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Original Article

Radionuclide imaging and therapy in malignant melanoma after survivin promoter-directed sodium iodide symporter gene transfer in vitro and in vivo

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Abstract: This study aimed to develop a gene expression targeting method specific for the imaging and therapy of malignant melanoma A375 cells using the sodium iodide symporter gene under control of the survivin promoter (Ad-Sur-NIS). When compared to control Ad-Sur-GFP-treated cells, Ad-Sur-NIS resulted in significantly higher iodide uptake in all 50, 100, or 150 MOIs examined cells (P<0.001). In vitro clonogenic assay showed the inhibition rates induced by ^{131}I were 94.8±12.4% in Ad-Sur-NIS, which was significantly higher than that in Ad-Sur-GFP infected cells (12.5±2.3%, P<0.001) or untreated cells (11.1±1.8%, P<0.001). In biodistribution studies, the tumor-to-muscle ratio in Ad-Sur-NIS infected tumors was higher than that in Ad-Sur-GFP infected tumors (16.34±4.43 vs 1.44±0.39, P<0.001). Moreover, mice that received the injection of Ad-Sur-NIS and ^{131}I showed a significant delay in tumor growth. Taken together, Ad-Sur-NIS expresses functional NIS, resulting in intracellular accumulation of radionuclide in malignant melanoma A375 cells in vitro and in vivo.

Keywords: Malignant melanoma, sodium iodide symporter, survivin, pertechnetate imaging, radioiodine therapy

Introduction

Malignant melanoma is one of the most common cancer types and it has one of the most rapidly increasing incidences in many countries around the world [1]. In the United States, the 5-year (2010-2014) average annual percent change in melanoma increased 2.3% in males and 1.2% in females [2]. Currently, the main treatment is early surgical resection in patients with localized disease [3]. However, there have been limited options for effective systemic treatment of disseminated melanoma [4]. Therefore, there is a need to develop new antitumor approaches for disseminated or recurrent disease.

The sodium iodide symporter (NIS) is an integral plasma membrane glycoprotein mainly expressed in thyroid follicular cells [5]. The biologic function of NIS is to mediate active transport of iodine, which is a crucial component for thyroid hormone biosynthesis [6]. This transporting ability of NIS has been successfully used for more than 70 years in radioiodide therapy of differentiated thyroid carcinoma, where radioactive iodide molecules (^{131}I) are used to internally radiate cancer cells of thyroid origin. Based on its characterization as a novel diagnostic and therapeutic gene, the cloning of the NIS gene has paved the way for the development of a novel gene therapy strategy for the treatment of cancers of nonthyroid origin based on targeted NIS gene transfer followed by radioiodine imaging and therapy [7-9].

Survivin, a novel member of the inhibitor of apoptosis protein family, is highly expressed in tumors and embryonic tissues but is expressed at low levels in normal terminally differentiated adult tissues [10]. Overexpression of survivin correlates with poor clinical outcome, tumor recurrence, and therapeutic resistance [11]. These unique characteristics of survivin make it an exciting potential transcriptional activation for cancer treatment [12, 13]. In previous studies, we have proven the feasibility of nonthyroidal radioiodine therapy after induction of iodide uptake by local adenoviral NIS gene transfer using tumor-specific survivin promoters (Ad-Sur-

NIS), to specifically target NIS expression to prostate, liver, and lung cancer cells [14-16].

In the current study, we therefore examined accumulation and therapeutic efficacy of radioactive iodide in malignant melanoma A375 cells following Ad-Sur-NIS gene transfer in vitro and in vivo.

Materials and methods

Cell culture

The malignant melanoma cell line A375 was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured maintained in RPMI 1640 medium supplemented 10% calf serum (Gibco, Carlsbad, CA, USA) and 1% penicillin/streptomycin. Cells were maintained at 37°C and 5% CO_2 in an incubator with 95% humidity.

Production of recombinant adenovirus and cell infection

The recombinant adenovirus Ad-Sur-NIS, which uses the survivin promoter to drive NIS expression, was used as previously described [16]. The recombinant adenovirus Ad-Sur-GFP, which uses the survivin promoter to drive GFP expression, was used as a negative control [16]. The A375 cells were added into 6-well plates with density of 1×10^6 cells per well, and then incubated for 48 h in RPMI 1640 medium prior to assay. The cells were then infected with 50, 100 or 150 multiplicities of infection (MOI) Ad-Sur-NIS or Ad-Sur-GFP. After 2 h infection, the media were replaced with fresh culture media, and virus-infected cells were further maintained.

