Radioiodine therapy for castration-resistant prostate cancer following prostate-specific membrane antigen promoter-mediated transfer of the human sodium iodide symporter

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Radioiodine therapy, the most effective form of systemic radiotherapy available, is currently useful only for thyroid cancer because of the thyroid-specific expression of the human sodium iodide symporter (hNIS). Here, we explore the efficacy of a novel form of gene therapy using prostate-specific membrane antigen (PSMA) promoter-mediated hNIS gene transfer followed by radioiodine administration for the treatment of castration-resistant prostate cancer (CRPC). The androgen-dependent C33 LNCaP cell line and the androgen-independent C81 LNCaP cell line were transfected by adenovirus. PSMA promoter-hNIS (Ad.PSMApro-hNIS) or adenovirus.cytomegalovirus–hNIS containing the cytomegalovirus promoter (Ad.CMV-hNIS) or a control virus. The iodide uptake was measured in vitro. The in vivo iodide uptake by C81 cell xenografts in nude mice injected with an adenovirus carrying the hNIS gene linked to PSMA and the corresponding tumor volume fluctuation were assessed. Iodide accumulation was shown in different LNCaP cell lines after Ad.PSMApro-hNIS and Ad.CMV-hNIS infection, but not in different LNCaP cell lines after adenovirus.cytomegalovirus (Ad.CMV) infection. At each time point, higher iodide uptake was shown in the C81 cells infected with Ad.PSMApro-hNIS than in the C33 cells (P < 0.05). An in vivo animal model showed a significant difference in 131I radioiodine uptake in the tumors infected with Ad.PSMApro-hNIS, Ad.CMV-hNIS and control virus (P < 0.05) and a maximum reduction of tumor volume in mice infected with Ad.PSMApro-hNIS. These results show prostate-specific expression of the hNIS gene delivered by the PSMA promoter and effective radioiodine therapy of CRPC by the PSMA promoter-driven hNIS transfection.

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INTRODUCTION

The human sodium iodide symporter (hNIS) is an intrinsic membrane glycoprotein with 13 putative transmembrane domains. hNIS is responsible for the ability of the thyroid gland to transport and concentrate iodide at levels approximately 20–40-fold above plasma concentration. Recently, the mechanism mediating active iodide transport across the basolateral membrane of the thyroid follicular cells has been clarified by cloning and characterization of hNIS. The expression of hNIS in thyroid cancer cells allows effective therapy with radioiodine, even in cases of advanced disease with distant metastases. This type of therapy contributes to the improved prognoses of these patients. A novel form of cytoreductive cancer gene therapy, using hNIS gene transfer to induce radioiodine accumulation activity in cancer cells, would extend the application of carrier-free radioiodine and the extensive experience with radioiodine in thyroid cancer therapy to the treatment of non-thyroidal cancer. Advanced prostate cancer (PCa) is initially sensitive to endocrine therapy, but easily becomes castration resistant with long-term endocrine therapy. When progressing to a castration-resistant stage, the PCa cells also acquire radiation resistance. At present, there is no curative therapy for castration-resistant PCa (CRPC). Previous reports have shown tissue-specific iodide uptake activity in PC cells in vitro and in vivo following adenovirus-mediated hNIS gene delivery. In this study, the androgen-independent human prostatic adenocarcinoma cell line LNCaP was stably transfected with the hNIS gene under the control of the prostate-specific membrane antigen (PSMA) promoter and prostate-specific iodide uptake activity, and the therapeutic response to the accumulated 131I were investigated.

MATERIALS AND METHODS

Cells and cell culture

The parental androgen-sensitive human LNCaP cell line was kindly donated by Prof. Jung Klaus (Department of Urology, University Hospital Charité, Humboldt University, Berlin, Germany) and cultured in Rosewell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, Carlsbad, California, USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Carlsbad, USA) at 37°C in a...
5% CO₂ environment. The LNCaP cells were fed twice per week and split once per week with trypsinization as one passage. The passage number of LNCaP cells was designated as C-33 when the passage number was less than 33 and C-81 the passage number was greater than 80. For the steroid-reduced conditions, the LNCaP cells were grown in phenol red-free RPMI 1640 medium supplemented with 10% charcoal/dextran-treated FBS serum (CDS) (Life Technologies, Carlsbad, USA).

cDNA cloning of human sodium iodide symporter
The hNIS cDNA (2000bp) was cloned from human thyroid tissue by the reverse transcription-polymerase chain reaction (PCR) kit (TAKARA, DaLian, ShenYang, China). Total RNA was isolated from human thyroid tissue. Single-stranded oligo (dT)-primed cDNA was generated using superscript II reverse transcriptase. A PCR primer set was designed to amplify the entire coding region of the hNIS cDNA (forward, 5'-CGCGTCGACATGGAGCCGTCGGAGACCC-3'; reverse, 5'-CGAACGTTTCAAGAGTTT TCTCCTGTCCG-3').

