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Original Article

Telomerase reverse transcriptase promoter-driven expression of iodine pump genes for targeted radioiodine therapy of malignant glioma cells

Jian Tan, Wei Li and Peng Wang

Abstract

Radioiodine is a routine therapy for differentiated thyroid cancers. Non-thyroid cancers can intake radioiodine after transfection of the human sodium iodide symporter (*hNIS*) gene. The human telomerase reverse transcriptase (*hTERT*) promoter, an excellent tumor-specific promoter, has potential value for targeted gene therapy of glioma. We used the *hTERT* promoter to drive the expression of the *hNIS* and human thyroid peroxidase (*hTPO*) gene as a primary step for testing the effects of radioiodine therapy on malignant glioma. The U87 and U251 cells were co-transfected with two adenoviral vectors, in which the *hNIS* gene had been coupled to the *hTERT* promoter and the *hTPO* gene had been coupled to the CMV promoter, respectively. Then, we performed Western blot, ^{125}I intake and efflux assays, and clonogenic assay with cancer cells. We also did ^{99m}Tc tumor imaging of nude mice models. After co-transfection with Ad-hTERT-hNIS and Ad-CMV-hTPO, glioma cells showed the ^{125}I intake almost 1.5 times higher than cells transfected with Ad-hTERT-hNIS alone. Western blots revealed bands of approximately 70 kDa and 110 kDa, consistent with the hNIS and hTPO proteins. In clonogenic assay, approximately 90% of co-transfected cells were killed, compared to 50% of control cells after incubated with 37 MBq of ^{131}I. These results demonstrated that radioiodine therapy was effective in treating malignant glioma cell lines following induction of tumor-specific iodide intake by the *hTERT* promoter-directed hNIS expression *in vitro*. Co-transfected *hNIS* and *hTPO* genes can result in increased intake and longer retention of radioiodine. Nude mice harboring xenografts transfected with Ad-hTERT-NIS can take ^{99m}Tc scans.

Key words Malignant glioma, sodium iodide symporter, hTERT promoter, tumor imaging

Glioma, which has the highest incidence among primary brain tumors, is very difficult to cure, resulting in a very high mortality in patients[1]. Tumor-targeted vectors driven by tumor-specific promoters can express therapeutic genes specifically in tumor tissues. But the transcription efficiency of most tumor-specific promoters is usually weaker than that of commonly used promoters such as the cytomegalovirus (*CMV*) promoter and the *simian virus 40* (*SV40*) promoter. As we all know, the telomerase reverse transcriptase (*TERT*) is highly expressed in most human cancer cells but not in normal somatic cells. Thus, it may be a target spot in anti-tumor therapy. The *hTERT* promoter has high G/C content but does not have TATA or CAAT boxes[2]. The *hTERT* promoter has been used to drive the expressions of many therapeutic genes, such as *caspase-8, Bax, diphtheria toxin A, thymidine kinase, rev-caspase-6, FADD,* and *TRAIL*[3]. Therefore, the *hTERT* promoter is a promising tumor-specific promoter in targeted cancer therapy.

The human sodium iodide symporter (*hNIS*), a membrane glycoprotein, facilitates transportation of iodide into thyroid follicular cells. Through targeted transfection and expression of the *hNIS* gene,

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non-thyroid cancers can intake iodine and then respond to radioiodine therapy in the same matter as thyroid cancer. Moreover, because of the crossfire effect of radiation therapy, radioiodine can kill not only the NIS-expressing cells but also adjacent tumor cells [4]. Researches on tumor-specific *hNIS* gene expression have been carried out in a variety of cancers, such as thyroid cancer, prostate cancer, colon cancer, breast cancer, liver cancer, and lung cancer [4]. Although the *hNIS* gene expression can lead to rapid intake of iodine in non-thyroid cells, this process is also accompanied by rapid outflow of iodine, which limits its killing effect. And the *hNIS* gene has been suggested as a possible reporter gene[5]. The human thyroid peroxidase (*hTPO*) is an enzyme that catalyzes iodine oxidation and tyrosine iodination at the initial step of thyroid hormone synthesis. Co-transfection of the *hTPO* and *hNIS* genes increases iodine intake and retention and enhances apoptosis of tumor cells[6]. Therefore, we hypothesized that co-expression of the *hTPO* and *hNIS* genes in glioma cells can overcome the rapid outflow of iodine caused by the *hNIS* gene expression alone.

