Targeted Radioiodine Therapy of Neuroblastoma Tumors following Systemic Nonviral Delivery of the Sodium Iodide Symporter Gene

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Abstract Purpose: We recently reported the significant therapeutic efficacy of radioiodine therapy in various tumor mouse models following transcriptionally targeted sodium iodide symporter (NIS) gene transfer. These studies showed the high potential of NIS as a novel diagnostic and therapeutic gene for the treatment of extrathyroidal tumors. As a next crucial step towards clinical application of NIS-mediated radionuclide therapy we aim at systemic delivery of the NIS gene to target extrathyroidal tumors even in the metastatic stage.

Experimental Design: In the current study, we used synthetic polymeric vectors based on pseudodendritic oligoamines with high intrinsic tumor affinity (G2-HD-OEI) to target a NIS-expressing plasmid (CMV-NIS-pcDNA3) to neuroblastoma (Neuro2A) cells.

Results: Incubation with NIS-containing polyplexes (G2-HD-OEI/NIS) resulted in a 51-fold increase in perchlorate-sensitive iodide uptake activity in Neuro2A cells in vitro. Through 123I-scintigraphy and ex vivo gamma counting Neuro2A tumors in syngeneic A/J mice were shown to accumulate 8% to 13% ID/g with a biological half-life of 13 hours, resulting in a tumor-absorbed dose of 247 mGy/MBq 131I after i.v. application of G2-HD-OEI/NIS. Nontarget organs, including liver, lung, kidneys, and spleen revealed no significant iodide uptake. Moreover, two cycles of systemic NIS gene transfer followed by 131I application (55.5 MBq) resulted in a significant delay in tumor growth associated with markedly improved survival.

Conclusions: In conclusion, our data clearly show the high potential of novel pseudodendritic polymers for tumor-specific NIS gene delivery after systemic application, opening the prospect of targeted NIS-mediated radionuclide therapy of nonthyroidal tumors even in metastatic disease. (Clin Cancer Res 2009;15(19):6079–86)

The exact mechanism by which iodide is actively transported across the basolateral membrane of thyroid follicular cells was clarified by the cloning and characterization of the sodium iodide symporter (NIS) 13 years ago (1–3). NIS, an intrinsic transmembrane glycoprotein with 13 putative transmembrane domains, is responsible for the ability of the thyroid gland to concentrate iodide, the first and rate-limiting step in the process of thyroid hormonogenesis (4, 5). Moreover, due to its expression in follicular cell–derived thyroid cancer cells, NIS provides the molecular basis for the diagnostic and therapeutic application of radioiodine, which has been successfully used for more than 60 years in the treatment of thyroid cancer patients and therefore represents the most effective form of systemic anticancer radiotherapy available to the clinician today (6). Since its cloning in 1996 NIS has been identified and characterized as a novel promising target gene for the treatment of extrathyroidal tumors following selective NIS gene transfer into tumor cells that allows diagnostic and therapeutic application of radioiodine and alternative radionuclides, such as 188Re and 211At (6–9). We have proven the feasibility of extrathyroidal radioiodine therapy after induction of iodide uptake by ex vivo
Translational Relevance

Based on the effective administration of radioiodine in the management of thyroid cancer, cloning of the sodium iodide symporter (NIS) has paved the way for the development of a novel gene therapy strategy based on targeted NIS expression in cancer cells followed by therapeutic application of $^{131}$I. Our pioneer studies have convincingly shown the oncology communities the enormous potential of NIS as a novel reporter and therapy gene, and allowed the approval of a first phase I clinical trial for radioiodine therapy of prostate cancer after local adenoviral NIS gene transfer. The next crucial step towards clinical application in metastatic cancer has to be the evaluation of gene transfer methods that have the potential to achieve sufficient tumor-selective transgene expression levels after systemic application.

