviral hexon expression has been evaluated in the liver sections from animals treated with Ad-IL-24, AdCN205-EGFP, AdCN205-IL-24, and Ad-Wt. Our data showed that expression of adenoviral hexon was only found in the liver sections from animals treated with Ad-Wt. No signal could be found in the liver sections treated with Ad-IL-24, AdCN205-EGFP, and AdCN205-IL-24 (Fig. 6). This study indicated that treatment of oncolytic adenoviral vectors did not induce viral replication in the normal tissues.

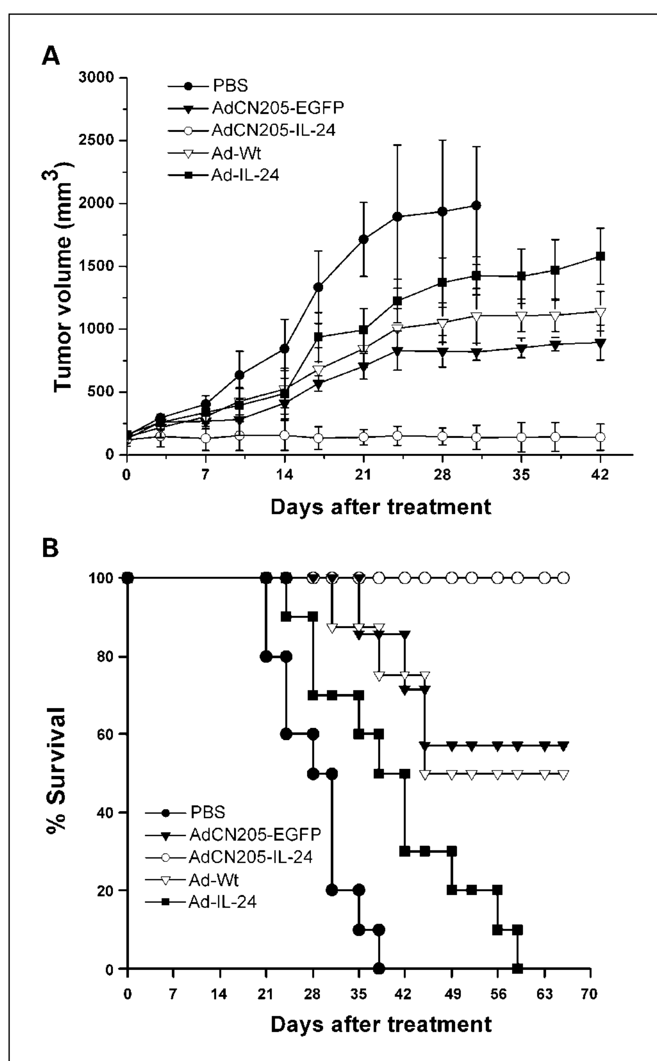

Fig. 5. Antitumor activity of oncolytic adenoviral vectors in the established tumor in animal model. Tumors were established by injecting BEL7404 cells s.c. into the right flank of nude mice. The tumor-bearing animals were treated with either PBS or 2×10^8 plaque-forming units of different vectors by intratumor injection. *A,* the size of tumor was measured twice weekly and tumor volume was calculated. Points, mean of tumor volume (n = 8-10); bars, SD. *B,* Kaplan-Meier survival.

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Discussion

Because cancer cells are genetically and phenotypically complex and frequently harbor multiple abnormalities (36), it is reasonable that single agent such as oncolytic adenovirus-alone is not sufficient for complete eradication of malignancies. Arming oncolytic adenovirus with therapeutic genes has become an important issue in the war against cancers (11). Among different therapeutic genes, *IL-24* exerted strong antitumor activity. Clinical trial with replication-defective adenovirus expressing *IL-24* has shown a safe profile and some partial responses in patients with cancers (37, 38). Therefore, we chose

