

Determination of the optimal time for radioiodine therapy in anaplastic thyroid carcinoma using the adenovirus-mediated transfer of sodium iodide symporter gene

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Abstract. Gene therapy using human sodium iodide symporter (hNIS) and radioiodine has been considered promising in a variety of gene therapy trials. However, the optimal timing of radioiodine application following hNIS gene transfer remains unknown. The present study aimed to investigate the serial expression of hNIS following adenovirus-mediated hNIS gene transfer into anaplastic thyroid carcinoma (ARO) to determine the optimal timing of radioiodine application. Recombinant adenovirus encoding the hNIS gene (rAd-hNIS) was generated using a homologous recombination reaction. The iodine uptake of rAd-hNIS-transfected ARO cells gradually increased until 120 min post-¹²⁵I application but the fold increase, reflecting the relative uptake of rAd-hNIS-transfected compared to non-transfected ARO cells, reached plateau at 60 min post-¹²⁵I application. For the *in vivo* analysis, rAd-hNIS was injected intratumorally into ARO cell xenografts in the thighs of nude mice (n=12). Two, 3, 4 and 6 days after rAd-hNIS injection, γ -scintigraphic images were obtained 60 min following injection of 5.5 MBq of ¹³¹I intraperitoneally. Treated/non-treated (T/NT) xenograft count ratios were the highest at day 2 post-rAd-hNIS injection (2.85 ± 0.61), and gradually decreased thereafter (2.54 ± 0.65 , 2.31 ± 0.42 and 2.18 ± 0.90 at days 3, 4 and 6 post-rAd-hNIS injection, respectively). Real-time poly-

merase chain reaction (RT-PCR) and immunohistochemical staining demonstrated that hNIS expression was the highest at day 2 following rAd-hNIS injection. In conclusion, the optimal timing for radioiodine administration is day 2 after adenovirus-mediated hNIS gene transfer into anaplastic thyroid carcinoma.

Introduction

Radioiodine plays a key role in the diagnosis and treatment of differentiated thyroid cancers, and is used to diagnose recurrent and metastatic disease and to treat differentiated thyroid cancers (1-3). The success of radioiodine for the treatment of differentiated thyroid cancers lies in the iodine-concentrating ability of the cancer, whereas most non-iodine-concentrating thyroid cancers result in treatment failure. However, if the iodine-concentrating function of thyroid cancers is restored, then radioiodine therapy would become feasible (4,5).

Sodium iodide symporter (NIS) is an integral membrane glycoprotein that mediates the active transport of iodine into thyroid follicular cells, the first step of thyroid hormone synthesis (6-8). The ability of the thyroid to concentrate iodine via NIS, provides the bases for thyroid diagnostic scintigraphic imaging using radioiodine and radioiodine therapy in hyperthyroidism and thyroid cancer.

Since NIS was cloned and characterized in 1996 (6,7), a number of studies have been conducted on NIS (9-13). At present, NIS is considered a novel therapeutic gene (4,14), since it offers a way of restoring the therapeutic effect of ¹³¹I in anaplastic and poorly differentiated thyroid carcinomas (15,16). Thus, coupling the delivery of the NIS gene into tumor cells by ¹³¹I administration may open new avenues of radionuclide gene therapy. However, the majority of previous studies have used *ex vivo* NIS gene transfer to produce engineered cancer cell lines stably expressing NIS (11-14). In such a condition of constitutive NIS expression, there is no restriction of radioiodine application with regard to the timing. However, such situations do not apply to gene therapy in clinic, in which the expression of NIS is limited in terms of time and place

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since the NIS gene should be exogenously delivered to the target tumor. Radioiodine application should be matched with NIS expression accordingly. In this regard, we believe that the *in vivo* transfection of NIS gene is a more reasonable means of emphasizing the application of radioiodine gene therapy in clinical practice.

The aim of this study was to investigate the optimal timing of radioiodine therapy during adenovirus-mediated human sodium iodide symporter (hNIS) gene transfer into anaplastic thyroid cancer (ARO) cells.