Because the *hTERT* promoter has tumor specificity and co-transfection of the *hNIS* and *hTPO* genes may ensure accumulation of radioiodine in tumor tissues, we generated recombinant adenoviruses expressing the *hNIS* gene under the control of the *hTERT* promoter and the *hTPO* gene under the control of the *CMV* promoter. Then we transfected them into glioma cells (U87 and U251), did some cell level experiments *in vitro* and tumor imaging *in vivo*.

Materials and Methods

Cell lines

U251 and U87 (glioma cell lines) and MRC-5 (human embryonic lung fibroblast cells) were from our laboratory inventory. All cell lines were cultured in DMEM containing 10% FBS (Gibco BRL, Germany) supplemented with 100 U/mL penicillin/streptomycin in a 37℃ incubator containing 5% CO_2. ^{125}I and ^{131}I were from the China Nuclear High-Tech Company.

Construction of recombinant adenovirus[7]

Full-length *hNIS* cDNA was amplified from the cDNA library (forward primer with *Nco* I site underlined: 5'-CCATGGATGGAGGCCGTGGAG-3' and reverse primer with *Xba* I site underlined: 5'-TCTAGACTCCTGC-TGGTCTCG-3') and cloned into pGL3-hTERT-204[8]. The pGL3-hTERT-hNIS contained the −204 to +56 *hTERT* promoter region and the *hNIS* gene. The *hTERT* promoter and the *hNIS* gene regions were transferred from pGL3-hTERT-hNIS into pShuttle to construct Ad-hTERT-hNIS. The *hNIS* gene was also inserted into pShuttle-CMV to construct recombinant adenovirus Ad-CMV-hNIS. The *hTPO* gene was amplified from the pcDNA-TPO plasmid (forward primer 5'-GCGGCCGC-CACCATGAGAGCGCTCGCTGTGCT-3' and reverse primer 5'-AAGCTTACTAATGATGATGATGATGATGGA-GGGCTCTCGGCAGCCTGTGAGTATCCC-3') and cloned into pShuttle-CMV to construct Ad-CMV-hTPO. The recombinant adenoviruses were generated with AdEasy™ Adenoviral Vector System (Stratagene, USA) and stored at −80℃. Ad-CMV-EGFP served as negative control which can not take up iodide.

Adenoviral infection of glioma cells

The cells were seeded into 6-well plates and transfected when fusion reached 5×10^5 to 1×10^6 cells/well. The cells were transfected with recombinant adenoviruses at 50 MOI. After 6 h incubation with the viruses, cells were replenished with fresh 10% FBS-DMEM and then incubated for another 24 h before measurement.

Western blot[9]

EPS 2A200 electrophoresis system (Amersham Biosciences Inc. USA) was used for Western blot. Total cellular protein was loaded on 10% SDS-PAGE gel for electrophoresis and then transferred onto PVDF membrane. The membrane was blocked with 5% non-fat milk overnight at 4℃. Anti-NIS polyclonal antibody (sc-48055, Santa Cruz Biotechnology, USA) was diluted at 1:200. Anti-His monoclonal antibody (clone 4D11, Millipore, Germany) was used to detect His-tagged TPO at 1:1000 dilution. After incubated with primary antibodies for 2 h at room temperature, the membranes were incubated with secondary antibodies (goal anti-rabbit antibody sc-2004 and mouse anti-rabbit antibody sc-2357, both from Santa Cruz Biotechnology, diluted at 1:2000) at room temperature for another 2 h. After 5 min × 4 washes with TBS-T, membranes were covered with ECL solution (Thermo Fisher Scientific, USA) at room temperature for 1 min and exposed to Fuji X-ray film for 1–3 min in the darkroom.