The present report is the first preclinical study to convincingly show the high potential of polycations based on polypropyleneimine dendrimers for tumor-specific delivery of the NIS gene after systemic application, resulting in a significant therapeutic effect of $^{131}$I in a neuroblastoma mouse model. This translational study therefore opens the exciting prospect of NIS-targeted radionuclide imaging and therapy of metastatic cancer using polyplex-mediated systemic NIS gene delivery.

stable NIS transfection or local adenoviral NIS gene transfer using tissue-specific promoters, such as the prostate-specific antigen promoter, a fetoprotein promoter, carcinoembryonic antigen promoter, and the calcitonin promoter to specifically target NIS expression to prostate, liver, colon, and medullary thyroid cancer cells, respectively (10–17). Further, the cloning of NIS has provided us not only with a powerful therapeutic gene, but also with one of the most promising reporter genes available today, which allows direct, noninvasive imaging of functional NIS expression by $^{123}$I-scintigraphy and $^{124}$I-PET-imaging, as well as exact dosimetric calculations before proceeding to therapeutic application of $^{131}$I (7, 18). Therefore, in its role as reporter gene NIS provides a direct way to monitor the in vivo distribution of viral and nonviral vectors, as well as the biodistribution, level, and duration of transgene expression – all critical elements in the design of clinical gene therapy trials (7, 18).

As a logical consequence of our pioneer studies in the NIS gene therapy field, the next crucial step towards clinical application of the promising NIS gene therapy concept has to be the evaluation of gene transfer methods that have the potential to achieve sufficient tumor-selective transgene expression levels, not only after local or regional but also after systemic application, to be able to reach tumor metastases.

Viral vectors are the most commonly used gene transfer systems employed in clinical trials due to their high potency in gene transfer (19). However, the limitations associated with viral vectors, including induction of immune and inflammatory responses, limited transgene loading size, potential toxicity and tumorigenicity, as well as high production costs have encouraged researchers to focus on alternative gene transfer vehicles.

Delivering genes to target organs with synthetic vectors is a vital alternative to virus-based methods. For systemic delivery, polycationic molecules are used to condense DNA into submicrometer particles termed polyplexes, which are efficiently internalized into cells, while DNA is protected from nucleases. Several polycations, like polyethylenimine (PEI), bear an intrinsic endosomolytic mechanism that allows the transition of the polyplex from the endosome to the cytoplasm (20). Nonviral vectors can be easily synthesized and convince especially by their absent immunogenicity and enhanced biocompatibility. The “golden standard” of PEI-based gene carriers is LPEI, the linear form of polyethylenimine, with a molecular weight of 22 kDa, also known as the commercially available JetPEI. The major drawback of LPEI is its significant toxicity after systemic application due to acute and long-term toxic effects (21). Therefore, several biodegradable polymers were developed for gene transfer (22, 23), aiming to reduce the toxicity profile while maintaining high transduction efficiency comparable with the standard synthetic gene vectors. We have recently developed a series of biodegradable carriers based on low molecular weight polycations cross-linked either via ester or disulfide bonds (24–26), showing very promising toxicity profiles and similar or even superior transfection efficiency in comparison with LPEI (25). As a continuation of this work, a novel class of branched polycations was synthesized based on oligoethylenimine (OEI)-grafted polypropyleneimine dendrimers (G2-HD-OEI; ref. 26). Low toxicity in association with high transfection efficiency was observed in different tumor cell lines in vitro using these polymers. Moreover, polyplexes formed by these biodegradable polymers prevented aggregation with erythrocytes and toxic side effects after systemic administration in vivo. Transgene expression was almost exclusively detected intratumorally in tumor-bearing mice, whereas with polyplexes based on linear PEI transgene expression in lung was >100 times higher than in the tumor (25–27). Apparently, polyplexes based on branched polycations exhibit a high intrinsic tumor affinity, significantly improving tumor-specific targeting of transgene expression, one of the major hurdles of gene therapy. In this context, Dufes et al. showed that systemic injection of polypropyleneimine dendrimer nanoparticles containing a tumor necrosis factor-α expression plasmid regulated by telomerase gene promoter leads to tumor-specific transgene expression, resulting in tumor regression and improved survival in various tumor models (28).

In the current study, we applied the above described OEI-grafted polypropyleneimine dendrimers (G2-HD-OEI) as novel biodegradable and highly efficient nonviral gene delivery vehicles for systemic NIS gene transfer in a syngeneic neuroblastoma mouse model. Based on its dual function as reporter and therapy gene, NIS was used for noninvasive imaging of vector biodistribution by $^{125}$I-scintigraphy followed by assessment of the therapy response after application of $^{131}$I.

Materials and Methods

Cell culture. The murine neuroblastoma (Neuro2A) cell line (ATCC-CCL-131) was cultured in DMEM (1 g/L glucose) supplemented with 10% fetal bovine serum (v/v; PAA) and 1% penicillin/streptomycin. Cells were maintained at 37°C and 5% CO2 in an incubator with
95% humidity. The cell culture medium was replaced every second day and cells were passaged at 85% confluency.

