Human sodium iodide symporter gene adjunctive radiotherapy to enhance the preventive effect of hMUC1 DNA vaccine

Yong Hyun Jeon1,2,3, Yun Cho1,2,3, Hyun Joo Kim1,2,3, Chul Woo Kim1,2,3, Jae Min Jeong1,2,3, Dong Soo Lee1,4 and June-Key Chung1,2,3*

1Department of Nuclear Medicine, Seoul National University College of Medicine, Seoul, Korea
2Tumor Immunity Medical Research Center, Seoul National University College of Medicine, Seoul, Korea
3Cancer Research Institute, Seoul National University College of Medicine, Seoul, Korea
4Department of Pathology, Seoul National University College of Medicine, Seoul, Korea

We demonstrate the use of combination therapy to overcome the limitations of cancer DNA vaccines by adding radioiodine gene therapy in an animal cancer model. We established a stable cell line (CT26/hMUC1-hNIS-FLuc: CMNF) expressing the hMUC1, hNIS and Fluc genes using a retro- and lentivirus system. The survival rates (% of CMNF cells were determined using clonogenic assays after 131I treatment. After i.m. immunization to 4 groups of Balb/c mice (pcDNA3.1, pcDNA3.1+131I, pcDNA3-hMUC1+PBS and pcDNA3-hMUC1+F Luc groups) with pcDNA3-hMUC1 or pcDNA3-FLuc one week for 2 weeks, 1 × 106 CMNF cells were injected s.c. into the right thighs of mice in each group. Twenty-one days after tumor transplantation, 131I was administered i.p. to the pcDNA3.1+131I and pcDNA3-hMUC1+F Luc groups. Tumor progression was monitored in the 4 groups by bioluminescent and scintigraphic imaging and by taking caliper measurements. Tumor masses were extracted and weighed at 39 days post-tumor challenge. We confirmed that CMNF cells highly express hMUC1, hNIS and Fluc by FACS, 125I uptake, and luciferase assay. The survival rates of CMNF were markedly reduced to (14.6 ± 1.5)% after 131I treatment compared with the survival rates of parental cells (p < 0.001). Tumor growth inhibition was significant only in the pcDNA3-hMUC1+131I group at 39 days post challenge. Tumor masses in pcDNA3-hMUC1+F Luc group were smaller than those of the other groups. This study shows that the weak preventive effects of cancer DNA vaccine can be overcome by radioiodine gene therapy utilizing sodium iodide symporter.

Key words: radioiodine gene therapy; immunotherapy; CT26/ hMUC1-hNIS-FLuc (CMNF); hMUC1; sodium iodide symporter; cancer DNA vaccine

Cancer DNA vaccine researchers have added various genes to cancer DNA vaccines to increase therapeutic effects, and these various gene supplements have resulted in significant tumor regression in living subjects.22–25 Since radioiodine gene therapy has been shown to be a powerful tool for cancer gene therapy, we attempted to develop a new combination therapy to enhance the preventive effects of cancer DNA vaccines by utilizing a NIS approach. In this study, we attempted to enhance the tumor growth inhibition induced by hMUC1 DNA vaccination by using radioiodine gene therapy of human NIS.

Material and methods

Animals

Specific pathogen-free 6-week-old female BALB/c mice were obtained from SLC (Hamamatsu, Japan).26 All experimental animals were housed under specific pathogen-free conditions and were handled in accord with the guidelines issued by the Seoul National University Animal Research Committee.

Generation of cDNA constructs and plasmid preparation

The human pancreatic mucin1 gene, hMUC1 (accession no. J05582), was cloned into the BamHI site of pcDNA3 vector (Invitrogen, Carlsbad, CA). Plasmid DNA was amplified in E. coli DH5α and purified by large-scale plasmid preparation using endotoxin-free Giga Prep columns (QIAGEN, Chatsworth, CA). DNA was dissolved in endotoxin-free TE buffer for storage.