Intake and efflux of radioiodine[10]

Cells were seeded in 6-well plates and infected with recombinant adenoviruses for 6 h, then replenished with fresh medium and incubated for another 24 h. Iodine intake was measured with the LKB 1261 Gamma Counter (LKB Co., Sweden). The cells (5×10^5 to 1×10^6 cells/well) were incubated with 18.5 kBq of $Na^{125}I$ for 50 min and then washed twice with PBS. Cells were

lysed with 0.3 mol/L NaOH and ^{125}I intake was measured. To measure the ^{125}I efflux, cells were cultured for 1 h with 18.5 kBq of $Na^{125}I$, then the old medium was decanted and cells were cultured for 0, 5, 10, 15, and 20 min. All experiments were repeated 3 times.

The clonogenic assay for ^{131}I-uptaking cells[11]

After infected with 50 MOI Ad-hTERT-hNIS, Ad-CMV-hNIS, Ad-hTERT-hNIS and Ad-CMV-hTPO, Ad-CMV-hTPO and Ad-CMV-hNIS, and Ad-CMV-EGFR (the control adenovirus), cells were washed once with PBS and treated with 3700 kBq of ^{131}I for 12 h. Then cells were seeded in 24-well plates at a density of 100 cells/well and incubated for 1 week. The cells were washed twice with PBS, fixed with 0.5 mL Carnoy's solution, and then counted after crystal violet staining. Cells were divided into 5 groups (control group transfected with Ad-CMV-EGFP, Ad-hTERT-hNIS transfected group, Ad-CMV-hNIS transfected group, Ad-hTERT- hNIS/Ad-CMV-hTPO co-transfected group, and Ad-CMV-hNIS/Ad-CMV-hTPO co-transfected group), with 6 wells for each group.

^{99m}Tc imaging of tumor burden in nude mice[10,12]

U87 cells were injected subcutaneously into 4-week–old nude mice at the right scapular. When tumor volume reached about 10 mm^3, 5 × 10^9 PFU of Ad-hTERT-hNIS and Ad-CMV-EGFP were injected into tumors, respectively. At 24 h after transfection, mice were fixed on hard plastic board in prone positions and given 37 MBq of ^{99m}Tc solution via intraperitoneal injection. SPECT imaging was conducted 30 min after injection. As U87 and U251 glioma cells behaved similarly in *in vitro* experiments, we conducted ^{99m}Tc imaging only on mice with U87 tumors.

Statistical analysis

Data are presented as mean ± standard deviation and were compared with variance analysis. Sample means were compared with *q* test (Newman-Keuls method). All statistical analyses were done using the SPSS 15.0 software package.

Results

The expression of hNIS and hTPO proteins

Expression of the hNIS and hTPO proteins was detected by Western blot. In U87 and U251 cells, the hNIS protein expression was detected as the 70 kDa band and the hTPO protein expression as the 110 kDa band. But the expression of the hNIS and hTPO proteins was relatively low (Figure 1).

Radioiodine intake and efflux

Co-transfection of the *hNIS* and *hTPO* genes caused rapid increase of iodine intake. U251 and U87 cells exhibited the highest level of iodine intake after 30 min of iodine exposure. In U87 cells, compared with that in the control group, iodine intake increased by 74 folds in Ad-CMV-hNIS/Ad-CMV-hTPO co-transfected group, by 63 folds in Ad-CMV-hNIS transfected group, by 31 folds in Ad-hTERT-hNIS/Ad-CMV-hTPO co-transfected group, and by 22 folds in Ad-hTERT-hNIS transfected group. In corresponding U251 groups, iodine intake increased by 65, 54, 34, and 27 folds of control, respectively. These results showed that co-transfection of the *hNIS* and *hTPO* genes can increase the iodine reservoir compared with transfection of the *hNIS* gene alone and that the *hTERT* promoter could lead to effective iodine intake, although the iodide intake leading by the *hTERT* promoter (Ad-hTERT-hNIS) were only a half compared with that leading by the *CMV* promoter (Ad-CMV-hNIS) (Table 1). As we have proved that U87 and U261 cells could express the *hTERT* gene (telomerase-positive) and MRC-5 cells could not express the *hTERT* gene (telomerase-negative) [8], the U87 and U251 cells