**Plasmids and polycation.** The expression vector CMV-NIS-pcDNA3 (pCMV-NIS) containing the full-length NIS cDNA coupled to the cytomegalovirus (CMV) promoter was kindly provided by Dr. S.M. Jhiang, Ohio State University, Columbus, OH. As control, NIS cDNA was removed using EcoRI and religated into the same expression vector in antisense direction (pCMV-antisense-NIS).

G2-HD-OEI was synthesized as described previously (26) and used as a 5 mg/ml stock solution.

**Polyplex formation.** Plasmid DNA was condensed with polymers at indicated conjugate/plasmid (c/p) - ratios (w/w) in HEPES buffered glucose [20 mmol/L HEPES, 5% glucose (w/v), pH 7.4] as described (26) and incubated at room temperature for 20 min prior to use. Final DNA concentration of polyplexes was 4 μg/ml for in vitro studies and 200 μg/ml for in vivo studies.

**Transient transfection.** For in vitro transfection experiments, Neuro2A cells were grown to 60% to 80% confluency. Cells were incubated for 4 h with polyplexes in the absence of serum and antibiotics followed by incubation with growth medium for 24 h. Transfection efficiency was evaluated by measurement of iodide uptake activity as described below.

**125I uptake assay.** Following transfections, iodide uptake of Neuro2A cells was determined at steady-state conditions as described by Weiss et al. (29). Results were normalized to cell survival measured by cell viability assay (see below) and expressed as cpm/A 490 nm.

**Cell viability assay.** Cell viability was measured using the commercially available MTS assay (Promega Corp.) according to the manufacturer’s recommendations as described previously (30).

**Establishment of Neuro2A tumors.** Neuro2A tumors were established in syngeneic male A/J mice (Harlan Winkelmann) by s.c. injection of 1 × 10⁶ Neuro2A cells suspended in 100 μl PBS into the flank region. The animals were maintained under specific pathogen-free conditions with access to mouse chow and water ad libitum. The experimental protocol was approved by the regional governmental commission for animals (Regierung von Oberbayern).

**NIS gene transfer and radioiodide biodistribution studies in vivo.** Experiments started when tumors had reached a tumor size of 8 to 10 mm after a 10-d pretreatment with i.p. injection of 2 μg L-T4/d (Henning, Sanofi-Aventis), diluted in 100 μl PBS, to suppress thyroidal iodine uptake. For systemic in vivo gene transfer, polyplexes (c/p 2) were applied via the tail vein at a DNA dose of 2.5 mg/kg (i.e., for a 20-g mouse, 250 μl polyplex in HEPES buffered glucose at 200 μg/ml DNA); these were either NIS-containing polyplexes (G2-HD-OEI/NIS) or polyplexes with the control vector (G2-HD-OEI/antisense-NIS). Two groups of mice were established and treated as follows: (a) i.v. injection of G2-HD-OEI/NIS (n = 6); (b) i.v. injection of G2-HD-OEI/antisense-NIS (control vector; n = 6). As an additional control, in a subset of mice treated with G2-HD-OEI/NIS (n = 9) the specific NIS-inhibitor sodium-perchlorate (NaClO₄ 2 mg/per mouse) was injected i.p. 30 min prior to ¹²⁵I administration. Twenty-four hours after polyplex application, the mice were injected i.p. with 18.5 MBq (0.5 μCi) ¹²⁵I, and iodide biodistribution was assessed using a gamma camera equipped with UXHR collimator (Ecami, Siemens) as described previously (8, 9). Regions of interest were quantified and expressed as a fraction of the total amount of applied radionuclide per gram tumor tissue. The retention time within the tumor was determined by serial scanning after radionuclide injection, and dosimetric calculations were done according to the concept of medical internal radiation dose, with the dosis factor of RADAR-group (www.doseinfo-radar.com).