Materials and methods

Cell lines. The human ARO cell line was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). ARO cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% CO₂, and when 40–80% confluent, they were transfected with recombinant adenoviral vector.

Human embryonic kidney (HEK) 293 cells were also obtained from ATCC, and adenovirus-transformed HEK 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA), containing 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin.

Cloning of a recombinant adenoviral vector for hNIS gene transfer. Recombinant adenoviral vector encoding hNIS (rAd-hNIS) was produced using a homologous recombination reaction. hNIS cDNA was kindly provided by Dr Sissy M. Jhiang of the Ohio State University (7) and was cloned using the AdEasy™ system (Qbiogene, Montréal, Canada), which contains a green fluorescent protein (GFP) gene, and uses a homologous recombination of a shuttle vector and a backbone bacteria plasmid. Briefly, the hNIS cDNA gene was first cloned into a shuttle vector, pAdTrack-CMV, and the resultant plasmid was linearized by digestion with restriction endonuclease *PmeI* and subsequently cotransformed into *E. coli*. BJ5183 recombinants containing the adenoviral backbone plasmid, pAdEasy-1, were selected for kanamycin resistance, and recombination was confirmed by restriction endonuclease analysis. The linearized recombinant plasmid was then transfected into an adenovirus packaging cell line, HEK 293. Recombinant adenovirus-producing foci were easily confirmed using fluorescence microscopy following HEK 293 transfection by observing GFP expression (17).

Adenovirus-mediated hNIS gene transfer in ARO cells *in vitro*. ARO cells (2×10^5) were added to each well in 24-well plates, and then incubated for 24 h in 0.5 ml RPMI media. The cells in each well were then transfected with rAd-hNIS at multiplicities of infection (MOIs) of 0, 2.5, 5 or 10. Forty-eight hours after transfection, 0.1 µCi (3.7 MBq) of ¹²⁵I in 10 µM of cold iodine was applied to each well and incubated for 10, 30, 60, 90 or 120 min in quadruplicate. To perform inhibition assays, we inhibited hNIS activity by adding 50 M potassium perchlorate to a separate 10 MOI quadruplicate. Following incubation, wells were washed with cold Hank's

balanced salt solution (HBSS) and radioactivities were counted using a γ-counter. A protein assay was also performed to calculate iodine uptake (pmol) per mg of protein in each well. The values quoted were the means of experiments performed in quadruplicate.

Adenovirus-mediated hNIS gene transfer in ARO cell xenografts *in vivo*. Three-week-old male BALB/c nude mice (n=12) were obtained from the Charles River Laboratories (Yokohama, Japan). The experiments were approved by our Institutional Animal Research Committee. Levothyroxine sodium (50–100 µg/kg/day) was supplemented in drinking water to block thyroid ¹³¹I uptake.

Fifteen days after 2×10^6 ARO cells were subcutaneously injected in 200 µl of sterile phosphate-buffered saline (PBS) into both thighs of 12 nude mice (when ARO cell xenografts had reached 8–10 mm in diameter), and 1.5×10^8 plaque-forming units (pfu) of rAd-hNIS in 50 µl PBS was injected into the ARO cell xenografts in the right thighs (n=12) (T, treated tumor). The same amount of normal saline was injected into ARO cell xenografts in the left thighs (NT, non-treated tumor). rAd-hNIS and normal saline were injected into 4 sites within each xenograft using 30-gauge insulin syringes.

Scintigraphic ¹³¹I images of adenovirus-mediated hNIS gene transformed ARO cell xenografts. Two, 3, 4 or 6 days following intratumoral injection of rAd-hNIS, ¹³¹I images were captured using a γ-camera (Sigma 410 Radioisotope Camera, Ohio-Nuclear, Inc., Solon, OH, USA) equipped with a pinhole collimator. Nude mice were anesthetized with an intraperitoneal (i.p.) injection of 53 mg/kg ketamine and 12 mg/kg xylazine and placed under the collimator in a prone position. Sixty minutes after an i.p. injection of 5.5 MBq of ¹³¹I, 5-min static images of the 12 mice (3 mice per day) were captured. Treated/non-treated (T/NT) count ratios were calculated at 60 min post-¹³¹I injection for each mouse. ARO xenografts were excised in all the cases after images were captured and preserved at -70°C until the following experiment.