DNA cloning of the prostate-specific membrane antigen promoter
The PSMA promoter DNA sequence was cloned from the total DNA isolated of the androgen-sensitive human prostatic adenocarcinoma cell line LNCaP. A PCR primer set was designed to amplify the entire region of the PSA promoter sequence (forward, 5'-CGGTTACCCTACTCAGCTGGCCCATGG-3'; reverse, 5'-CGCCGCTCGACTGTGCTGC TGCTCTACTGCG-3').

Recombinant adenovirus production
The pShuttle.PSMAPro-hNIS and pShuttle.CMV-hNIS were generated using the rapid DNA ligation kit and the sequence of the insert was determined by DNA sequencing.

Iodide uptake studies in vitro
The uptake of 131I by the virus-infected LNCaP cells was determined at steady-state conditions as described by Weiss et al. There were four wells in each group. In brief, 2–6 days after infection, the iodide uptake studies were performed in Hank's balanced salt solution (HBSS) supplemented with 10 μmol l⁻¹ NaI, 0.1 μCi g⁻¹ per ml and 10 μmol l⁻¹ HEPES at pH 7.3. The trapped iodide was removed from the cells by 20 min incubation in 1 mol l⁻¹ NaOH and was measured by γ-counting.

Adenovirus-mediated human sodium iodide symporter gene delivery in LNCaP cell xenografts in vivo
A total of 15, 4-week-old male castrated BALB/c nude mice (SPF) weighing 13–17 g (Centre of Experimental Animal, the Second Military Medical University, Shanghai, China) were used in this study. All animal experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act of 1986 and its associated guidelines, the EEC Directive of 1986 (86/609/EEC). The study was approved by the Ethical Committee of Changhui Hospital. The xenotransplants derived from the C-81 cells were established in mice by subcutaneous injections of 1 × 10⁶ cells suspended in 0.25 ml of phenol red-free RPMI 1640 medium supplemented with 10% CDS medium and 0.25 ml of Matrigel basement membrane matrix. Following 6–8 weeks of tumor growth, once the tumor volume became palpable and measurable by callipers (approximately 5 mm), a total volume of 150 μl (3 × 10⁴ plaque forming units per ml in 3% sucrose/phosphate buffered saline) of a recombinant Ad.PSMApro-hNIS and Ad.CMV-hNIS or control virus Ad.CMV was injected directly into the tumors using tuberculin syringes with a 28-gauge needle. The needle was moved to various sites within the tumor during the injection to maximize the area of virus exposure. Before the injections, the mice received a low-iodine diet and T4 supplementation (5 mg l⁻¹) in their drinking water for 2 weeks to maximize the radioiodine uptake in the tumor and reduce the iodide uptake by the thyroid gland.

Iodide uptake studies in vivo
Four days after the intratumoral injection of Ad.PSMAPro-hNIS and Ad.CMV-hNIS or control virus, five mice in each group were injected intraperitoneally with 500 μCi of 131I (Xinke, Shanghai, China), and radioiodine imaging was performed using a gamma camera equipped with a low energy general-purpose collimator (Toshiba, Tokyo, Japan). Every week, the radioiodine uptake by the tumors was monitored and quantified by imaging with a gamma camera. The tumor volume fluctuation was also recorded.

Statistics
We calculated descriptive statistics for each variable. Before the analysis, we examined each variable for its distributional characteristics. All the data are shown as the means ± standard deviation (s.d.). Statistical significance was defined as P < 0.05 for a two-tailed test. The calculation was performed using Statistical Analysis Software (SAS) 9.1.

RESULTS
Cell morphological features of the different LNCaP cells
The C-81 subgroup cells were established by continuous passaging of the C-33 LNCaP parental cells in RPMI 1640 medium. Compared to the C-33
parental cells, the cell body of the C-81 subgroup cells became smaller and more rounded (Figure 1). Even in the steroid-reduced conditions, the C-81 cells still survived and were able to be continuously passaged.

Iodide uptake studies in vitro
At each time point (2, 4 and 6 days) after adenovirus infection, the iodide uptake was measured (Table 1). Iodide accumulation was shown in the different LNCaP cell lines after Ad.PSMApro-hNIS or Ad.CMV-hNIS infection, but was not shown in the different LNCaP cell lines after Ad.CMV infection. At each time point, higher iodide uptake was shown in the C81 cells (FBS or CDS culture) infected with Ad.PSMApro-hNIS than in the C33 cells (P = 0.023). No difference was observed in the iodide uptake in the different LNCaP cell lines infected with Ad.CMV-hNIS (P = 0.762). The maximum iodide accumulation was shown at 4 days in the different LNCaP cells.