**Analysis of radioiodide biodistribution ex vivo.** For ex vivo biodistribution studies, mice were injected with G2-HD-OEI/NIS (n = 9) or G2-HD-OEI/antisense-NIS (n = 9) as described above followed by i.p. injection of 18.5 MBq ¹²⁵I 24 h later. A subset of NIS-transduced mice (n = 9) was treated with sodium-perchlorate prior to ¹²⁵I administration as an additional control. Two, six, and twelve hours after ¹²³I injection, the mice were sacrificed and organs of interest were dissected and weighed, and radioiodide uptake was measured in a gamma counter, with five NIS-transduced animals per time point (G2-HD-OEI/NIS) and three mice of each control. Results were reported as percentage of injected dose per organ (% ID/organ).

**Analysis of NIS mRNA expression using quantitative real-time PCR.** Total RNA was isolated from Neuro2A tumors or other tissues using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s recommendations. Single-stranded oligo (dT)-primer cDNA was generated using Superscript III Reverse Transcriptase (Invitrogen). The following primers were used: hNIS: (5′-TGGCGGACTTGGCAGTACATT-3′) and (5′-TGCGAGATAATTCCGGTGGAACA-3′), GAPDH: (5′-GAGAAGGCCTGGGCTCATT-3′) and (5′-CAGTGGG-GACACGGAAAGG-3′). Quantitative real-time PCR (qPCR) was done with the cDNA from 1 μg RNA using the SYBR green PCR master mix (Quiagen) in a Rotor Gene 6000 (Corbett Research). Relative expression levels were calculated using the comparative ΔΔCt method and internal GAPDH for normalization.

**Immunohistochemical analysis of NIS protein expression.** Immunohistochemical staining of frozen tissue sections derived from Neuro2A tumors after systemic gene delivery was done as described previously (13).

**Radioiodine therapy study in vivo.** Following a 10-d L-T4 pretreatment as described above, two groups of mice were established receiving 55.5 MBq ¹³¹I as a single i.p. injection 24 h after systemic application of G2-HD-OEI/NIS (n = 6) or G2-HD-OEI/antisense-NIS (n = 6), respectively. As control, two further groups of mice were treated with saline instead of ¹³¹I after injection of either G2-HD-OEI/NIS (n = 6) or G2-HD-OEI/antisense-NIS (n = 6). A further control group was injected with saline only (n = 6). The treatment consisting of systemic polyplex application followed by ¹³¹I or saline application after 24 h was repeated once on days 3 and 4, respectively. Tumor sizes were measured before treatment and daily thereafter for up to 4 wk. Tumor volume was estimated using the equation: tumor volume = length × width × height / 0.52. Experiments were repeated twice, and tumor volumes are expressed as means of 12 mice per group.

**Indirect immunofluorescence assay.** Indirect immunofluorescence was done on frozen sections as described previously (8).

**Statistical methods.** All in vitro experiments were carried out in triplicates. Results are represented as mean ± SD of triplicates. Statistical significance was tested using Student’s t test.
Results

Iodide uptake studies in vitro. Transfection conditions using G2-HD-OEI/NIS were optimized in Neuro2A cells by measurement of perchlorate-sensitive iodide uptake activity 24 hours following polyplex application (data not shown). We found an optimal c/p ratio of 2, which resulted in highest transfection efficiency at low cytotoxicity. This ratio was used in all subsequent experiments. Twenty-four hours after transfection with G2-HD-OEI/NIS, Neuro2A cells showed a 51-fold increase in 125I accumulation as compared with cells incubated with empty G2-HD-OEI (Fig. 1). Furthermore, no perchlorate-sensitive iodide uptake above background level was observed in cells transfected with the control vector G2-HD-OEI/antisense-NIS. Polyplex-mediated NIS gene transfer did not alter cell viability as measured by MTS assay (Fig. 1).

In vivo radioiodine biodistribution studies. To investigate the iodide uptake activity in Neuro2A tumors after systemic in vivo NIS gene transfer, 125I biodistribution analysis confirmed significant iodide uptake in tumors following systemic NIS gene transfer (Fig. 3). Although NIS-transduced Neuro2A tumors accumulated 6% to 8% ID/organ accumulation of 8% to 13% ID/g in NIS-transduced tumors with a biological half-life of 13 hours. Considering a tumor mass of 1 g and an effective half-life of 12 hours for 131I, a tumor-absorbed dose of 247 ± 94 mGy/MBq 131I was calculated (Fig. 2D). To confirm that tumoral iodide uptake was indeed NIS-mediated, a subset of G2-HD-OEI/NIS injected mice (n = 9) received sodium-perchlorate 30 minutes prior to 131I administration. In all experiments a single injection of 2 mg sodium-perchlorate completely blocked tumoral iodide accumulation in addition to abolishing physiologic iodide uptake in stomach and thyroid gland (Fig. 2B). Moreover, no significant iodide uptake was observed in nontarget organs, including lung, liver, kidneys, or spleen, which confirms the tumor-specificity of nanoparticle-mediated NIS gene delivery.

