

# Therapeutic effects of adenovirus-mediated CD and NIS expression combined with Na<sup>131</sup>I/5-FC on human thyroid cancer

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**Abstract.** Thyroid cancer is the most common type of malignant endocrine tumor diagnosed. Previous studies have indicated that gene therapy is the most promising and effective therapeutic method for thyroid cancer. Therefore, in the present study, Na<sup>131</sup>I/5-fluorocytosine (5-FC) treatment was combined with cytosine deaminase (CD, encoded by the *CDA* gene) and sodium iodide symporter (NIS, encoded by the *SLC5A5* gene) to act together as a therapeutic tool for thyroid cancer. The present study explored the combined cytotoxic effects of adenovirus-mediated CD and NIS under the control of the progression elevated gene-3 (*PEG-3*) promoter (Ad-*PEG-3-CD-NIS*) with Na<sup>131</sup>I/5-FC against the human thyroid cancer TT cell line *in vitro*. The *PEG-3* fragment was obtained by polymerase chain reaction (PCR) using rat genomic DNA as the template, and then Ad-*PEG-3-CDA-SLC5A5* was constructed using *Xba*I. TT cells were transfected by recombinant adenovirus. The method of reverse transcription-quantitative PCR was performed to test the expression of CD and NIS at the level of transcription. The morphological change was assessed by fluorescence microscopy and investigated by western blot analysis. An MTT assay was used to determine the number of living cells inhibited by single or combination therapies on TT cells. The results indicated that the *PEG-3* was successfully cloned, and was also positively regulated in 293 cells. *CDA* and *SLC5A5* genes were highly expressed in TT cells. Na<sup>131</sup>I combined with 5-FC significantly decreased the human thyroid cancer cells. In conclusion, combination therapy of Ad-*PEG3-CDA-SLC5A5* and Na<sup>131</sup>I/5-FC induces significantly more apoptotic characteristics than either single treatment with Ad-*PEG-3-CDA-SLC5A5* or Na<sup>131</sup>I/5-FC, and low doses of Ad-*PEG-3-CDA-SLC5A5* enhanced the cytotoxic effects.

## Introduction

Thyroid cancer was the most common malignant endocrine tumor diagnosed in 2006 in the USA (1,2). Thyroid cancer is also the seventh most common type of cancer in Canadians, and there were ~5,650 cases of thyroid cancer diagnosed in 2012 (1,2). Concurrently, equal trends in the increase in incidence rate have been identified all over the world (3-14). The age-standardized incidence rate of thyroid cancer has increased from 1.1/100,000 to 6.1/100,000 for males, and from 3.3/100,000 to 22.2/100,000 for females, from 1970 to 1972 in the USA (1,15). A previous study indicated that the thyroid cancer incidence rate in Canada was the fastest increasing rate in the world, trends in the incidence rate of thyroid cancer have demonstrated a 6.8% increase for males and 6.9% increase for females per annum between 1998 and 2007 (16-18). Most recently, the number of new cases of thyroid cancer is estimated to be 12.9 per 100,000 men and women annually in 2015 in the US (19,20).

At present, previous studies (17,19,21) have suggested that gene therapy is the most promising and effective therapeutic method for thyroid cancer. The principle of gene therapy depends on the intracellular conversion of a relatively non-toxic pro-drug (or drug gene) to a toxic drug (therapeutic protein) through gene transcription and translation processes. The gene therapy method exhibits more advantages than conventional chemotherapy, as it limits the pro-drug-induced toxicity to the targeted cells (17,19,21-23). The surrounding cells and tissues are not affected by systemic toxicity. In previous years, the cytosine deaminase (*CD*) and sodium iodide symporter (*NIS*) genes have been employed as therapeutic genes in certain studies. Bentires-Alj *et al* (24) investigated the feasibility of *CDA* suicide gene therapy in a model of peritoneal carcinomatosis. Kogai and Brent (23) used the *NIS* gene to target cancer cells as an effective therapeutic method. Therefore, the present study used the *CD* and *NIS* genes to treat thyroid cancer cells.

With the exception of gene therapy, 5-fluorocytosine (5-FC) and Na<sup>131</sup>I have also been used in cancer therapy combined with gene therapy: Kucerova *et al* (25) utilized CD-mesenchymal stromal cells/5-FC as an effective gene therapeutic tool. Zimmer *et al* (26) also used Na<sup>131</sup>I to mediate radiochemical therapy. Therefore, in the present study, 5-FC and Na<sup>131</sup>I were combined together to act as an assistant therapy tool for thyroid cancer.