Iodide uptake studies in vivo
Six weeks following the subcutaneous injection of the C-81 cells, when the tumors had reached a tumor size of approximately 5 mm in diameter, an intratumoral injection of Ad.PSMApro-hNIS and Ad.CMV-hNIS or control virus was performed. Four days after the adenovirus injection with the maximum iodide accumulation, the mice were given 500 µCi of 131I by intraperitoneal injection. The 131I radiiodine uptake in the tumors, thyroid, liver and spleen is shown in (Table 2). There was a difference in the 131I radiiodine uptake in the tumors infected with Ad.PSMApro-hNIS, Ad.CMV-hNIS and control virus (P = 0.0075), but no difference was observed in the 131I radiiodine uptake in the thyroid (P = 0.892). There was no iodide accumulation in the liver or the spleen. The maximum reduction in the tumor volume occurred in the mice infected with Ad.PSMApro-hNIS, (Figure 2) and the maximum reduction in the tumor volume in the Ad.PSMApro-hNIS and Ad.CMV-hNIS groups was 60.4% and 41.4%, respectively (P = 0.0222).

DISCUSSION
hNIS can mediate the active transport of iodide in a variety of extrathyroidal tissues. We characterized hNIS as a novel therapeutic gene for potential use against cancers outside the thyroid gland, including breast cancer, melanoma tumors, cervical cancer, human glioma and hepatoma cell lines. As early as 2000, Spitzweg et al.14 reported radioiodine accumulation and therapeutic effectiveness of 131I in an NIS-transfected LNCaP cell line.

To avoid radiation exposure to additional organs, tissue-specific expression of hNIS is important. Thus, the strategy of transfecting hNIS DNA under control of the prostate-specific promoter was used. Prostate-specific antigen (PSA) promoter is the most common, but has limited packaging capacity of the adenoviral vectors.12 The surviving promoter and the probasin promoter are also recommended for driving prostate-specific expression of hNIS.16,17 Few reports have disclosed the introduction of such exogenous promoters in CRPC.

In contrast to the above promoters, the PSMA promoter is highly expressed in a variety of PC cell lines, including both androgen-dependent or castration-resistant lines. The PSMA gene maps to a region of chromosome 11p11–12, with the promoter region containing a 1244 nt fragment that consists of approximately 1 kb of the 5’ flanking sequence from the most proximal start site and 200 nt of the 5’ untranslated region (5’UTR). Wright et al.18 disclosed the upregulation of PSMA in LNCaP cells after androgen deprivation treatment. Our study also found upregulation of PSMA in the C81 cells cultured in CDS medium compared with the C33 cells cultured in FBS-containing medium (data not shown). The fine prostate specificity of PSMA has been demonstrated, and less expression of PSMA in other tissues such as the normal duodenum, kidney, salivary gland and brain has been reported.19-21 In this study, we also found a higher iodide accumulation in the LNCaP cells infected with Ad.PSMApro-hNIS than those infected with Ad.CMV-hNIS, which further shows the fine prostate specificity of the PSMA promotor.

In this study, the C33 cells were androgen-sensitive LNCaP cells, but became androgen-independent C81 cells after continuous passage over 80 generations. The C81 cells can survive in the presence or absence of androgen. The differential iodide accumulation in the different LNCaP cells after Ad.PSMApro-hNIS infection demonstrates that the PSMA promotor is a good driver of the expression of hNIS in androgen-independent PCa. In an in vivo animal model from this study, the intratumoral injection of Ad.PSMApro-hNIS can greatly reduce the volume of tumor arising from the androgen-independent C81 cells. The above result suggests the possibility of PSMA promoter-driven hNIS for targeted radioiodine therapy of CRPC. We did not further evaluate

![Figure 1: C-81 (a) cells (derived from C-33 parental cells) (b) had a smaller, rounded cell body. Scale bars = 10µm](image1)

![Figure 2: Four days following the intratumoral injection of Ad.PSMApro-hNIS and Ad.CMV-hNIS or control virus, five mice in each group were injected intraperitoneally with 500µCi 131I. Every week, the radioiodine uptake by the tumors was monitored and quantified by imaging with a γ camera, and the tumor volume fluctuation is presented as the mean ± standard deviation (s.d)](image2)