Ex vivo radioiodine biodistribution studies. Ex vivo biodistribution analysis confirmed significant iodide uptake in tumors following systemic NIS gene transfer (Fig. 3). Although NIS-transduced Neuro2A tumors accumulated 6% to 8% ID/organ accumulation of 8% to 13% ID/g in NIS-transduced tumors with a biological half-life of 13 hours. Considering a tumor mass of 1 g and an effective half-life of 12 hours for 131I, a tumor-absorbed dose of 247 ± 94 mGy/MBq 131I was calculated (Fig. 2D). To confirm that tumoral iodide uptake was indeed NIS-mediated, a subset of G2-HD-OEI/NIS injected mice (n = 9) received sodium-perchlorate 30 minutes prior to 131I administration. In all experiments a single injection of 2 mg sodium-perchlorate completely blocked tumoral iodide accumulation in addition to abolishing physiologic iodide uptake in stomach and thyroid gland (Fig. 2B). Moreover, no significant iodide uptake was observed in nontarget organs, including lung, liver, kidneys, or spleen, which confirms the tumor-specificity of nanoparticle-mediated NIS gene delivery.

Fig. 2. 123I gamma camera imaging of mice harboring Neuro2A tumors 4 h following i.p. injection of 18.5 MBq 123I after G2-HD-OEI-mediated NIS gene delivery. Treatment with G2-HD-OEI/NIS induced significant tumor-specific iodide accumulation in Neuro2A tumors with accumulation of 8% to 13% ID/g (A), which was completely abolished upon pretreatment with NaClO4 (B). Mice treated with control vectors (G2-HD-OEI/antisense-NIS) showed no tumoral iodide uptake (C). Iodide was also accumulated physiologically in thyroid, stomach, and bladder (A and C). D, time course of 123I accumulation in Neuro2A tumors after systemic polyplex-mediated NIS gene delivery followed by injection of 18.5 MBq 123I as determined by serial scanning. Maximum tumoral radioiodine uptake was 8% to 13% ID/g tumor with an average effective T1/2 of 12 h for 123I.

Fig. 3. Evaluation of iodide biodistribution ex vivo 2, 6, and 12 h following injection of 18.5 MBq 125I. Although tumors in NIS-transduced mice showed high perchlorate-sensitive iodide uptake activity (up to 6-8% ID/organ), nontarget organs revealed no significant iodide accumulation. No iodide accumulation was measured after injection of control vector. Results were reported as percent of injected dose per organ ± SD.
**Analysis of NIS mRNA expression by qPCR analysis.** In order to assess NIS mRNA expression after systemic NIS gene transfer, mRNA of various tissues was extracted and analyzed by qPCR with a pair of NIS-specific oligonucleotide primers 24 hours after NIS gene transfer. Only a low background level of NIS mRNA expression was detected in untreated tumors or tumors after application of G2-HD-OEI/antisense-NIS. In contrast, a significant level of NIS gene expression was induced in Neuro2A tumors after systemic injection of G2-HD-OEI/NIS (Fig. 4A). As expected, administration of the competitive NIS inhibitor sodium-perchlorate had no influence on NIS mRNA expression in NIS-transduced tumors. Furthermore, no significant NIS mRNA expression above background level was detected in nontarget organs, like liver and lung, after systemic application of G2-HD-OEI/NIS or G2-HD-OEI/antisense-NIS (Fig. 4A).