In vitro ^{125}I uptake experiments

After 48 h infection, the cells were incubated with 3.7 kBq of ^{125}I in 1 mL of medium without serum for 30 min. Cells were washed twice with cold PBS followed by lysis using 0.5 mL trypsin for each sample. Radioactivity was quantified with a γ-counter (No.262 Nuclear Instrument Factory, Xi'an, China).

To perform the inhibition experiments, the cells were incubated with both 300 μM $KClO_4$ and ^{125}I for 30 min followed by the quantification of ^{125}I uptake as described above.

In vitro clonogenic assay

The procedure was performed as previously described [15]. In brief, the A375 cells transfected with Ad-Sur-NIS or Ad-Sur-GFP were incubated in RPMI-1640 medium containing 370 kBq/ml ^{131}I for 7 h. After incubation, cells were then seeded onto six-well plates at a density of 1000 cells per well. After 1 week, colonies containing more than 30 cells were counted. All experiments were performed in triplicate. Results were expressed as the percentage of inhibited cells.

In vivo scintigraphic images

Animal experiments were approved by the Sichuan University Animal Care and Use Committee. Tumors were established in 6-week-old nude mice by subcutaneous injection of 1×10^7 A375 cells per mouse. The experiments started until the tumors achieved a diameter of 5 mm. The Ad-Sur-NIS (1×10^9 PFU) or Ad-Sur-GFP (1×10^9 PFU) were injected intratumorally by group (3 mice per group) for gene transfecting in tumors.

At 2 days after adenovirus transfection, scintigraphic images were acquired. Mice received intravenously 18.5 MBq $^{99m}TcO_4^-$ followed by imaging with a γ camera (Philips Medical Systems, Milpitas, CA) 2 h after pertechnetate exposure. The used matrix size was 256×256, and this made the pixel size 1.08×1.08 mm.

Biodistribution of ^{125}I in the tumor-bearing mice

For biodistribution studies, mice were injected with Ad-Sur-NIS or Ad-Sur-GFP as described above followed by intravenous injection of 370 kBq ^{125}I 48 h later. Two hours after ^{125}I injection, the mice were sacrificed, tumor and muscle were dissected and weighed, and radioiodide uptake was measured in a γ-counter. The results were reported as the tumor-to-muscle (T/M) ratio.

In vivo ^{131}I therapeutic experiments

Experiments started when tumors had reached 4 to 5 mm in diameter after a 10-d pretreatment with 5 mg/l thyroxine (Sigma-Aldrich, St Louis, MO) in the drinking water, to suppress thyroidal iodine uptake. The mice were random-

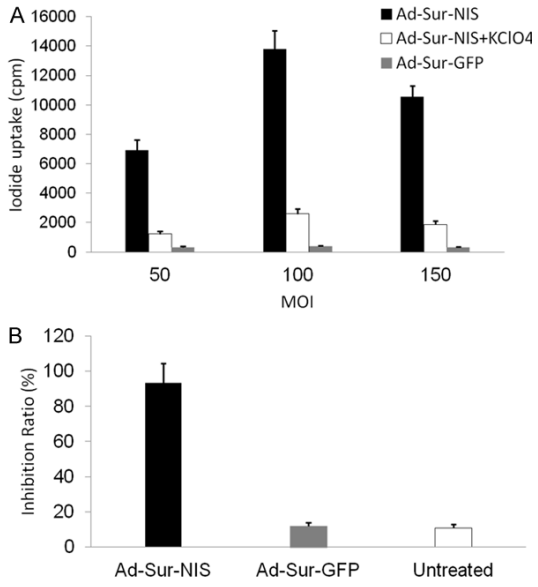

Figure 1. In vitro experiments. A: A375 cells were infected with 50, 100, or 150 MOIs Ad-Sur-NIS or Ad-Sur-GFP, and exposed to ^{125}I 48 h post-infection. When compared to control Ad-Sur-GFP-treated cells, Ad-Sur-NIS resulted in significantly higher iodide uptake in all 50, 100, or 150 MOIs examined cells (P<0.001). In comparison with 50 or 150 MOI infection, 100 MOI Ad-Sur-NIS caused up to 1.8 or 1.3 times higher iodide accumulation in cells. B: In vitro clonogenic assay, the inhibition rates induced by ^{131}I were 94.8±12.4% in Ad-Sur-NIS, which was significantly higher than that in Ad-Sur-GFP infected cells (12.5±2.3%, P<0.001) or untreated cells (11.1±1.8%, P<0.001).

