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BRIEF REPORT

A Pilot Study of Combined Suicide/Cytokine Gene Therapy in Two Patients with End-Stage Anaplastic Thyroid Carcinoma

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This study represents the first report of gene therapy for anaplastic thyroid carcinoma, one of the most aggressive solid tumors in humans. Two patients with end-stage anaplastic thyroid carcinoma were treated by direct intratumor injection of retroviral vector producer cells followed by ganciclovir. The retroviral vector carried the human IL-2 gene and the suicide gene thymidine kinase of herpes simplex virus type 1. Treatment was safe and associated with only mild adverse events. Transduction of tumor cells and production of T helper type 1 cytokines was demonstrated in tumor biopsies. Gene therapy led also to a marked increase in T helper type 1 cytokine expression in peripheral blood mononuclear cells. Radiological evaluation of injected tumor masses demonstrated local tumor necrosis. (*J Clin Endocrinol Metab* 90: 2831–2834, 2005)

ANAPLASTIC THYROID CARCINOMA (ATC) remains one of the most aggressive and lethal solid tumors in humans (1). Because of the lack of an effective therapy, development of new therapeutic options, such as gene therapy, is needed (2, 3). After our clinical experience of polygene therapy in patients with recurrent glioblastoma multiforme based on combined, retroviral vector-mediated, delivery of a suicide gene (thymidine kinase of herpes simplex virus type 1, *HSV-TK*) and a cytokine gene (human IL-2, *hIL-2*) (4), we tested this gene therapy strategy in experimental models of thyroid cancer and demonstrated that retroviral vectors could efficiently transduce ATC cells and that delivery of *IL-2/HSV-TK* plus ganciclovir (GCV) eradicated tumor masses *in vivo* with a marked bystander effect (5, 6).

In this study we assessed the feasibility and safety of this gene therapy approach in two patients with end-stage ATC. ATC is a suitable target for gene therapy protocols based on direct intratumor delivery of retroviral vectors or retroviral vector-producing cells (RVPCs). The reasons are 2-fold: 1) the anatomical location of the tumor, which allows easy access; and 2) the high proliferation rate of neoplastic cells, which can be selectively transduced by retroviral vectors.

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Abbreviations: ATC, Anaplastic thyroid carcinoma; CT, computed tomography; GCV, ganciclovir; MRI, magnetic resonance imaging; PBMC, peripheral blood mononuclear cell; RCR, replication-competent retroviral particle; RVPC, retroviral vector-producing cell.

Patients and Methods

Patients

The clinical protocol was approved by the ethics committees of University of Padova and University of Ancona, Italy. Patients gave informed consent in accordance with the University of Ancona institutional review board using Declaration of Helsinki guidelines.

Patient 1 was a 65-yr-old female with a 30-yr history of nontoxic multinodular goiter, who in April 2001 showed a rapid enlargement of the left thyroid lobe due to a 6 × 5-cm cold mass and underwent left hemithyroidectomy. Histological examination showed ATC arising from papillary carcinoma (follicular variant) with extracapsular invasion and lymph node metastases. After palliative radiotherapy for local tumor recurrence and neck lymph node metastases, in November 2001, because of clinical worsening and increase in tumor mass size, the patient was admitted to University of Ancona, where she underwent tracheal resection and was submitted to gene therapy.

Patient 2 was a 67-yr-old female with a 30-yr history of nontoxic multinodular goiter, who in November 2001 showed a sudden increase in the right thyroid lobe and in January 2002 underwent total thyroidectomy and dissection of cervical right lymph nodes. Histological examination demonstrated a 3.5-cm nodule of ATC in the right lobe, showing invasion of extracapsular and prethyroid tissues and the right common carotid artery. A 1-cm nodule of papillary thyroid cancer was demonstrated in the left thyroid lobe. In April–May 2002, the patient underwent external radiotherapy and laser thermoablation of the tumor mass, but due to clinical worsening and increase in tumor mass, in July she was admitted to the Division of Endocrinology in Ancona, where she underwent gene therapy.

Retroviral vector and vector-producing cells

The RVPCs used in this study were derived from a single clone of the PA317 packaging cell line (ATCC CRL 9078, American Type Culture Collection, Manassas, VA), producing the LIL-2-TK retroviral vector at high titer (>1 × 10^6 colony-forming units/ml) (4). Construction and preclinical application of the LIL-2-TK vector, which expresses both *hIL-2* and *HSV-TK* therapeutic genes, have been previously reported (7). RVPCs for injection were grown in a biosafety level 3 Good Manufac-

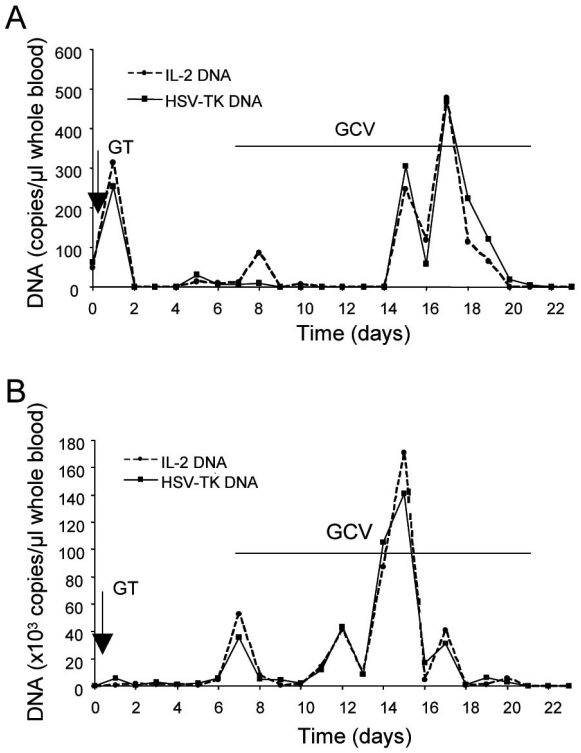

FIG. 1. Quantitative real-time PCR analysis of retroviral vector sequences in peripheral blood samples from patient 2 during the first (A) and the second (B) cycle of gene therapy. Day 0 is the day of RVPC injection. The *horizontal bar* represents the period of GCV treatment.

turing Practice facility and tested for viability, vector titer, and the presence of replication-competent retroviral particles (RCR) as previously reported (4).