**Radioiodine therapy studies after in vivo NIS gene transfer.** Twenty-four hours after systemic administration of G2-HD-OEI/NIS or G2-HD-OEI/antisense-NIS polyplexes, a therapeutic dose of 55.5 MBq (1.5 mCi) $^{131}$I or saline was injected i.p. This cycle consisting of systemic NIS gene transfer followed by radioiodine or saline administration was repeated once on day 3 and 4 (Fig. 5A). As an additional control, tumor growth of mice injected with saline only was assessed ($n=6$). Mice treated with G2-HD-OEI/NIS or G2-HD-OEI/antisense-NIS followed by application of saline and mice treated with G2-HD-OEI/antisense-NIS followed by application of $^{131}$I as well as saline-treated mice showed an exponential tumor growth. In contrast, NIS-transduced (G2-HD-OEI/NIS) and $^{131}$I-treated tumors showed a significant delay in tumor growth (Fig. 5A). Although all mice in the control groups had to be killed within 2 weeks after the onset of the experiments due to excessive tumor growth, 70% of the mice treated with $^{131}$I after injection showed no significant iodide uptake. In both groups the thyroid gland and the stomach accumulated approximately 40% and 39% ID/organ (data not shown). It is worth noting that the average tumor weight in this experiment was approximately 0.7 g. Further, a single perchlorate injection prior to radioiodine application significantly blocked iodide uptake in NIS-transduced tumors and in physiologically NIS-expressing tissues, including thyroid and stomach, throughout the observation period up to 12 hours. In addition, no significant iodide uptake above background levels was observed in nontarget organs, including lung, liver, kidneys, or spleen, confirming tumor-specific NIS gene delivery (see also Fig. 2A).

$^{131}$I 2 hours after radioiodine injection, mock-transduced tumors showed no significant iodide uptake. In both groups the thyroid gland and the stomach accumulated approximately 40% and 39% ID/organ (data not shown). It is worth noting that the average tumor weight in this experiment was approximately 0.7 g. Further, a single perchlorate injection prior to radioiodine application significantly blocked iodide uptake in NIS-transduced tumors and in physiologically NIS-expressing tissues, including thyroid and stomach, throughout the observation period up to 12 hours. In addition, no significant iodide uptake above background levels was observed in nontarget organs, including lung, liver, kidneys, or spleen, confirming tumor-specific NIS gene delivery (see also Fig. 2A).
of G2-HD-OEI/NIS survived approximately 4 weeks (Fig. 5B). Importantly, none of these mice showed major adverse effects of radionuclide or polyplex treatment in terms of lethargy or respiratory failure. However, a minor body weight loss of 3% to 5% was observed in mice after systemic administration of polyplexes.

**Immunofluorescence analysis.** Three to four weeks after treatment, the mice were sacrificed, and tumors were dissected and processed for immunofluorescence analysis. Immunofluorescence analysis using a Ki67-specific antibody (green) and an antibody against CD31 (red, labeling blood vessels) showed striking differences between NIS-transduced (Fig. 6A) and mock-transduced tumors (Fig. 6B). As compared with mock-transduced tumors (G2-HD-OEI/antisense-NIS), NIS-transduced tumors (G2-HD-OEI/NIS) exhibited a significantly lower intratumoral blood vessel density and proliferation index after 131I therapy.

**Discussion**

The cloning and characterization of NIS, as one of the oldest and most successful targets of molecular imaging and therapy, has provided us with a powerful new reporter and therapy gene that allowed the development of a promising cytoreductive gene therapy strategy based on NIS gene transfer in extrathyroidal tumors followed by targeted radionuclide therapy (6).

Many of the characteristics of NIS, which have been confirmed by our work to date, suggest that it represents an ideal therapy gene due to several advantages. NIS as a normal human gene and protein implies that its expression in cancer cells is unlikely to be toxic or to elicit a significant immune response that could limit its efficacy. In addition, NIS gene therapy is associated with a substantial bystander effect based on the crossfire effect of the β-emitter 131I with a path length of up to 2.4 mm. A bystander effect is desirable for any kind of gene therapy strategy, because it reduces the level of transduction efficiency required for a therapeutic response (18). In its dual role as reporter and therapy gene, NIS allows direct, noninvasive imaging of functional NIS expression by 123I-scintigraphy and 124I-PET-imaging, as well as exact dosimetric calculations before proceeding to therapeutic application of 131I (7, 18). Moreover, NIS is already being used clinically as a molecular basis of 131I therapy, an already approved anticaner therapy in thyroid cancer with a well-understood therapeutic window and safety profile (6).