ized into two groups (5 mice per group): the first group received the injection dose of Ad-Sur-NIS at 1×10^9 PFU; the second group received the injection dose of Ad-Sur-GFP at 1×10^9 PFU. On 2 days after the injection of Ad-Sur-NIS or Ad-Sur-GFP, all mice received 111 MBq ^{131}I. Tumor size was measured at 5-day intervals and the volume was calculated using the formula: 0.5×length×width^2. All mice were investigated over a total of 25 days and then euthanized.

Immunohistochemical analysis of NIS and Ki67 expression

To detect the NIS and Ki67 expression, resected tumors from nude mice were fixed in 4% paraformaldehyde for routine histopathological examination with immunohistochemical (IHC) examination with anti-NIS monoclonal antibody (Novus, Littleton, USA) and anti-Ki67 monoclonal antibody (Abcam, Cambridge, UK) [16, 17].

Statistical analysis

All data were represented as mean ± standard derivation. For in vitro cell and in vivo experiments, statistical significance was tested using Student's t-test, and statistical significance was achieved when the *P* value was <0.05.

Results

^{125}I uptake studies in vitro

To assess the functionality of the NIS expressed from the virus, A375 cells were infected with 50, 100, or 150 MOIs Ad-Sur-NIS or Ad-Sur-GFP followed by exposureto ^{125}I and quantification of iodide accumulation (**Figure 1A**). When compared to control Ad-Sur-GFP-treated cells, Ad-Sur-NIS resulted in significantly higher iodide uptake in all different MOIs examined in cells (P<0.001). The peak of ^{125}I uptake was observed at cells with transfection of 100 MOI Ad-Sur-NIS, in which cells' ^{125}I accumulation was 1.8 or 1.3 times higher than 50 or 150 MOI infected cells. Therefore, 100 MOI was used in all subsequent experiments. This uptake in Ad-Sur-NIS-infected cells was nearly eliminated when $KClO_4$ was administered (**Figure 1A**), which is a known competitive inhibitor of NIS, confirming that iodide uptake was mediated by NIS expressed from Ad-Sur-NIS.

Clonogenic assay of A375 cells

Cell viability assay was conducted for evaluation of the radioiodine sensitivity in these transgene cells by incubating cells with infected with 370 kBq ^{131}I for 7 h. The inhibition rates induced by ^{131}I were 94.8±12.4% in Ad-Sur-NIS, which was significantly higher than that in Ad-Sur-GFP infected cells (12.5±2.3%, P<0.001) or untreated cells (11.1±1.8%, P<0.001) (**Figure 1B**). These results demonstrated that coupling Ad-Sur-NIS infection and ^{131}I treatment specifically and efficiently led to A375 cell death in vitro, indicating potential for iodine-131 diagnosis and radiotherapy.

$^{99m}TcO_4^-$ scintigraphic imaging and biodistribution studies

The specific NIS expression was demonstrated in scintigraphic images. The A375 tumors infected with Ad-Sur-NIS showed significant $^{99m}TcO_4^-$ uptake, with the exception of normal