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Following the enzyme/pro-drug systems developed and applied in clinical practice, herpes simplex virus-1 thymidine kinase (HSV-tk) has been used in previous years. HSV-tk is an enzyme that may convert pro-drugs to toxic products in targeted cells (21). In the absence of the drug, constitutive expression of the *HSV-tk* gene does not exert any harmful effects on normal cell growth. A previous study has also suggested that transgenic animals transfected with the *HSV-tk* gene have not suffered toxicity effects (21). A minimal promoter region may be located in the progression elevated gene-3 (*PEG-3*), which is associated with malignant transformation and tumor progression (26). *PEG-3* may initiate the expression of other genes in tumor cells (27,28). Therefore, in the present study, the *PEG-3* gene was used as the promoter for *CDA* and *SLC5A5* gene expression in tumor cells.

The present study attempted to develop *CDA* and *SLC5A5* therapy through a replication-defective adenovirus encoding human *CDA* and *SLC5A5* (Ad-*CDA-SLC5A5*) genes to treat human thyroid cancer cells.

## Materials and methods

**Cell lines and cell culture.** The human thyroid cancer TT cell line and the adenovirus-transformed human embryonic kidney 293 cell line (used as an expression tool for recombinant proteins) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All these cells were grown in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) at 37°C in the presence of 5% CO<sub>2</sub>.

**PEG-3 gene clone and adenoviral vector construction.** *PEG-3* was amplified using rat genomic DNA (cat. no. 69238; EMD Millipore, Billerica, MA, USA) as the template with forward primer 5'-TATAGTCAGCTCTAGAAGCCATCTCACCAGCCCAG-3' and reverse primer 5'-CCGGGGATCCTCTAGAGTGTCTGGCCTAGAAAGGG-3' (SBS Genetech Co., Ltd., Beijing, China). *pSB539-4* (22) was ligated into the pAV-murine cytomegalovirus-green fluorescent protein (GFP)-3FLAG vector (VB161208-1123ehs; Cyagen Biosciences, Santa Clara, CA, USA) for the generation of the recombinant Ad-*PEG-3-CDA-SLC5A5* digested by *Xba*I. A diagrammatic sketch for the double-cistron vector under the regulation of the tumor-specific promoter *PEG-3* gene is presented in Fig. 1.

**Adenovirus infection.** On the day prior to viral infection, TT cells (3.6x10<sup>5</sup> cells/well) were plated in each well of 6-well plates. When the cells reached 70-90% confluence, the culture medium was aspirated and the cell monolayer was washed with pre-warmed sterile PBS.

The recombinant generation of Ad-*PEG-3-CDA-SLC5A5* was additionally amplified in 293 low-passage cells. Viral particles were purified using cesium chloride density gradient ultracentrifugation (54,645 x g for 20 h at 4°C). 293 cells in serum-free DMEM were transfected with Ad-GFP to identify the optimal conditions using Lipofectamine<sup>®</sup> 2000 (cat. no. 18324-111; Invitrogen; Thermo Fisher Scientific, Inc.).

The uptake of Ad-*PEG-3* vector was detected by fluorescence microscopy (magnification, x100) following transfection. Additionally, the transfected TT cells were co-cultured with PHH, Hep3B, HuH7 and CCLP1 cell lines (purchased from ATCC), with the aforementioned culture conditions, to study interactions between cell populations in respect to targeting gene expression (29).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** One-Step SYBR<sup>®</sup> PrimeScript<sup>™</sup> RT-PCR kit II was purchased from Clontech Laboratories, Inc., (Mountainview, CA, US). Total RNA was isolated from cultured cells using an RNAiso Plus kit (1 ml/5x10<sup>6</sup> cells; Takara Bio, Inc.). The concentration and purity of RNA were detected by an ultraviolet spectrometer. cDNA was generated according to the One-Step SYBR<sup>®</sup> PrimeScript<sup>™</sup> RT-PCR kit II protocol. *CDA* fragments were amplified with forward primer, 5'-GGAAAACGGGAAAGTTGCATCA-3' and reverse primer, 5'-GCCTTCTCCCGCTTAGAGAC-3'. Primers for the qPCR of the mouse *SLC5A5* gene were: Forward, 5'-AGCAGGCTTAGCTGTATCCC-3' and reverse, 5'-AGCCCCGTAGTAGAGATAGGAG-3', to yield 235-bp products. Primers for the reference gene, rat β-actin, were as follows: Forward 5'-ATCTGGCACCACACCTTC-3' and reverse 5'-AGCCAGGTCCAGACGCA-3'. DNA amplification was conducted in a PerkinElmer thermocycler 2400 (PerkinElmer, Inc., Waltham, MA, USA) using an initial denaturation step at 95°C for 8 min, followed by 30 cycles of amplification with denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 30 sec, ending with a final extension at 72°C for 7 min. The 2<sup>-ΔΔC<sub>q</sub></sup> method was used to quantify the expression levels (30).