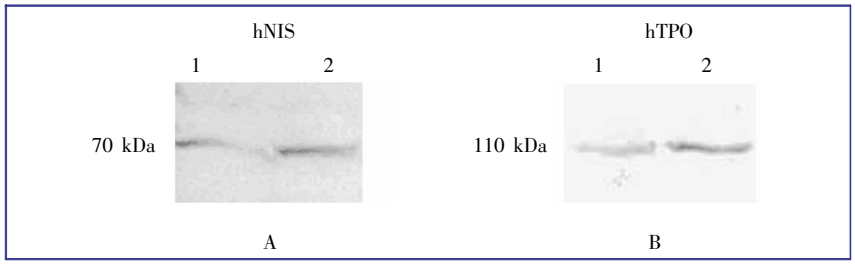

Figure 1. **Western blots for hNIS and hTPO protein levels.** A1 and B1, Ad-hTERT-hNIS and Ad-CMV-hTPO co-transfected U87 cells; A2 and B2, Ad-hTERT-hNIS and Ad-CMV-hTPO co-transfected U251 cells. The hTPO protein was observed as a major band of molecular weight at 110 kDa. The hNIS protein was observed as a major band of molecular weight at 70 kDa.

transfected with Ad-hTERT-hNIS could intake iodide, but the iodine intake of the MRC-5 cells transfected with Ad-hTERT-hNIS was the same as the cells transfected with Ad-CMV-EGFP (as negative control which can not take up iodide) (do not shown in Table 1).

After removal of iodine, rapid iodine loss within the cells was observed. In U251 and U87 cells co-transfected with the *hNIS* and *hTPO* genes, the effective half-life of iodine was 10 min, whereas in cells transfected with the *hNIS* gene, the effective half-life of iodine was 8 min. All transfected cell groups had a relatively short effective half-life of iodine (Figure 2).

T he clonogenic assay for ^{131}I-intaking cells *in vitro*

After radioiodine treatment, colony formation rates of U251 and U87 cells transfected with control virus were (48.3 ± 6.3)% and (40.0 ± 4.9)%, respectively. In U87 cell groups, clone formation rates of cells were (13.7 ± 1.1)% in Ad-hTERT-hNIS transfected group, (8.6 ± 1.6)% in Ad-hTERT-hNIS/Ad-CMV-hTPO co-transfected group, (6.2 ± 1.7)% in Ad-CMV-hNIS transfected group, and (5.7 ± 0.9)% in Ad-CMV-hNIS/Ad-CMV-hTPO co-transfected group. In corresponding U251 cell groups, the rates were (16.0 ± 2.0)%, (8.9 ± 1.0)%, (7.0 ± 1.3)% , and (5.9 ± 1.1)% , respectively. This result demonstrated that transfection of the *hNIS* and *hTPO* genes can more effectively kill tumor cells and suppress colony formation relative to the control. However, co-transfection of Ad-hTERT-hNIS and Ad-CMV-hTPO failed to provide a better killing effect when compared with transfection of Ad-hTERT-hNIS alone in U251 cells (P = 0.59, t = 3.4). Similarly, co-transfection of Ad-hTERT-hNIS and Ad-CMV-hTPO provided only a slight enhancement in killing U87 cells relative to transfection

Table 1. ^{125}I intake assays in U87 and U251 cell lines

Group	Maximal ^{125}I iodide intake per well (cpm)	
	U87 cells	U251 cells
Ad-CMV-EGFP	480.0 ± 25.7	415.3 ± 7.1
Ad-hTERT-hNIS	10569.3 ± 203.2*	11208.0 ± 404.5*
Ad-hTERT-hNIS and Ad-CMV-hTPO	14886.3 ± 506.2*	14527.3 ± 179.7*
Ad-CMV-hNIS	29971.0 ± 753.6*	22419.0 ± 242.9*
Ad-CMV-hNIS and Ad-CMV-hTPO	35072.3 ± 234.9*	26996.0 ± 587.8*

Data are expressed as mean ± standard deviation (SD) of 3 experiments. *P < 0.01, vs. Ad-CMV-EGFP.