Generation of Lentivirus

To construct hNIS expressing lentiviral vector under the control of ubiquitin C promoter, the hNIS gene was cloned into pLenti6/UbC/V5-DEST (Invitrogen, Carlsbad, CA). Replication-incompetent lentivirus was produced by cotransfecting lentiviral vector carrying hNIS and a ViraPowerTM Packaging Mix (Invitrogen, Carlsbad, CA) into 293FT producer cell line. 293FT cells (6 × 106) were transient transfected using 36 μl Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in 10 cm tissue culture plates. Cells were cotransfected with 5 μg of lentiviral vector and 10 μg of packaging plasmids (gag, pol, vsv-g, rev). Growth medium (DMEM containing 10% FBS, and 1% penicillin/streptomycin) was changed at 24 hr post-transfection and lentivirus-containing

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The first two authors contributed equally to this paper.

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*Correspondence to: Department of Nuclear Medicine, Seoul National University, College of Medicine, 28 Yongdong-dong, Chongno-gu, Seoul, 110-744, Korea. E-mail: jkchung@plara.snu.ac.kr

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supernatant was harvested at 48 hr post-transfection. Harvested supernatants were centrifuged at 3,000 rpm for 15 min at 4°C to pellet cell debris and stored at -80°C for later use. To assess the activity of recombinant virus encoding hNIS reporter gene, HT1080 human fibrosarcoma cells were infected by adding thawed lentivirus-supernatant containing 10 μg/ml of Polybrene. Human sodium-iodide symporter (hNIS) expression was confirmed by examining 125I uptake.

**Generation of Retrovirus**

Firefly luciferase gene under EF-1α promoter was cloned into pMSCVneo (BD Bioscience Clontech, CA). Retrovirus was produced by cotransfection into a human 293FT producer cell line using retroviral vector carrying the luciferase gene and packaging plasmids (gag, pol, vsv-g) (BD Bioscience Clontech, CA). Transient transfection of 293FT cells (6 x 10⁶) was performed using 36 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in 10 cm tissue culture plates. Cells were cotransfected with 5 μg of retroviral vector and 10 μg of packaging plasmids. Growth media (DMEM containing 10% FBS and 1% penicillin/streptomycin) were changed at 24 hr post-transfection and retrovirus-containing supernatants were harvested at 48 hr post-transfection. Harvested supernatants were centrifuged at 3,000 rpm for 15 min at 4°C to pellet cell debris and stored at -80°C for later use. To assess the activity of recombinant virus encoding firefly-luciferase reporter gene, HT1080 human fibrosarcoma cells were infected by adding thawed retrovirus-containing supernatant and 10 μg/ml of Polybrene. Cell luciferase expression was confirmed by checking luciferase activity using a luminometer (Applied Biosystems, Forster, CA).

**Murine tumor cell line expressing hMUC1, hNIS and firefly luciferase**

hMUC1 expressing CT26 (CT26/hMUC1) was kindly provided by Dr. Jung-Ah Cho. Viral supernatants were transduced into CT26/hMUC1 of the H-2d MHC type. Stable clones (CT26/hMUC1-hNIS-Fluc) were also subjected to luciferase assays and 125I uptake using a microplate luminometer (Applied Biosystems, Forster, CA) and gamma counter (GMI, Ramsey, MN).

To examine hMUC1 expressions on CMNF cell surfaces, cells were harvested and suspended in 0.1% BSA-containing PBS. Primary antibodies were added to these suspensions, and anti-MUC1 (Biomeda, CA) mouse antibody was used as the primary antibody. After 1 hr incubation on ice, cells were washed and pelleted to remove unbound antibodies. FITC-tagged anti-mouse antibody was used as a secondary antibody and incubated for 30 min on ice. Cold 2% PFA-containing PBS was used to fix the
cells, and finally, fluorescence intensities were measured using a Coulter FACScan. FACS analysis showed that CMNF cells highly expressed hMUC1 tumor antigen. In addition, we observed that bioluminescent signals increased with cell numbers.