**Western blot analysis.** Transfected TT cells were lysed using radioimmunoprecipitation assay lysis buffer (Abcam, Cambridge, MA, USA). After centrifugation at 12,000 x g for 20 min at 4°C, protein concentrations were determined using a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology, Haimen, China). Total protein (5 μg/ml/lane) was denatured in protein Laemmli loading buffer (Abcam), separated by 10% SDS-PAGE, and then transferred to a polyvinylidene difluoride membranes (EMD Millipore). Tris-buffered saline-Tween 20 (TBST) solution supplemented with 10% non-fat dry milk (Abcam) was used to block the membrane for 2 h at room temperature. The blots were then incubated with primary CD antibody [AID antibody (2D3); cat. no. sc-101417; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA] and NIS (NIS-G-5) antibody (cat. no. sc-514487; 1:1,000; Santa Cruz Biotechnology, Inc.) overnight at 4°C. The blots were washed three times, for 10 min each, in TBST followed by incubation for 1 h at room temperature with goat horseradish peroxidase-conjugated anti-mouse secondary antibodies (cat. no. 31430; 1:10,000; Thermo Fisher Scientific, Inc.). Blots from three independent trials were developed using enhanced chemiluminescent reagents (Beyotime Institute of Biotechnology). β-actin (anti-β-actin; cat. no. ab8229; 1:1,000; Abcam) was used as a control. Band intensities were quantified by scanning densitometry using the Quantity One software v. 4.6 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

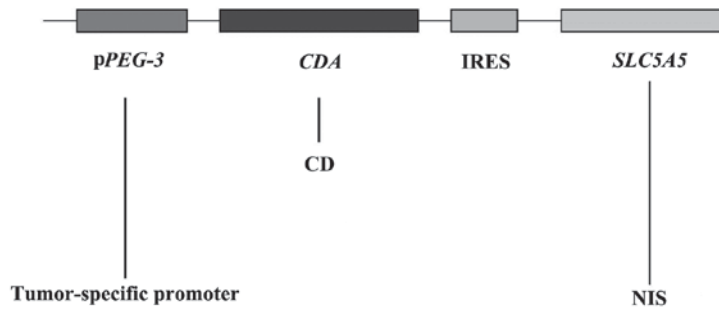


Figure 1. Diagrammatic sketch of a double-cistron plasmid under the regulation of the tumor-specific promoter *PEG-3* gene. *PEG-3*, progression elevated gene-3; p*PEG-3*, *PEG-3* promoter; CD, cytosine deaminase encoded by *CDA*; IRES, internal ribosome entry site; NIS, sodium iodide symporter encoded by *SLC5A5*.