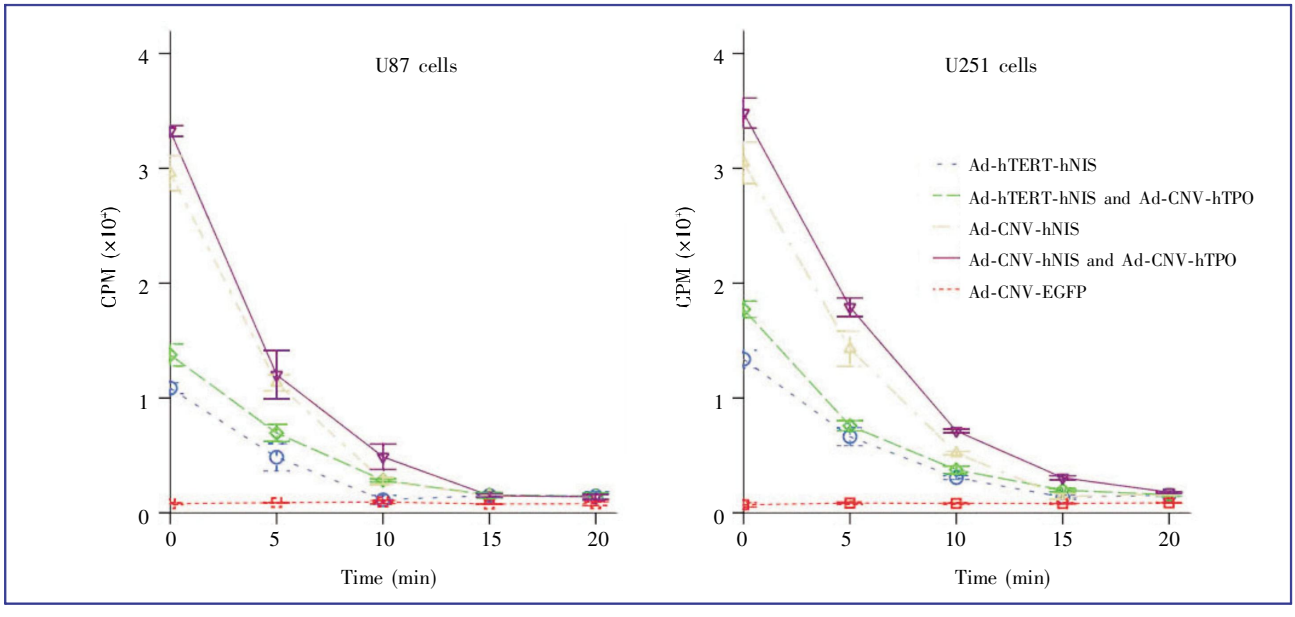

Figure 2. **^{125}I efflux assays in U87 and U251 cells.** When the radioactive medium was replaced, iodide was rapidly released from U87 and U251 cells. The half-life of iodide in all transfected cells (except those transfected with Ad-CMV-EGFP) was short and about 8 to 10 min. The U87 and U251 cells transfected with Ad-CMV-EGFP could not intake iodide.

of Ad-hTERT-hNIS alone (P = 0.25, t = 3.5). Likewise, co-transfection of Ad-CMV-hNIS and Ad-CMV-hTPO was not more effective than singular Ad-CMV-hNIS transfection (P = 0.71, t = 0.94 for U251 cells; P = 0.89, t = 1.21 for U87 cells) (Figure 3).

^{99m}Tc imaging of tumors in nude mice

At 30 min after ^{99m}Tc injection, tumor tissues with Ad-hTERT-hNIS could intake radioiodine, but tumor tissues without Ad-hTERT-hNIS transfection did not, suggesting that the *hNIS* expression in tumor tissues mediated by the *hTERT* promoter can facilitate iodine intake *in vivo* (Figure 4).