In vitro clonogenic assay

The procedure used, with minor modifications, has been described previously. Briefly, cells were grown in a 75-cm² flask and incubated for 7 hr at 37°C in 5 ml HBSS containing 37 MBq/10 ml (1 mCi/10 ml) Na¹³¹I. The reaction was terminated by removing the radioisotope-containing medium and washing the cells twice with HBSS. The cells were then trypsinized, counted and plated at densities of 250 or 1,000 cells per well in DMEM in 6-well plates. Cells were grown for 10 days, fixed with 3:1 methanol/acetic acid, stained with crystal violet and macroscopic colonies numbers were counted. Survival rates were defined as colony numbers in radionuclide treated plates expressed as percentage of numbers in plates treated with HBSS only.

Monitoring of tumor growth inhibition in living mice

The IVIS200 imaging system (Xenogen, Alameda, CA), which includes an optical CCD camera mounted on a light-tight specimen chamber, was used for data acquisition and analysis. Firefly D-luciferin potassium salt (Fluc substrate), was diluted to 3 mg/100 ul in PBS before use, and mice were injected i.p. with 100 µl of this D-luciferin solution. Mice were placed individually in the specimen chamber containing the CCD camera, and then cooled to ~10°C. Light emitted by luciferase in mice was then measured. Gray scale photographic images and bioluminescent color images were superimposed using LIVINGIMAGE V. 2.12 (Xenogen, Alameda, CA) and IGOR image analysis software (WaveMetrics, Lake Oswego, OR). Bioluminescent signals were expressed in units of photons per cm² per second per steradian (P/cm²/s/sr).

pcDNA3.1 (50 µg/100 µl) or pcDNA3-hMUC1 (50 µg/100 µl) was injected i.m. into quadriceps muscles of right hind legs once a week for 2 weeks. One week after the final immunization, each group was inoculated s.c. with 1.3×10⁵ CMNF cells in right thighs. Mice had received a low-iodine diet and T4 supplementation in their drinking water for 2 weeks post CMNF challenge to maximize radioiodine uptake in tumors and to reduce iodide uptake by thyroid glands. Each animal was administered 111 MBq (3 mCi) of¹³¹I or saline i.p. Mice were repeatedly imaged at 7, 13, 21, 28, 35 days post CMNF challenge using an optical CCD camera to acquire photons, 10 min after injecting D-luciferin. To quantify emitted light, regions of interest were drawn over the tumor region and total photon effluxes over an exposure time of 10 sec were determined. For scintigraphic imaging, ⁹⁹mTc-pertechnetate was injected i.p. and mice were imaged using a γ-ray camera (ON-410) at 21 days postchallenge. Tumor size was measured using a caliper at 17, 21, 28, 32, 39 days and tumor weights were measured at 39 days.


Statistical analysis

Statistical significances were determined using an unpaired Student's t test, and Kaplan Meier curves were generated for survival analysis. The survival curves of 2 groups were compared using the log rank test. P values of < 0.05 were considered significant.

Results

Establishment of a cancer cell line expressing high levels of hMUC1, hNIS and firefly luciferase.

To determine hMUC1, hNIS and Fluc gene expressions in selected stable cell lines (CT26/hMUC-hNIS-Fluc, referred to as a CMNF), we performed FACS analysis, and luciferase and bioluminescence signals and 131I uptake assays, as shown in Figure 1. FACS analysis showed that stable transfectants highly expressed the hMUC1 gene (Fig. 1a), and bioluminescence signals and 131I uptake were found to correlate well with cell numbers (Figs. 1b and 1c).

In vitro clonogenic assay

As shown in Figure 2, the survival rates of CT26/hMUC-hNIS-Fluc cells were markedly reduced to (14.6 ± 1.5)% in response to 131I vs. CT26 cells (p < 0.001).