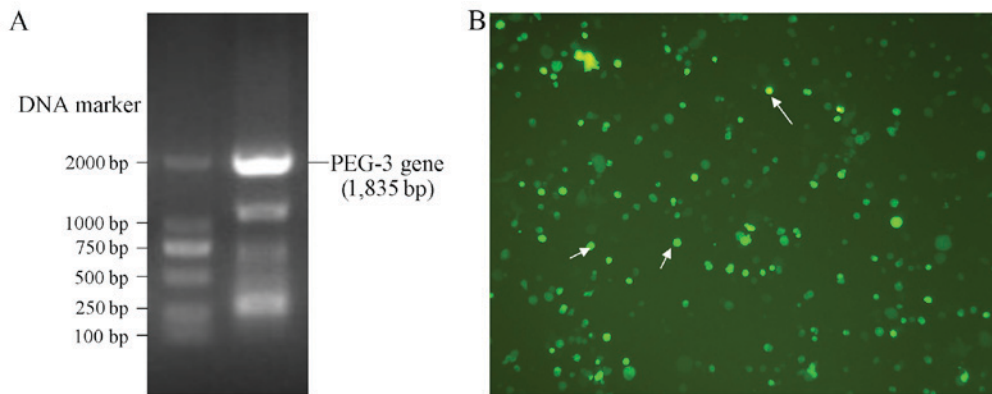


Figure 2. Transfected 293 cells expressing the *CDA-SLC5A5* gene. (A) *PEG-3-CDA-SLC5A5* gene detection in 293 cells using a polymerase chain reaction assay. (B) *PEG-3-CDA-SLC5A5* identification of multiplicity of infection in 293 cells using fluorescence microscopy (magnification, x100). Arrows indicate 293 cells that were successfully transfected and that expressed the *PEG-3-CDA-SLC5A5* gene.

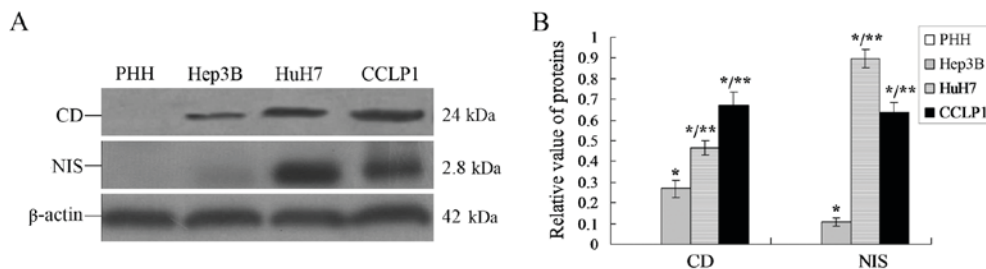


Figure 3. Western blot analysis of CD and NIS expression in TT cells co-cultured with the PHH, Hep3B, HuH7 or CCLP1 cell lines. (A) Western blot assay for CD and NIS expression. (B) Quantification of western blotting results. No CD or NIS protein was detected in the PHH cell line. \* $P < 0.05$  vs. PHH; \*\* $P < 0.05$  vs. Hep3B. CD, cytosine deaminase; NIS, sodium iodide symporter.

**MTT assay.** MTT assay was performed to evaluate the cell viability in culture. The cells were seeded onto a 96-well plate at a concentration of  $1.0 \times 10^5$  cells/ml and a volume of  $90 \mu\text{l}$ /well. Different concentrations of adenovirus ( $2 \times 10^5$ - $1 \times 10^6$  PFU/ml) were applied to culture wells in triplicate. Dimethyl sulfoxide was used as a negative control. Following incubation at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  for 48 h, a mixture of 0.1 ml phenazine methosulfate and MTT (5 mg/ml) was added to each well with a volume of  $50 \mu\text{l}$ . The plates were additionally incubated at  $37^\circ\text{C}$  for 2 h to allow MTT formazan production. The absorbance was determined with an ELISA reader (Thermo Fisher Scientific, Inc.) at a test wavelength of 450 nm and a reference wavelength of 690 nm.

**Statistical analysis.** Statistical analyses were performed using SPSS v.16.0 software (SPSS, Inc., Chicago, IL, USA). Values were reported as the mean  $\pm$  standard deviation. Kruskal-Wallis tests followed by Mann-Whitney U tests were used to determine the statistical significance of the data.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

*PEG-3* gene cloning and determination of multiplicity of infection (MOI) in 293 cells. *pSB539* is highly homologous to the *PEG-3* promoter (1,835 bp), which targets cancer cell

Table I. Examination of the percentage of living cells in transfected and untransfected TT cells treated with Na<sup>131</sup>I and 5-FC.