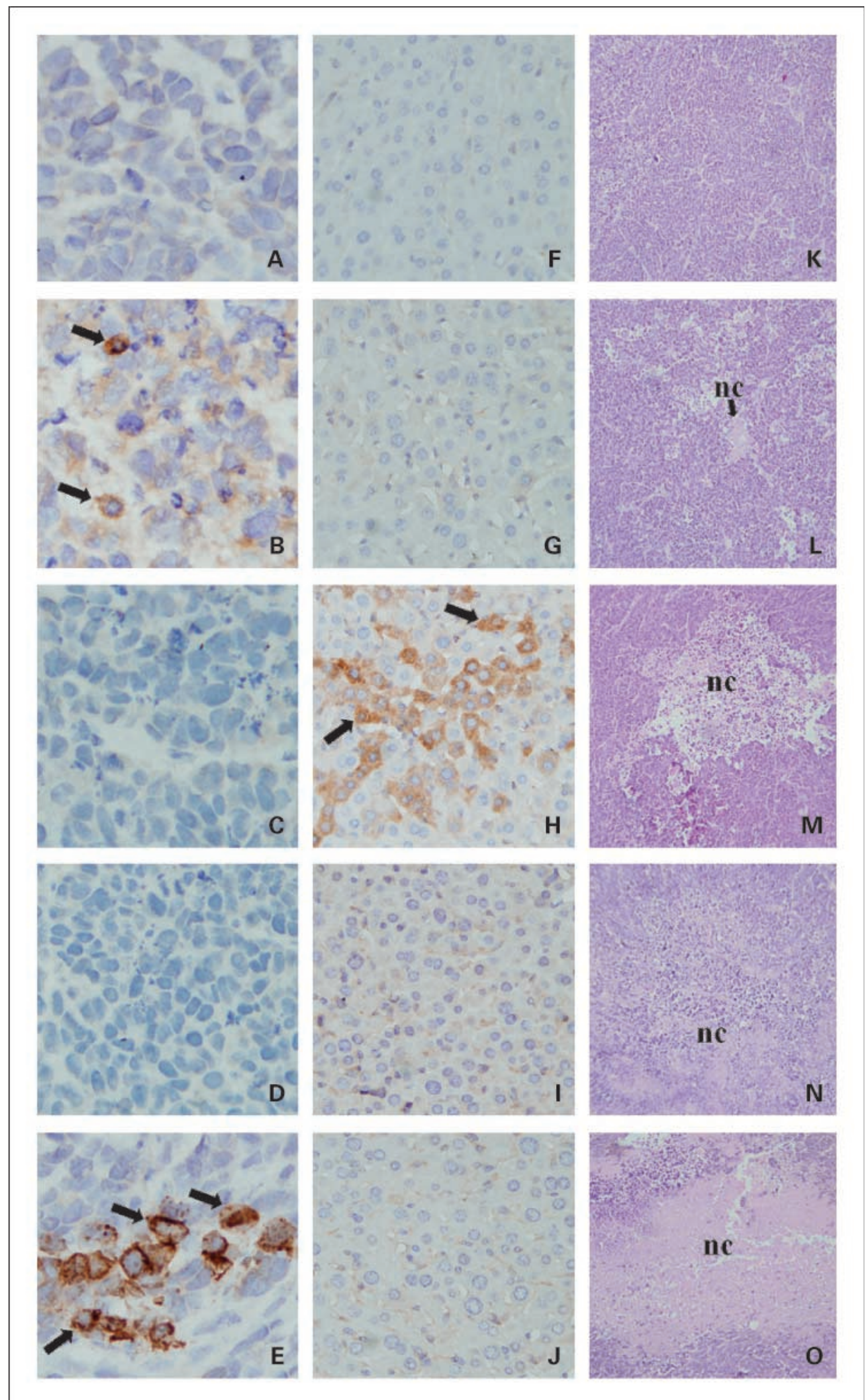

Fig. 6. Histologic analysis of tumor or liver sections from mice whose tumors were treated with PBS (*A, F*, and *K*), Ad-IL-24 (*B, G*, and *L*), Ad-Wt (*C, H*, and *M*), AdCN205-EGFP (*D, I*, and *N*), and AdCN205-IL-24 (*E, J*, and *O*). *A* to *E*, immunohistochemical staining for mda-7/IL-24 protein in tumor sections (*brown staining*). Original magnification, ×400. *B* and *E*, arrows, cells expressing IL-24. *F* to *J*, immunohistochemical staining for adenoviral hexon protein on the liver sections (*brown staining*). Original magnification, ×400. The liver from mice treated with Ad-Wt (*H*) stained positive. *K* to *O*, H&E staining of tumor sections, indicating large areas of necrosis (*nc*) in tumors treated with Ad-Wt, AdCN205-EGFP, and AdCN205-IL-24. Original magnification, ×100.

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IL-24 as therapeutic gene in this newly constructed oncolytic adenovirus.

In the present work, we describe a dual gene therapy approach with a cancer cell–specific adenoviral replication and oncolysis and expression of apoptosis-inducing cytokine gene *IL-24*. Because transgene expression from the endogenous E3 native promoter was dependent on the viral replication, the tumor specificity of oncolytic adenovirus should be very important. So, we used double-controlled oncolytic adenovirus AdCN103, which targets TERT and retinoblastoma dysfunctions, for this purpose because our previous study has shown that it exerted restrictive viral replication in hepatocellular carcinoma tumor cells (35).

The most important issues of our new constructs would be that transgene could be expressed in a predicted manner and substitution of deleted E3 6.7K/gp19K with transgene could interfere with adenoviral replication. To answer these questions, we have determined transgene expression and viral progeny assay. Our data showed that IL-24 expression could be detected at 6 h, reached the maximal level at 48 h, and maintained the high level until 96 h after infection of tumor cells. The most important is that expression level of IL-24 is 14 times higher than that induced by cytomegalovirus promoter. However, only low level of IL-24 could be found in normal cells after 72 h of infection. In addition, there was no difference on virus production in tumor cells after infection with AdCN205-EGFP or AdCN205-IL-24. These data indicated that our newly constructed oncolytic adenoviral vectors not only maintained their intrinsic capacity of viral replication in tumor cells but also induced high and sustained expression of therapeutic gene.

Our results indicated that AdCN205-IL-24 exerted much strong cytotoxicity to tumor cells compared with that induced by control vector AdCN205-EGFP or Ad-Wt. AdCN205-EGFP and Ad-Wt induced similar cytotoxicity to tumor cells. In contrast, both AdCN205-EGFP and AdCN205-IL-24 did not induce cytopathic effect to normal cells. In addition, our data further showed that a strong antitumor activity was observed in the animal model with the established tumors. These data indicated that incorporation of reporter gene into oncolytic adenovirus did not affect cytotoxicity and arming oncolytic adenovirus with therapeutic gene *IL-24* induced additional or synergistic antitumor activity. Recent studies have also shown that arming oncolytic adenovirus with suicide gene or cytokine gene can also significantly increase therapeutic index (12, 16, 17, 19).

In conclusion, we have successfully created adenoviral replication-based transgene expression system. Our data showed that expression of transgenes was restricted to tumor cells. The high level of expression of IL-24 and adenoviral replication greatly improved the oncolytic effects of this vector under both *in vitro* and *in vivo* conditions. These data indicated that this novel AdCN205-IL-24 can provide potent and safe vector for the therapy of cancers.

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Jingjing Luo, Qingmei Xia, Rui Zhang, et al.

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