The capacity of the NIS gene to induce radiiodine accumulation in nonthyroidal tumors has been investigated in a variety of tumor models by several groups including our own (6–17, 31). In our initial studies in the prostate cancer model, we used the prostate-specific antigen promoter to achieve prostate-specific iodide accumulation, which resulted in a significant therapeutic effect after application of 131I and alternative radionuclides such as 188Re and 211At even in the absence of iodide organification (8, 9, 15, 16, 32, 33). Taken together, our pioneer work in the prostate cancer model and consecutive work in other tumor models, such as medullary thyroid, colon, and hepatocellular cancer (10, 12, 13, 17), have convincingly shown the enormous potential of NIS as a novel reporter and therapy gene. Based on our promising preliminary work and the proof-of-principle of tumor-specific NIS gene therapy in prostate cancer, including extensive toxicity studies, a first phase I clinical trial was approved at the Mayo Clinic for radiodine therapy of locally recurrent prostate cancer after local adenoviral NIS gene transfer (34). One of the major hurdles on the way to efficient and safe application of the NIS gene therapy concept in the clinical setting, particularly in metastatic disease, is optimal tumor-specific targeting in the presence of low toxicity and high transduction efficiency of gene delivery vectors, with the ultimate goal of systemic vector application. Only a limited number of studies have investigated systemic NIS gene delivery approaches with the aim of NIS-targeted radionuclide therapy of metastatic disease. An oncolytic measles virus encoding human NIS was applied systemically in a multiple myeloma mouse model and allowed to enhance the oncolytic potency of the virus after 131I application (35). In a more recent study, an oncolytic vesicular stomatitis virus was designed to express NIS to be able to monitor virus replication by 123I scintigraphic imaging in addition to stimulation of the oncolytic potency by the combination with 131I therapy, which was successfully investigated in a multiple myeloma mouse model after systemic vesicular stomatitis virus application (36).

In the current study we utilized a nonviral gene delivery system for tumor-targeted NIS gene transfer in the neuroblastoma mouse model Neuro2A. The syngeneic Neuro2A mice model develops well-vascularized tumors with leaky vasculature, thereby allowing intratumoral accumulation of polyplexes with subsequent diffusion from the blood vessel into the tumor tissue (37). Similar effects were observed with hepatoma models.
like the human xenografts HepG2 and Huh7, whereas other xenograft models such as the A549 lung carcinoma model were less susceptible to polyplex-mediated gene delivery (37). Branched polycations based on OEI-grafted polypolylyamine dendrimers (G2-HD-OEI) have recently been characterized as biodegradable synthetic gene delivery vectors with high in vivo transduction efficiency and remarkable intrinsic tumor affinity in the presence of low toxicity (26). G2-HD-OEI complexed with the human NIS cDNA under the control of the unsppecific CMV promoter revealed high transfection efficiency in vitro resulting in a 51-fold increase in iodide uptake activity in Neuro2A cells at an optimal polymer to plasmid w/w ratio of 2 that provided highest transfection efficiency at low cytotoxicity. Following systemic application of NIS-conjugated G2-HD-OEI via the tail vein in vivo, 85% of Neuro2A tumors in a syngeneic mouse model showed tumor-specific 123I accumulation with approximately 8% to 13% ID/g, a biological half-life of 13 hours, and a calculated effective half-life of 12 hours for 131I. In contrast, mice pretreated with the competitive NIS-inhibitor sodium-perchlorate or mice injected with control vectors showed no tumor iodide uptake, confirming that the observed radiiodine accumulation in the tumors was mediated by functional NIS expression. These data are consistent with a recently published study by Chisholm et al. showing tumor-specific targeting of NIS in various xenograft tumor mouse models by nano–single-photon emission computed tomography/computer tomography imaging of radioiodine biodistribution using polypropyleneimine dendrimers for systemic NIS gene delivery (38).

In addition, in our study in vivo 123I scintigraphic imaging studies were confirmed by ex vivo biodistribution experiments revealing significant tumoral radioiodine accumulation, whereas no iodide uptake was measured in nontarget organs like lung, liver, spleen, or kidneys. Tumoral NIS expression was further confirmed by real time qPCR as well as NIS-specific immunoreactivity, which was primarily membrane-associated and occurred in clusters. The patchy staining pattern nicely correlates with experiments using PEI-based polyplexes carrying the β-galactosidase reporter gene, in which a heterogeneous and patchy distribution of transgene activity in transduced tumors was observed (39). These data are also consistent with previously reported studies using luciferase as reporter gene for the evaluation of transduction efficiency and tumor specificity of various olioethyleneimine acrylate ester-based pseudo-dendrimers including G2-HD-OEI. After systemic polyplex application high luciferase activity was found selectively in tumor tissue, whereas no significant expression was detected in nontarget organs concomitant with absent or low toxicity (25, 26). In contrast, when standard LPEI were used as gene delivery vectors, high luciferase activity was observed in the lung, and acute or long-term toxic effects were observed (21, 25, 40). In our study, even after repeated injection of G2-HD-OEI followed by administration of 131I no major side effects occurred. Data from previous studies suggest that polyplexes formed with branched structures like G2-HD-OEI do not show pronounced aggregation with erythrocytes that usually results in high transgene expression in the first vascular bed encountered, namely the lung. Consequently, such polymers are able to deliver the nucleic acid payload toward the tumor site, most probably due to passive tumor targeting, that occurs due to the imperfect and leaky tumor vasculature combined with an inadequate lymphatic drainage (41).