Treatment	Percentage of living cell in transfected TT cells				Percentage of living cell in untransfected TT cells			
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
Na <sup>131</sup> I+5-FC (KBq/ml + $\mu$ g/ml)								
3,700+5.0	7.7 $\pm$ 0.4	23.2 $\pm$ 3.5	23.2 $\pm$ 3.5	79.1 $\pm$ 6.1	2.5 $\pm$ 1.8	3.5 $\pm$ 1.5	6.4 $\pm$ 4.3	7.9 $\pm$ 4.9
370+0.5	6.2 $\pm$ 1.8	14.5 $\pm$ 2.7	35.1 $\pm$ 4.8	47.2 $\pm$ 7.1	1.7 $\pm$ 0.8	3.2 $\pm$ 1.6	5.1 $\pm$ 3.5	5.1 $\pm$ 4.1
37+0.05	3.4 $\pm$ 1.2	7.9 $\pm$ 3.1	18.7 $\pm$ 3.3	35.4 $\pm$ 6.2	1.7 $\pm$ 1.6	3.2 $\pm$ 1.9	4.1 $\pm$ 3.5	5.2 $\pm$ 2.8
3.7+0.005	1.1 $\pm$ 0.4	3.8 $\pm$ 2.8	11.8 $\pm$ 4.5	20.1 $\pm$ 3.8	1.8 $\pm$ 0.7	2.8 $\pm$ 1.2	2.9 $\pm$ 1.8	3.3 $\pm$ 1.7
Na <sup>131</sup> I (KBq/ml)								
3,700	5.2 $\pm$ 0.8	11.8 $\pm$ 2.2	30.1 $\pm$ 5.6	41.2 $\pm$ 4.7	1.7 $\pm$ 0.6	1.7 $\pm$ 0.8	5.5 $\pm$ 2.9	6.1 $\pm$ 3.5
370	2.7 $\pm$ 1.0	3.3 $\pm$ 1.1	8.8 $\pm$ 2.7	19.7 $\pm$ 3.8	0.8 $\pm$ 0.3	1.0 $\pm$ 0.5	5.0 $\pm$ 1.3	4.1 $\pm$ 2.8
37	1.6 $\pm$ 0.8	1.4 $\pm$ 0.5	4.2 $\pm$ 2.4	8.7 $\pm$ 3.1	0.7 $\pm$ 0.5	3.0 $\pm$ 2.1	4.5 $\pm$ 1.8	4.1 $\pm$ 1.4
3.7	0.6 $\pm$ 0.5	1.5 $\pm$ 0.8	2.5 $\pm$ 1.3	4.3 $\pm$ 0.2	1.1 $\pm$ 0.4	1.8 $\pm$ 0.9	2.3 $\pm$ 0.8	2.3 $\pm$ 0.8
5-FC ( $\mu$ g/ml)								
5.0	3.5 $\pm$ 0.5	8.6 $\pm$ 1.2	25.2 $\pm$ 4.0	32.3 $\pm$ 5.8	2.1 $\pm$ 0.9	2.0 $\pm$ 1.1	3.6 $\pm$ 2.0	3.7 $\pm$ 3.1
0.5	1.5 $\pm$ 0.6	2.5 $\pm$ 1.5	7.2 $\pm$ 2.3	11.2 $\pm$ 2.9	3.0 $\pm$ 2.1	2.5 $\pm$ 1.1	2.9 $\pm$ 1.2	3.9 $\pm$ 1.6
0.05	1.6 $\pm$ 0.9	2.6 $\pm$ 1.2	2.3 $\pm$ 1.8	3.9 $\pm$ 1.8	2.3 $\pm$ 0.9	2.5 $\pm$ 0.4	2.9 $\pm$ 1.8	3.8 $\pm$ 2.4
0.005	1.4 $\pm$ 0.6	2.7 $\pm$ 2.2	3.5 $\pm$ 2.0	3.6 $\pm$ 1.8	1.2 $\pm$ 0.5	3.8 $\pm$ 2.2	2.8 $\pm$ 1.4	2.9 $\pm$ 1.6

5-FC, 5-fluorocytosine.

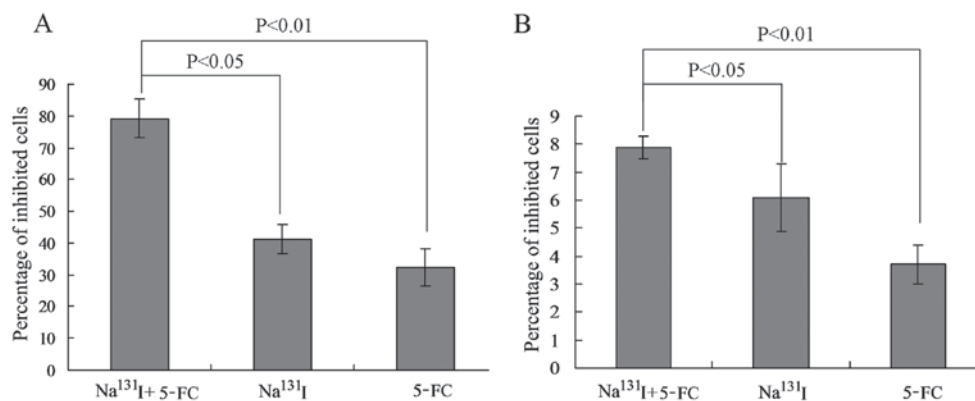


Figure 4. Examination of the living cell numbers for Na<sup>131</sup>I and 5-FC-treated TT cells. (A) Percentage of inhibited cells in transfected TT cells. (B) Percentage of inhibited cells in untransfected TT cells (control). The comparison of living cell numbers among the groups are indicated. 5-FC, 5-fluorocytosine.