Discussion

High-grade gliomas are prone to infiltration. Although new treatments have been developed, many deficiencies still exist in glioma treatment. Tumor-specific promoters, such as the *hTERT* promoter, have been confirmed to mediate gene expression only in tumor cells, achieving maximum tumor-specific toxicity and minimum damage to normal cells. The *hNIS* gene, as a transporter, facilitates iodide intake in thyroid cells. If expressed in tumor cells, the *hNIS* gene can enrich iodine in these cells and mediate radioiodine treatment[12]. Nevertheless, researchers found rapid iodine loss after the *hNIS* gene transfection in tumor cells, but this shortcoming may be overcome by co-transfection of the *hNIS* and *hTPO* genes. Co-transfection of the *hNIS* and *hTPO* genes resulted in increased intake and retention of radioiodine and enhanced tumor killing[6] when a balance was reached between hNIS-mediated iodine intake and hTPO-mediated inhibition of iodine outflow[13]. In contrast, other researchers have found that the *hNIS* and *hTPO* genes, when co-expressed, effectively mediated iodine assimilation but did not significantly improve iodine retention[14].

To explore radioiodine therapy of glioma mediated by the *hTERT* promoter-controlled *hNIS* expression, we constructed a recombinant adenovirus carrying the *hNIS* gene controlled by the *hTERT* promoter, Ad-hTERT-hNIS, and then co-transfected the *hNIS* and *hTPO* genes into glioma cell lines U251 and U87 to investigate whether expression of the *hNIS* and *hTPO* genes could enhance iodide intake. In the process of thyroid cells synthesizing thyroid hormone, the first step is guided by the *hNIS* gene-mediated iodine intake, the second step is guided by the *hTPO* gene-mediated organic iodine intake. The *hNIS* gene is a key factor that lead intake iodine into cells. If there is no intake of iodine, there is

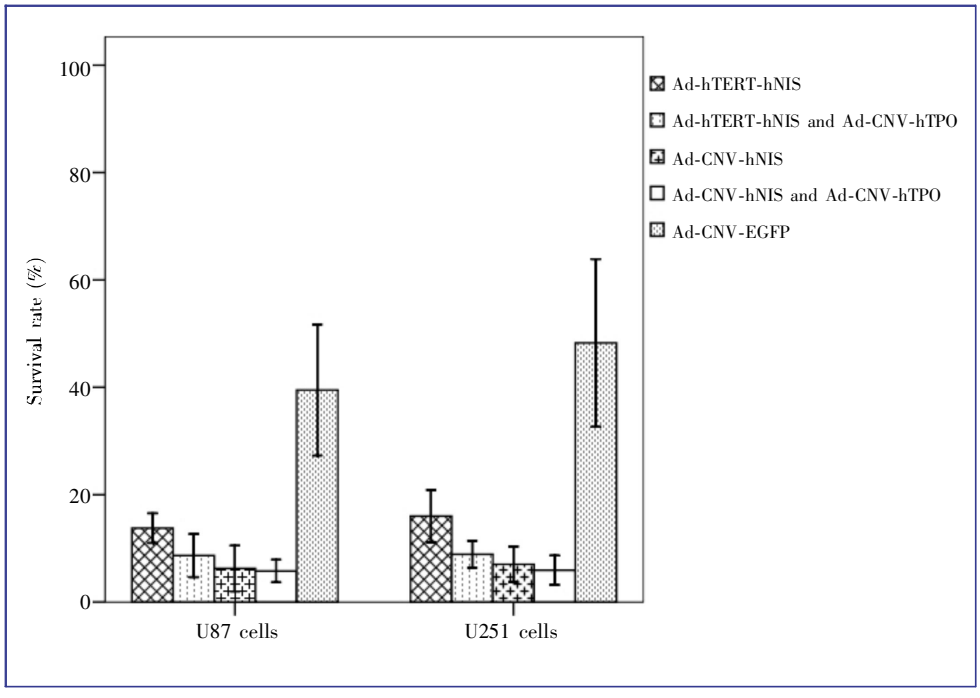

Figure 3. ***In vitro* clonogenic assay of U251 and U87 tumor cells.** The survival rates of U251 and U87 cells transfected with Ad-hTERT-hNIS and Ad-CMV-hTPO, Ad-hTERT-hNIS, Ad-CMV-hTPO and Ad-CMV-hNIS, or Ad-CMV-hNIS were lower than those transfected with Ad-CMV-EGFP. The survival rates of transfected U251 and U87 cells not incubated with ^{131}I were almost 90% (data not shown).