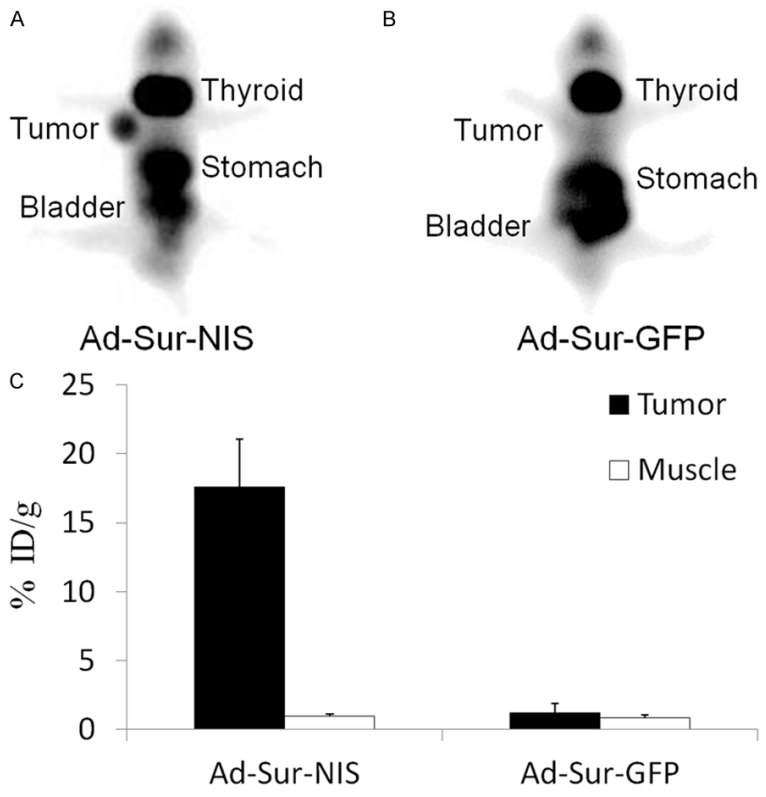

Figure 2. $^{99m}TcO_4^-$ scintigraphic imaging and biodistribution studies. The tumors infected with Ad-Sur-NIS showed significant $^{99m}TcO_4^-$ uptake (A), while only background activity was observed in tumors infected with Ad-Sur-GFP (B). (C) The T/M ratio in Ad-Sur-NIS infected tumors was higher than that in Ad-Sur-GFP infected tumors (16.34±4.43 vs 1.44±0.39, P<0.001).

physiological uptake in the thyroid, stomach, and bladder (**Figure 2A**). Only background activity was observed in the A375 tumors infected with Ad-Sur-GFP (**Figure 2B**).

The biodistribution data are provided in **Figure 2C**. The T/M ratio in Ad-Sur-NIS infected tumors was higher than that in Ad-Sur-GFP infected tumors (16.34±4.43 vs 1.44±0.39, P<0.001). The tumor specificity of Ad-Sur-NIS was confirmed by biodistribution, which exhibited selective uptake of ^{125}I in tumors.

Radionuclide therapy study in vivo

Mice that received the injection of Ad-Sur-NIS and ^{131}I showed a significant reduction in tumor growth. In contrast, mice receiving the injection of Ad-Sur-GFP and ^{131}I showed an exponential tumor growth (P<0.001) (**Figure 3A**). These results suggest that the injection of Ad-Sur-NIS and ^{131}I can reduce tumor growth in vivo, but the injection of Ad-Sur-GFP and ^{131}I showed no therapeutic effect.

Immunohistochemical staining of NIS and Ki67 expression

Three days after the start of treatment, mice were sacrificed and A375 xenografts were dissected and processed for immunohistochemical analysis using a NIS-specific antibody. Analysis showed high levels of NIS-specific immunoreactivity in Ad-Sur-NIS-infected tumors (**Figure 3B**). In contrast, the tumors infected with the injection of Ad-Sur-GFP exhibited no NIS-specific immunoreactivity (**Figure 3C**).

Eight days after the start of treatment, mice were killed and tumors were dissected and processed for immunohistochemical analysis using a Ki67-specific antibody. Ad-Sur-NIS-treated tumors exhibited a significantly lower proliferation index after ^{131}I therapy when compared with Ad-Sur-GFP-treated tumors (**Figure 3D**, **3E**).

Discussion

Recurrent or advanced malignant melanoma requires new treatment approaches [18, 19]. One developmental concept is combination of radiation with gene therapy. In our recent studies, Ad-Sur-NIS has been used as a transgene for concentrating radioiodide to cancers of non-thyroid origin, for example, in hepatocellular, lung, and prostate cancers [14-16]. This successful experience exemplified our efforts toward the application of Ad-Sur-NIS to malignant melanoma. Therefore, the present study aimed to investigate Ad-Sur-NIS mediating radioactive iodide uptake into malignant melanoma A375 cells.