lines (26,27). To verify the cloning of the *PEG-3* gene and the transfection efficiency of Ad-*PEG-3* vector in 293 cells, the *PEG-3* gene was amplified by PCR, and the uptake of Ad-*PEG-3* vector was detected by fluorescence microscopy following transfection. The PCR results indicated that *PEG-3* mRNA was successfully cloned into the Ad-vector, which was also transfected into the 293 cells (Fig. 2A). The results of microscopy observation demonstrated highly efficient transfection when the virus was diluted to a MOI of 10<sup>5</sup> (~1x10<sup>6</sup> cells/ml with virus at a MOI of 5; Fig. 2B).

*CD* and *NIS* proteins express highly in TT cells. From the results of Fig. 2, it was identified that the *PEG-3* gene had

been successfully expressed in TT cells, which may trigger the positive expression of downstream genes such as *CDA* and *SLC5A5*. Western blot analyses were performed and the results demonstrated that there were differences in *CD* and *NIS* protein expression levels in TT cells when they were co-cultured with different cell lines (PHH, Hep3B, HuH7 or CCLP1; Fig. 3).

*Na<sup>131</sup>I combined with 5-FC decreases living human thyroid cancer cell viability.* The effect of Ad-*PEG-3* vector transfection on human thyroid living cells was determined by MTT assay. The number of living cells was calculated as 1- the optical density reading at 600 nm. The MTT assay results



indicated that either Na<sup>131</sup>I or 5-FC could inhibit TT living cells significantly at 24, 48, 72 or 96 h when treated with different combinations (Table I and Fig. 4). Particularly, the Na<sup>131</sup>I combined with 5-FC group exhibited a significantly decreased number of living cells compared with that of the Na<sup>131</sup>I and 5-FC single treatment groups ( $P < 0.05$  and  $P < 0.01$ , respectively; Fig. 4A). Concurrently, the living cell numbers for untransfected TT cells, used as the control in the present study, were also significantly decreased when treated with Na<sup>131</sup>I and 5-FC in combination compared with that of the Na<sup>131</sup>I and 5-FC single treatment groups ( $P < 0.05$  and  $P < 0.01$ , respectively; Fig. 4B).

## Discussion

At present, the most significant problem for cancer gene therapy is the delivery of the therapeutic gene to the targeted tumor cells or tissues (17,21). Indeed, almost all clinical trials currently being performed depend on direct intra-tumor injection of the vector (27). In order to overcome this problem, scientists have created certain vectors such as engineered adenoviral vectors and cationic liposomes (14,15). However, some vectors are not able to be expressed in various types of human cancer (31). In the present study, the pAV-murine cytomegalovirus-GFP-3FLAG vector was used to transport the therapeutic genes. A previous study indicated that NIS expression is primarily controlled by the thyroid-selective transcription factors paired box gene 8 (Pax-8) and NK2 homeobox 1 (Nkx2.1) in thyroid cancer (31). Pax-8 and Nkx2.1 target the NIS upstream enhancer through the cardiac homeobox transcription factor Nkx2 (16,32).

Previous advances propose additional improvements to CDA suicide gene therapy (32). The uracil phosphoribosyl transferase (*UPRT*) gene from *Escherichia coli* encodes uracil phosphoribosyltransferase, which converts uracil and 5-phosphoribosyl-1-R-diphosphate to uridine monophosphate (UMP). This protein is a potential target in cancer therapy, but not present in mammalian genomes when combining with *UPRT* (33,34).

The limitation of the present study was that only one thyroid cancer cell line, the TT cell line, was employed, which may be not sufficient to support the function of a gene as part of a gene therapy cancer study. Therefore, in following studies, the same *in vitro* experiments of the present study should be attempted with different thyroid cancer cell lines.

To conclude, transfection with an Ad-*PEG-3* plasmid into human thyroid cancer cells may inhibit tumor growth *in vitro*. This may be a useful tool for gene therapy in human thyroid cancer and other types of cancer.

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