In our mouse studies 123I uptake of 40% ID was detected in the thyroid gland and 39% ID in the stomach 2 hours after 123I injection, resulting from endogenous NIS expression in thyroid and stomach, which has been described by several groups including our own (7). In the current study, however, 123I uptake in the stomach was significantly higher than that expected in humans, which may be the result of increased NIS expression in the murine gastric mucosa and may also be caused by pooling of gastric juices as mice were anesthetized for prolonged period for serial imaging. In addition, due to exquisite regulation of thyroidal NIS expression by TSH, 123I accumulation in the thyroid gland can effectively be downregulated by thyroid hormone treatment as shown in humans (42).

Most importantly, systemic polyplex-mediated NIS gene transfer resulted in tumor-specific iodide uptake activity in Neuro2A tumor-bearing mice, which was sufficiently high for a significant therapeutic effect of 131I. After two cycles of systemic polyplex application followed by 131I injection, tumor-bearing mice showed a significant delay of tumor growth associated with a significantly prolonged survival. In addition, immunofluorescence analysis showed markedly reduced proliferation associated with decreased blood vessel density inside and surrounding the tumor after systemic polyplex-mediated NIS gene transfer followed by 131I application, suggesting radiation-induced tumor stroma cell damage in addition to tumor cell death. The crossfire effect of 131I with a maximum path length of up to 2.4 mm might be responsible for stromal cell damage leading to reduced angiogenesis and secretion of growth-stimulatory factors, thereby enhancing therapeutic efficacy.

Following polyplex-mediated systemic NIS gene delivery, therapeutic efficacy of NIS-targeted radionuclide therapy could be further stimulated by application of alternative radionuclides, such as the β-emitter 188Re or the α-emitter 211At. Both are known to be also transported by NIS, but offer the possibility of higher energy deposition in a shorter time period due to their higher energy and shorter half-lives. This has convincingly been shown by several groups, including our own studies in the prostate cancer model described above (8, 9, 43, 44). In view of the patchy and heterogeneous expression pattern of NIS protein expression after polyplex-mediated systemic NIS gene transfer, 188Re might be a promising alternative radionuclide due to the longer path length of the β particles (mean range, 3.1 mm; maximum range 10.4 mm) and therefore superior crossfire effect, which will be addressed in future studies.

Moreover, tumor-specific targeting could be enhanced by coupling of tumor-targeting ligands, such as the serum glycoprotein transferrin (Tf) or epidermal growth factor (45, 46), and further optimized by application of tumor-specific promoters as shown in our earlier work (10–13, 15–17).

In conclusion, our data clearly show the high potential of branched polycations based on OEI-grafted polypolylyamine dendrimers for tumor-specific delivery of the NIS gene after systemic application. Based on the role of NIS as a potent and well characterized reporter gene allowing noninvasive imaging of functional NIS expression by 123I-scintigraphy and 124I-PET imaging, this study allowed detailed characterization of in vivo biodistribution of polyplex-mediated functional NIS expression by gamma camera imaging, which is an essential prerequisite for exact planning and monitoring of clinical gene therapy trials with the aim of individualization of the NIS gene.
therapy concept in the clinical setting. Tumor-specific iodide accumulation was further shown to be sufficiently high for a significant delay of tumor growth associated with increased survival in syngeneic mice bearing neuroblastoma tumors after two cycles of NIS-polyplex application followed by 131I therapy. This study therefore opens the exciting prospect of NIS-targeted radionuclide therapy of metastatic cancer using polyplexes based on biodegradable polymers for systemic gene delivery.

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No potential conflicts of interest were disclosed.


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