Tumor growth inhibition induced by hMUC1 vaccination plus radiiodine gene therapy

We performed following experimental procedures for radioiodine gene therapy and immunotherapy (Fig. 3). NIS gene expression was observed in all 4 mouse groups (Fig. 4a). We also observed tumor growth inhibition induced by hMUC1 vaccination in the two pcDNA3-hMUC1 vaccination groups (pcDNA3-hMUC1 + PBS, pcDNA3-hMUC1 + 131I) compared to the two pcDNA3.1 vaccination groups (pcDNA3.1, pcDNA3.1 + 131I groups) by bioluminescent imaging (Fig. 4b). Following 131I treatment to all groups, tumor progression was monitored by bioluminescent imaging and caliper measurements until 39 days post-challenge (Figs. 4b–4d), and a difference in tumor growth inhibition was observed between the pcDNA3-hMUC1 + PBS and pcDNA3-hMUC1 + 131I groups at 28 days postchallenge. Significant tumor growth inhibition was observed in the pcDNA3-hMUC1 + 131I group vs. the pcDNA3.1 + PBS, pcDNA3.1 + 131I and pcDNA3-hMUC1 + PBS groups (p < 0.05). At 39 days postchallenge, animals were euthanized and tumor masses were removed and weighed. Significant tumor growth inhibition was observed in the pcDNA3-hMUC1 + 131I group vs. the pcDNA3.1 + PBS, pcDNA3.1 + 131I and pcDNA3-hMUC1 + PBS groups (p < 0.05) (Fig. 4e). As shown in the Kaplan-Meier plots in Figure 4f, 80% of tumor bearing mice survived until 50 days postchallenge in the combination therapy group (pcDNA3-hMUC1 + 131I group) using hMUC1 vaccination + 131I treatment, whereas almost all animals in the other groups died (pcDNA3.1 + PBS, pcDNA3.1 + 131I and pcDNA3-hMUC1 + PBS group: 0, 10, 20 %, respectively).

Discussion

Immunotherapy using cancer DNA vaccines is now viewed as a means of inhibiting tumor growth in living organisms. Various strategies have been applied to increase the potency of DNA vaccines; for example, targeting tumor-associated antigens to facilitate rapid cellular degradation, directing tumor-associated antigens to chemokines, coexpressing cytokines or coadministering CpG motifs. However, immunotherapies based on cancer DNA vaccines in combination with these strategies have been found to be ineffective at inhibiting tumor growth. Thus, immunotherapy researchers have tried to utilize other modalities to overcome the preventive (or therapeutic) limitations of cancer DNA vaccines.

Sodium iodide symporter (NIS) has been used for radionuclide gene therapy as an adjunct to treatment in various types of cancer. Some investigators have found that effective gene expression is induced in living subjects by infecting various cancer cells with NIS, and have achieved good therapeutic effects in preclinical models. Other reports have shown that NIS gene transfer using tissue-specific promoter allows the targeting of NIS gene expression in specific cancer cells, thereby maximizing tissue-specific cytotoxicities and minimizing toxic side-effects in normal cells. NIS gene transfer into cancer cells causes therapeutic radionuclides (131I, 188Re) to concentrate in these cells, and thus facilitating the use of therapeutic radionuclides to induce cancer cell apoptosis. Our group has previously reported that NIS expressing human hepatocellular carcinoma can be selectively killed given 131I and 188Re accumulation via NIS gene expression, and demonstrated that therapy and imaging based on NIS gene transfer could be used to treat anaplastic thyroid carcinoma. It has also been shown that the human MUC1 mucin is over-expressed in an incompletely glycosylated form in various human cancers and because high expression of tumor associated antigen hMUC1 is related with rapid tumor progression and a poor prognosis in several types of human cancer, it is considered an attractive immunotherapeutic target. Moreover, cancer DNA vaccine encoding hMUC1 has been studied for the targeting of epithelial cancers expressing high levels of MUC1, and it has been shown that MUC1 DNA vaccination can inhibit MUC1 expressing tumor growth in a preclinical model.

In the present study, we developed a combination therapy based on an MUC1 vaccine and radionuclide gene therapy. To do this we established an adenocarcinoma colon cancer cell line stably expressing hMUC1, firefly luciferase and sodium iodide symporter by retro- or lentivirus infection, which we refer to as the CMNF cell line. Because CMNF cells express immunogenic target and therapeutic genes, we were able to perform combined cancer DNA immunotherapy and radionuclide gene therapy in living mice. In addition, bioluminescent signals emitted by CMNF allow the monitoring of therapeutic effects.