Detection of hNIS mRNA expression in ARO cell xenografts by real-time polymerase chain reaction (RT-PCR). The following primer pairs were used to detect hNIS mRNA using RT-PCR: 5'-GCT AAG TGG CTT CTG GGT TG-3' (hNIS gene sense primer); 5'-GTA AGC ACA GGC CAG GAA AA-3' (hNIS gene antisense primer). These hNIS gene primer pairs corresponded to the coding regions 941–960 and 1300–1319 and yielded a product of 379 bp for the hNIS gene. For comparison purposes, RT-PCR for the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was also performed using the primer pairs: 5'-ACC AGG GCT GCT TTT AAC TCT-3' (GAPDH gene sense primer); 5'-GAG TCC TTC CAC GAT ACC AAA G-3' (GAPDH gene antisense primer). The GAPDH gene primer pairs corresponded to the coding regions 130–150 and 576–597 and yielded a 468-bp product. Total RNA was isolated using RNeasy Midi kits (Qiagen Inc., Valencia, CA, USA) and a rotor-stator homogenizer. RT-PCR was performed over 27 amplification cycles using a GeneAmp® PCR System 9700 (Applied Biosystem, Inc., Foster City, CA). The PCR hNIS gene and GAPDH gene fragments were analyzed by 1.5% agarose/ethidium bromide gel electrophoresis.

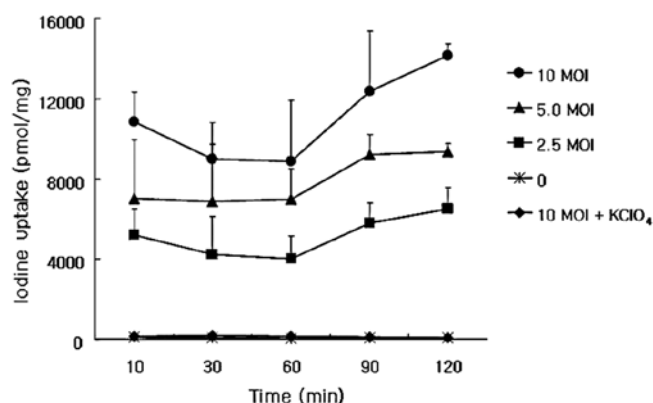


Figure 1. Iodine uptake analysis of adenovirus-mediated hNIS-transfected ARO cells. Iodine uptake increased until 120 min post-¹³¹I administration at viral titers of 2.5, 5.0 and 10 MOIs. Increased iodine uptake was inhibited by potassium perchlorate.

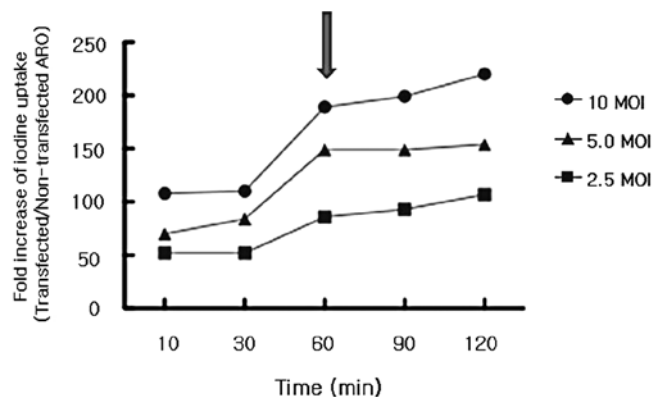


Figure 2. Fold increase of ¹²⁵I uptake by ARO cells transfected with recombinant adenovirus encoding the hNIS gene (rAd-hNIS) compared with non-transfected ARO cells. There was a leap forward of the relative uptake of iodine ~60 min post-iodine administration (arrow).