In our current study, A375 cells infected with Ad-Sur-NIS showed significantly higher ^{125}I

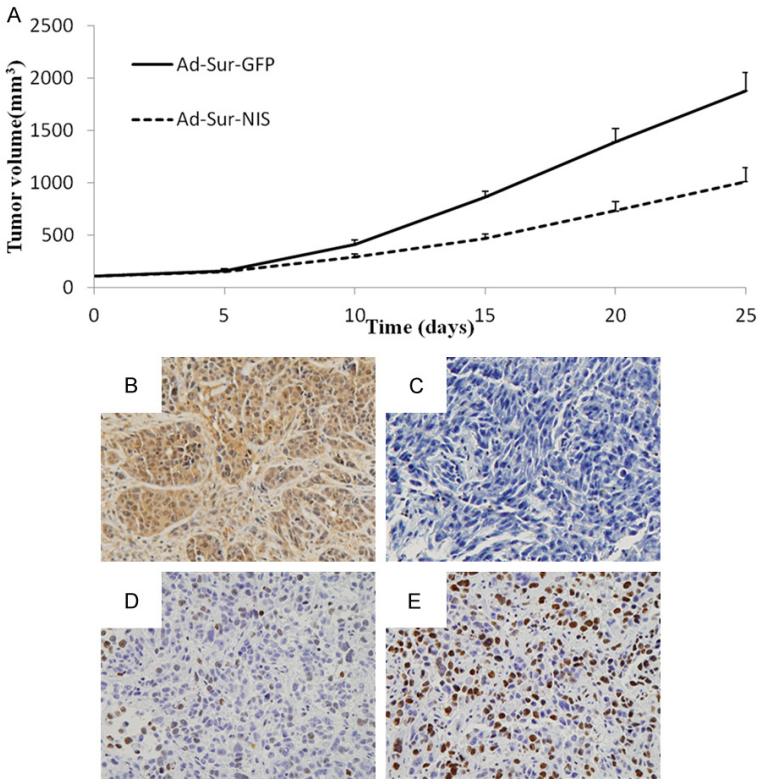

Figure 3. Therapeutic efficacy in vivo and immunohistochemical staining results (×400). A: Mice receiving an injection of Ad-Sur-NIS and ^{131}I showed a significant reduction in tumor growth. In contrast, mice receiving the injection of Ad-Sur-GFP and ^{131}I showed an exponential tumor growth ($P<0.001$). B-E: As compared with Ad-Sur-GFP-treated tumors, Ad-Sur-NIS-treated tumors exhibited a significantly higher NIS-specific immunoreactivity and lower proliferation index after ^{131}I therapy.

uptakes than Ad-Sur-GFP-infected cells, and 100 MOI Ad-Sur-NIS indicated the best transgene efficiency with the maximum ^{125}I uptake. The amount of accumulated ^{131}I has been shown to be sufficiently high to selectively kill Ad-Sur-NIS-transduced A375 cells in a clonogenic assay. The result thus demonstrated that coupling Ad-Sur-NIS and ^{131}I treatments in vitro efficiently and specifically led to cell killing. Scintigraphy and biodistribution studies confirmed that the specific accumulation of the radionuclide also occurred in the Ad-Sur-NIS-infected A549 tumors in vivo.

Most importantly, Ad-Sur-NIS gene transfer resulted in tumor-specific iodide uptake activity in A375 tumor-bearing mice, which was sufficiently high for a significant therapeutic effect of ^{131}I. After Ad-Sur-NIS application followed by ^{131}I injection, tumor-bearing mice showed a significant delay of tumor growth. In addition, immunofluorescence analysis showed markedly reduced proliferation after Ad-Sur-NIS gene transfer followed by ^{131}I application, suggesting radiation-induced tumor stromal cell damage in addition to tumor cell death. The crossfire effect of ^{131}I with a maximum path length of up to 2.4 mm might be responsible for stromal cell damage leading to reduced secretion of growth-stimulatory factors, thereby enhancing therapeutic efficacy [20].

In conclusion, radioiodine uptake was successfully increased in A375 tumors following tumor-specific survivin promoter-targeted NIS gene transfer in vitro and in vivo.

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Disclosure of conflict of interest

None.

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