This combination therapy was found to have the desired effects in vitro and in vivo, and the survival of NIS expressing CMNF cells was effectively reduced (14.6 ± 1.5)% of the control after 131I treatment. In addition, we observed effective in vivo tumor growth inhibition in the combination therapy group (the pcDNA3-hMUC1 + 131I treatment group) but not in mono therapy group (pcDNA3.1, pcDNA3.1 + 131I, pcDNA3-hMUC1 + PBS treatment group). Tumor growth inhibition occurred in mice immunized with pcDNA3-hMUC1 but not in mice immunized with pcDNA3.1 at 28 days post-CMNF challenge based on caliper and bioluminescent imaging findings (Fig. 4b). However, tumor growth inhibition was not sustained in pcDNA3-hMUC1 + PBS treatment group from 28 days postchallenge.

Although we did not investigate immunologic aspects, we believe that this phenomenon (reduction in tumor growth inhibition) may have been due to an immunogenic effect of the DNA vaccine, the hindrance regulator T-cells, and disruption of cyto-

**Figure 4** – In vivo visualization of tumor growth inhibition induced by combined radioiodine gene and immunotherapy. Mice were immunized with pcDNA3.1 or pcDNA3-hMUC1 once a week for 2 weeks. One week after the last immunization, each group was subcutaneously challenged with CMNF cells. (a) Mice were imaged using a γ-camera equipped with collimator at 21 days post-challenge before 131I treatment. This revealed an imaging showing iodide uptake in the thyroid and stomach, which both express NIS, and in the bladder where iodide was also cleared in the urinary T, thyroid, S, stomach; B, urinary bladder. (b) Bioluminescent images were obtained from seven days until day 32 post-challenge. (c) To quantify light intensities, regions of interest were drawn over tumor regions and total photon effluences were measured. (d) Tumor volumes were measured in these mice using caliper following time point. (e) Tumor masses were extracted and weighed on day 39 post-challenge. (f) Survivals are expressed as percentages. Experiments were performed in duplicate, and bars present means ± SD, n = 10 mice/group.
a Scintigraphic imaging for determination of NIS gene expression
(21 days after tumor challenge, pre-treatment with I-131)

b Days after tumor challenge

c Tumor measurement by bioluminescence

d Tumor mass measurement by using caliper

e Tumor mass weighting

f Survival rate of tumor bearing mice

Experimental Group

Percent surviving (%)
kinase balance in the host.8–11 Furthermore, we could suggest following 2 reasons for the possible mechanisms of more significant tumor growth inhibition effect after I-131 treatment in MUC1 vaccinated group than other groups. The one is the immune booster response to the MUC1 antigen, which was released from the vaccinated group than other groups. The other mechanism, which might be involved, is that the released hNIS itself from dead cells after radionuclide treatment could also raise immune response against viable CNMF cells with hNIS expression. We recently reported that hNIS vaccination with MIDGE plasmid vector could raise hNIS-associated cell mediated immune responses in mice tumor model.45

This study has a limitation that should be mentioned. We injected MUC1 vaccine before tumor inoculation, which is not accord with the normal situation in cancer patients, but which is consistent with cancer vaccine treatment in a postoperative setting after tumor resection. Thus, it would be more accurate to say that the described combination therapy helps prevent tumor recurrence. In terms of future studies, the repertoire of immune responses initiated by radioiodine gene therapy in living subjects requires investigation. Moreover, because recent sodium iodide symporter radioiodine gene therapies have concentrated on the adenoviral system, which is controlled by specific-promoter regulation, further efforts are required to broaden the scope of the radioiodine/immunotherapy concept.

Conclusion

We demonstrate for the first time that limited tumor growth inhibition achieved using a hMUC1 DNA vaccine can be augmented by radioiodine gene therapy based on sodium iodide symporter over an extended period (52 days) in a preclinical model. We hope that this combination therapy will find a place as a treatment for preventing the occurrence or recurrence of cancer.

References