Immunohistochemical staining of excised ARO cell xenografts. Immunohistochemical staining of paraffin-embedded tissue sections derived from ARO cell xenografts was performed using rabbit anti-rat thyroid iodide transporter IgG (TIT11-A, Alpha Diagnostic International, San Antonio, TX, USA). Tissue sections were deparaffinized by three passages in xylene, subjected to a graded series of ethanol washes (100, 95 and 90% ethanol solutions) and then washed in distilled water. Endogenous peroxidase activity was blocked by incubation in 3% H₂O₂/methanol for 10 min and sections were washed in Tris-buffered saline and Tween-20 (TBS Tween-20: pH 7.4±0.05, Tris 0.005 M, NaCl 0.15 M, Tween-20 0.05%). To expose antigens, slides were heated in 0.01 M citrate buffer for 12 min. After cooling to room temperature for 20 min, slides were incubated using primary antibodies (TIT11-A diluted to 1:100) for 60 min, and washed with TBS Tween-20, anti-rabbit secondary antibody (EnVision™+, K 4003 HRP, Rabbit, DakoCytomation Inc., Glostrup, Denmark) for 40 min. 3,3'-Diaminobenzidine was used as the chromogen. Slides were counterstained with Mayer's hematoxylin and observed under a light microscope.

Results

In vitro iodine uptake analysis of adenovirus-mediated hNIS gene-transfected ARO cells. The iodine uptake of adenovirus-mediated hNIS gene-transfected ARO cells increased for 120 min at viral titers of 2.5, 5.0 and 10 MOIs, and was completely inhibited when potassium perchlorate was administered (Fig. 1). The fold-increase of iodine uptake by ARO cells transfected with the rAd-hNIS versus non-transfected ARO cells also increased for 120 min, but there was a leap forward ~60 min post-iodine application (Fig. 2). Thus, after the radioiodine administration, the 60 min time point was selected for scintigraphic imaging studies.

Scintigraphic ¹³¹I images of adenovirus-mediated hNIS gene-transfected ARO cell xenografts. ARO cell xenografts in the right thighs of nude mice were readily visualized 60 min after administering ¹³¹I on days 2, 3, 4 and 6 following recombinant adenovirus injection (Fig. 3). ¹³¹I accumulation

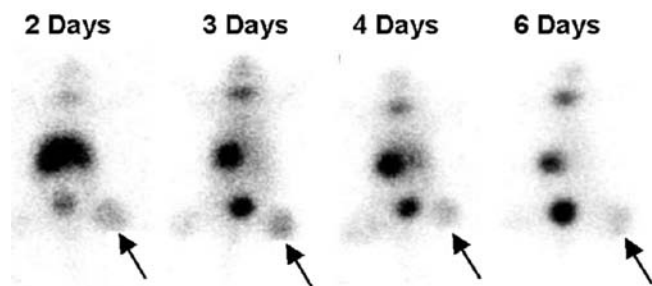


Figure 3. Representative 60-min ¹³¹I static images of BALB/c nude mice 2, 3, 4 and 6 days after the intratumoral injection of recombinant adenovirus encoding hNIS into ARO cell xenografts in right thighs (arrows). Transfected ARO cell xenografts were readily visualized in the day 2 and 3 images, however, ¹³¹I uptake using xenografts was gradually reduced thereafter. Images of mice are in prone position.

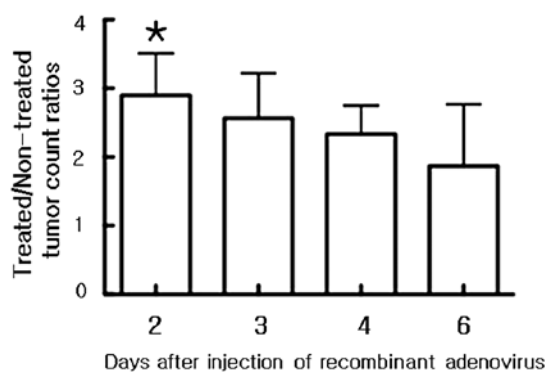


Figure 4. Treated/non-treated (T/NT) tumor count ratios of adenovirus-mediated hNIS-transfected ARO cell xenografts in ¹³¹I images captured 60 min post-injection. Mean count ratios on days 2, 3, 4 and 6 after intratumoral virus injection were 2.85±0.61, 2.54±0.65, 2.31±0.42 and 2.18±0.90, respectively. * The T/NT ratio at day 2 post-adenovirus injection was the highest of the other time points.