Table 1: Iodide uptake by different LNCaP cell lines after Ad.PSMApro-hNIS, Ad.CMV-hNIS or Ad.CMV infection

| Cell line | Ad. PSMApro-NIS | Ad. CMV-NIS | Ad. CMV |
|-----------|----------------|-------------|----------|
|           | 2 days | 4 days | 6 days  | 2 days | 4 days | 6 days  | 2 days | 4 days | 6 days  |
| C33       | 7135.6±524.9 | 9753.1±567.4 | 6435.6±403.0 | 5832.7±367.6 | 7878.7±453.2 | 4937.4±396.7 | 401.5±39.7 | 412.4±29.9 | 397.4±43.9 |
| C81       | 8337.5±296.6 | 12961.0±341.4 | 7203.8±367.3 | 5644.3±288.9 | 8255.6±243.3 | 4792.7±249.7 | 387.3±42.2 | 414.5±48.0 | 367.5±50.2 |
| C81(CDS)  | 8969.4±483.7 | 15377.0±410.3 | 8965.7±463.4 | 5537.4±342.7 | 8194.7±523.7 | 5084.3±357.0 | 381.7±50.2 | 397.4±39.8 | 408.6±47.8 |
| P         | 0.023 | 0.762 | 0.774 |
Table 2: Iodide uptake by tumors derived from C81 cells infected with Ad.PSMApro-hNIS, Ad.CMV-hNIS or Ad.CMV and iodide uptake by the thyroid, liver and spleen of castrated nude mice

|                  | Ad.PSMApro-hNIS (c.p.m. mg⁻¹) | Ad.CMV-hNIS (c.p.m. mg⁻¹) | Ad.CMV (c.p.m. mg⁻¹) | P   |
|------------------|-------------------------------|---------------------------|-----------------------|-----|
| **Tumor**        | 2846.03±1888.29               | 982.77±107.99             | 9.19±1.00             | 0.0075 |
| **Thyroid**      | 1407.67±143.66                | 1394.63±147.70            | 1501.33±147.07        | 0.892 |
| **Liver**        | 10.03±0.45                    | 8.03±0.78                 | 5.83±0.68             | 0.661 |
| **Spleen**       | 8.19±0.70                     | 11.80±0.72                | 7.78±0.42             | 0.595 |

PSMA promoter-driven hNIS for metastatic CRPC in this study. A recent study in a rat model showed that, instead of an intratumoral injection, intravenous administration is also safe and effective for transferring the hNIS gene to local and metastatic PC cells. The above study suggests the possibility of prostate-restricted hNIS gene transfer combined with ¹³¹I for treating metastatic CRPC in the clinic, although further work is needed.

In this study, the Ad.PSMApro-hNIS construct was able to cause the C81 cells to concentrate ¹³¹I by 2.9-fold over those infected with Ad.CMV-hNIS. This iodide concentrating ability exceeds that observed in normal thyroid cells and can explain the cytotoxic effect of radioactive iodine therapy in prostate tumors. High iodide uptake can cause potential damage to adjacent organs; however, in the in vivo animal model, no significant damage to the liver or the spleen was shown. Hypothyroidism is the most common complication of radioiodine therapy, but pretreating the patients with thyroid hormone might be advantageous because it would suppress the thyroid-stimulating hormone levels and thyroidal ¹³¹I uptake. Even in cases of hypothyroidism, the patients could be easily and inexpensively managed with thyroid hormone replacement therapy.

hNIS has been demonstrated to be a potentially therapeutic gene for human PC. Six men had clinically localised PCa and received an intraprostatic injection of Ad5-γCD/mutTK (SR39) rep-hNIS under transrectal ultrasound guidance. On multiple days after the adenovirus injection, the hNIS gene expression was detected in the prostate of all the men (100%). On average, the gene was expressed throughout 45% (range 18%-83%) of the prostate volume. Although hNIS transfer as a targeted radioiodine therapy is only introduced for local PCa and no prostate-specific promoter has been used in hNIS transfer, the encouraging clinical outcomes show the promising future for the application of PSA marker-driven hNIS transfer for treating CRPC. In summary, transfer of the Ad.PSMApro-hNIS can result in high levels of hNIS expression in androgen-independent LNCaP cells. Because of its tissue specificity, Ad.PSMApro-hNIS is capable of limiting the radioactive iodine accumulation in the prostate tissue, thereby minimizing extratumoral cytotoxicity. When combined with ¹³¹I radioiodine therapy, Ad.PSMApro-hNIS provides a potential therapy for CRPC.

AUTHOR CONTRIBUTIONS

XFG participated in conceiving of the study and drafted the manuscript. GHC conducted the cell culture and animal model experiments and was involved in drafting the manuscript. TZ was responsible for drafting the manuscript, study design and statistics. CLX participated in the design of the study. YLD carried out the gene delivery, DNA and cDNA cloning, recombinant adenovirus production, and participated in drafting the manuscript. YHS conceived of the study and participated in its design and coordination and revised it critically for important intellectual content. All of the authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare that there are no competing interests.

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