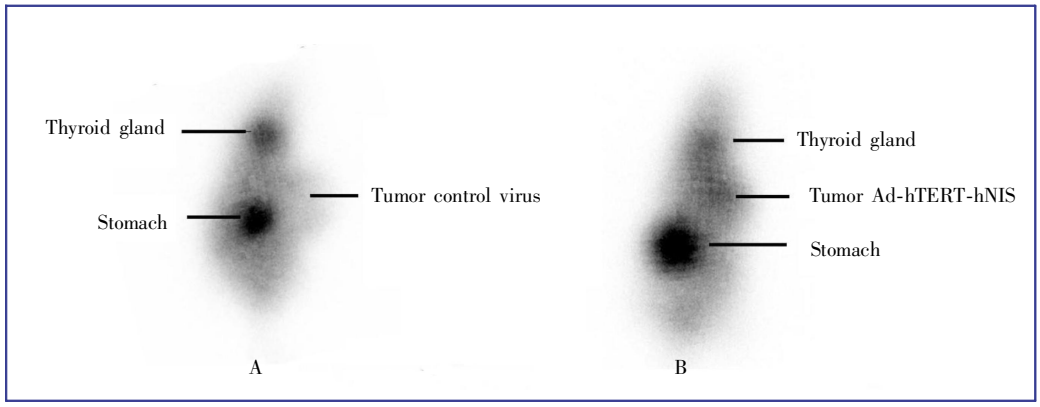

Figure 4. **The ^{99m}Tc scans of nude mice**. Nude mice harboring xenografts (right shoulder) injected Ad-hTERT-hNIS or Ad-CMV-EGFR 24h later, were imaged by scans taken 30 min after intraperitoneal injection of ^{99m}Tc (37 MBq). In contrast to the control tumor, it showed no in vivo accumulation of radionuclide. A, injection of Ad-CMV-EGFP; B, injection of Ad-hTERT-hNIS.

no step that guided by the *hTPO* gene. Whether the *TPO* gene is guided by the tumor-specific promoter has no practical meaning for targeted gene radioiodide therapy of cancer. So we only constructed the adenovirus expressed the *NIS* gene that guided by the tumor-specific *hTERT* promoter.

By comparing the effects of Ad-hTERT-hNIS and Ad-CMV-hNIS on iodine intake, we proved that the *hTERT* promoter can mediate the *hNIS* expression in telomerase-positive U87 and U251 cells, but not in telomerase-negative MRC-5 cells, so the *hTERT* promoter can achieve high tumor-specific expression, which provides insight for future research on *hNIS*-based tissue-specific radioiodine treatment. We also confirmed that the time to achieve iodine flow equilibrium in Ad-hTERT-hNIS and Ad-CMV-hNIS transfected cells was not significantly different (30 min in both groups). Although *hNIS* expression can up-regulate iodide intake, the difference in promoter activity leads to distinct expression patterns. Once equilibrium was achieved, iodine intake rates were different but the inflow times were constant. We also found that co-transfection of the *hNIS* and *hTPO* genes mediated by the CMV promoter and the *hTERT* promoter could slightly extend the time of iodine reservoir, but such a change was not sufficient to increase the cell-killing effect in the colony formation assay. We also performed ^{99m}Tc imaging on the tumor tissues transfected with Ad-hTERT-hNIS in nude mice, suggesting that the recombinant adenovirus Ad-hTERT-hNIS may be used to do the reporter gene imaging.

In summary, the *hNIS* gene expression mediated by the *hTERT* promoter was restricted only in telomerase-positive cells. Co-transfection of the *hNIS* and *hTPO* genes resulted in increased iodine intake and extension of iodine retention, but because the increase was not significant, co-transfection of the *hNIS* and *hTPO* genes did not effectively enhance cell killing in colony formation experiments. Nude mice harboring xenografts transfected with Ad-hTERT-NIS could take ^{99m}Tc scans.

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EBV VCA-IgA, Epstain-Barr virus viral capsid antigen-IgA antibody.

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