by xenografts was more prominent on the day 2 and 3 images compared to the day 4 and 6 images, which showed a gradual reduction. Mean T/NT count ratios of ARO cell xenografts of ¹³¹I images on days 2, 3, 4 and 6 were 2.85±0.61, 2.54±0.65, 2.31±0.42 and 2.18±0.90, respectively (Fig. 4).

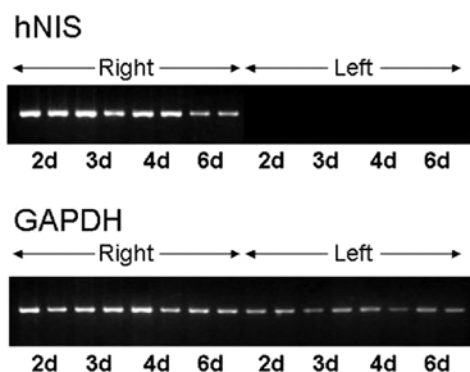


Figure 5. RT-PCR products of hNIS mRNA and GAPDH mRNA from ARO cell xenografts. Amplified 379-bp hNIS dsDNA band intensities on days 2, 3 and 4 post-recombinant adenovirus intratumoral injection were higher compared to those of amplified 468-bp GAPDH dsDNA bands in the same specimens. hNIS band intensities (379 bp) peaked on day 2 and then gradually decreased.

RT-PCR results of adenovirus-mediated hNIS gene-transfected ARO cell xenografts. RT-PCR for hNIS mRNA, which was extracted from adenovirus-transfected ARO cell xenografts excised from the right thighs of nude mice, produced a 379-bp hNIS dsDNA RT-PCR product, whereas RT-PCR for hNIS mRNA extracted from normal saline-injected ARO cell xenografts into left thighs did not yield any product. Amplified 379-bp hNIS dsDNA band intensities at 2, 3 and 4 days post-recombinant adenovirus injection were higher compared to those of amplified 468-bp GAPDH dsDNA bands in the same specimens. Moreover, the band intensities of hNIS mRNA RT-PCR products were the highest on day 2 and then gradually decreased, and on day 6 these intensities were lower compared to those of the GAPDH mRNA RT-PCR products (Fig. 5).

Immunohistochemical staining results of adenovirus-mediated hNIS gene-transfected ARO cell xenografts. Immunohistochemical staining of excised ARO cell xenografts was performed to determine the immunohistochemical localization of hNIS in adenovirus-mediated hNIS gene-transfected ARO cells. hNIS expression was the highest in ARO cell xenograft tissue specimens excised 2 days post-recombinant adenovirus injection, which then gradually decreased. Necrotic

areas were most abundant in ARO cell xenografts excised at 6 days post-recombinant adenovirus injection. hNIS expression was predominantly found in the cytoplasmic membranes of adenovirus-mediated hNIS gene-transfected ARO cells (Fig. 6), whereas no hNIS expression was observed in tissue specimens excised from ARO cell xenografts injected intratumorally with normal saline.

Discussion

In the present study, we successfully transferred the hNIS gene *in vivo* by intratumorally injecting recombinant adenovirus encoding the hNIS gene (rAd-hNIS), and serially measured transferred hNIS gene expression 2, 3, 4 and 6 days following recombinant virus injection using radioiodine imaging, RT-PCR and immunohistochemistry. The results showed that 2 days post-intratumoral injection is an optimal time for radioiodine therapy using the hNIS gene.

Thyroid cancer has been a target of gene therapy using NIS gene and radioiodine. Shimura *et al* (15) and Smit *et al* (16) reported that transfection of the NIS gene into thyroid cancer cells resulted in radioiodine accumulation. However, those authors used cell lines stably expressing the NIS gene. In the present study, we injected recombinant adenovirus encoding hNIS *in vivo* intratumorally to more accurately reflect perceived clinical applications.

In vivo NIS gene transfer has been investigated in a variety of cancer cells using adenoviral vectors. Boland *et al* (9) injected ^{131}I i.p. 3 days following intratumoral injection of recombinant adenovirus into a human cervical tumor cell xenograft. Cho *et al* (10) injected ^{125}I i.p. 43 h after recombinant adenovirus intratumoral injection into a human glioma cell xenograft, and Spitzweg *et al* (18) injected ^{123}I i.p. 4 days after recombinant adenovirus intratumoral injection into a human prostate cancer cell xenograft. However, those studies did not mention the optimal timing of radioiodine application since most were performed only at a single time point.

When it comes to the use of adenoviral vector as a vehicle, an initial high expression followed by a subsequent reduction in hNIS expression delivered by adenovirus has been reported in a non-tumor animal model (19). However, a thorough evaluation of adenovirus-mediated hNIS expression in terms of the optimal timing of radioiodine anti-tumor therapy has yet to be conducted.

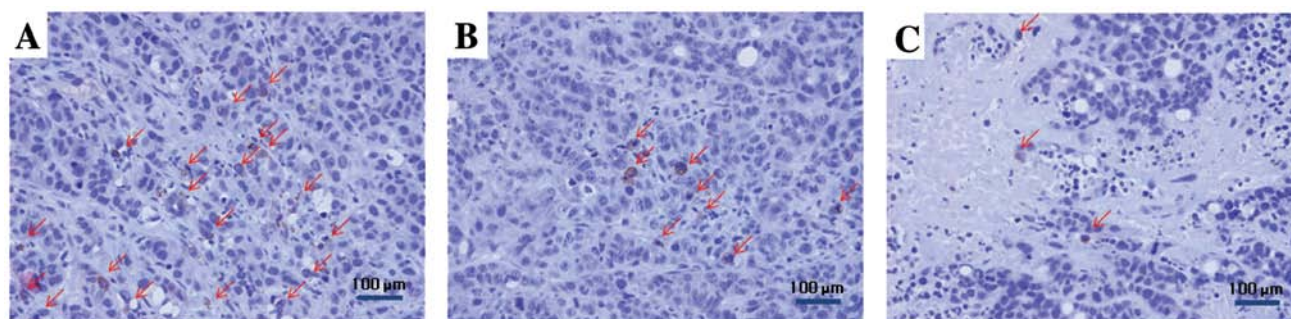


Figure 6. Immunohistochemical localization of hNIS expression in adenovirus-mediated hNIS gene-transfected ARO cell xenografts. hNIS expression was the highest in specimens excised on day 2 (A) after intratumoral recombinant adenovirus injection and decreased on day 3 (B) and was the lowest in specimens excised on day 6 (C) post-injection (original magnification, x400). Arrows indicate hNIS-expressing tumor cells.

Gene therapy using hNIS and ^{131}I administration have encountered several obstacles that need to be overcome. One of these obstacles is the rapid washout of delivered radioiodine. In their study, Spitzweg *et al* calculated that the average biological half-life of ^{131}I , which enters a tumor-expressing hNIS, is only 5.6 h (18). However, the average radioiodine half-life in metastatic thyroid cancer patients responding to radioiodine therapy was reported to be as long as 5.5 days (1). The application of tissue-specific promoters, or cotransfection of the thyroperoxidase gene, or the application of high-energy β -ray emitting radioisotopes such as ^{188}Re , may help resolve this problem (13,20-22). Increased knowledge of NIS and the development of gene therapy techniques should enable the identification of a role for the NIS gene in radionuclide gene therapy in the near future (4,5).

In conclusion, radioiodine uptake was successfully increased in ARO tumors by adenovirus-mediated hNIS gene transfer *in vitro* and *in vivo*. The optimal time for radioiodine administration (day 2 post-recombinant adenovirus injection) was determined by serial imaging and analysis. The results obtained during this study suggest the possibility of applying radioiodine therapy in iodine non-concentrating tumors by hNIS gene transfer.

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