Treatment protocol

The day before RVPC inoculation, patients underwent detailed mapping of the neck by magnetic resonance imaging (MRI) to identify suitable tumor targets for RVPC injection. The neoplastic mass was divided into four quadrants, and three or four RVPC inoculations were planned for each quadrant. Areas of solid tumor showing rapid growth and/or risk of infiltration of the vasculature or the trachea were preferentially chosen as targets for gene therapy. The procedure consisted of ultrasound-guided intratumor administration of about 10^9 RVPCs in a total volume of 6–10 ml, injected in 12–16 aliquots using a 19-gauge needle. Eight days after RVPC implantation, patients were treated by i.v. infusion of GCV (Cymevene, Recordati, Milan, Italy) at a dose of 5 mg/kg body weight over 1 h, twice daily for 14 d. Immediately before the beginning of GCV treatment, the site of RVPC implantation was biopsied by fine needle aspiration to ascertain transduction efficiency *in vivo*. Treatment with prednisone 25 mg/d, provided to both patients to prevent edema, was not discontinued during the gene therapy protocol.

Assessment of safety

Patients underwent daily clinical evaluation and standard laboratory analyses of blood, urine, and stools during hospitalization and at follow-up visits, scheduled at 15-d intervals. The rate and severity of side effects possibly related to gene therapy were recorded and scored according to the Common Toxicity Criteria published by NCI (http://ctep.cancer.gov/forms/CTCAEv3.pdf). Biosafety monitoring consisted of assays for the presence of RVPC and retroviral sequences in biological samples, including peripheral blood, urine, and stool specimens, and in cytological tumor samples. Detection of RCR was performed on d 7 after RVPC injection by a biochemical test for reverse transcriptase (8). The presence of vector DNA sequence and specific transcripts was assessed by a sensitive quantitative real-time direct and reverse PCR method, using oligonucleotide primers and probes specific for *HSV-1 TK*, *hIL-2*, *neo*, *gag*, and human and murine *GAPDH* genes.

Assessment of efficacy

The efficacy of treatment was assessed by clinical and radiological evaluation. Patients were studied by MRI or computed tomography (CT) scan the day before RVPC injection, after GCV treatment, and during follow-up.

Molecular assays of tumor biopsies and biological samples

Therapeutic gene expression was evaluated in tumor samples by real-time quantitative RT-PCR, using primers specific for *HSV-TK* and *hIL-2*, as reported above. Analysis of cytokine (*i.e. TNFα, IFNγ, IL-2, IL-1β*, and *IL-10*) gene expression in tumor biopsies and peripheral blood was performed by real-time quantitative RT-PCR using specific oligonucleotide primers and probes.

Results

Patient 1 received three consecutive cycles of gene therapy, whereas patient 2 received a less aggressive treatment, with two cycles of gene therapy spaced by a break of about 1 month. The duration of each gene therapy cycle, including GCV treatment, was about 21 d.

Treatment was well tolerated and associated only with grade 1 (mild) or 2 (moderate) adverse events, which were not attributed to RVPCs or to the injection procedure. Adverse events, which occurred during GCV treatment, included transient increases in creatinine and transaminase levels, anemia in both patients (patient 1 required

TABLE 1. Analysis of expression of therapeutic genes and cytokine genes by quantitative RT-PCR in tumor specimens obtained by fine needle aspiration from patients with ATC

Tumor samples[a]	*HSV-TK*[b]	*IL-2*[b]	*IFNγ*[b]	*TNFα*[b]	*IL-10*[b]	*IL-1β*[b]
Patient 1						
Before first RVPC injection	0	0	324 ± 48	45 ± 4	50 ± 7	9.1 ± 0.8
Before first GCV therapy	19,124 ± 875	11,450 ± 1,043	865 ± 63	71 ± 5	762 ± 57	19.4 ± 2.7
Before second GCV therapy	60,078 ± 1,204	45,574 ± 5,103	38,923 ± 1,776	10,923 ± 766	405 ± 31	10.5 ± 2.1
Not injected with RVPCs[c]	0	9 ± 3	89 ± 32	16 ± 2	24 ± 4	3.5 ± 0.4
Patient 2						
Before first RVPC injection	0	0	56 ± 19	13 ± 2	4 ± 0.6	1.6 ± 0.3
Before first GCV therapy	45,575 ± 6,346	48,058 ± 4,870	2,364 ± 541	188 ± 23	101 ± 9	13 ± 1.2
After first GCV therapy	128 ± 25	87 ± 23	348 ± 57	26 ± 3	16 ± 1.7	0.5 ± 0.1

[a] Cytological samples were obtained at different times during the gene therapy protocols.
[b] Data represent the mean ± SD of triplicate tests and are expressed as mRNA copies per micrograms of total RNA.
[c] Tumor sample collected after RVPC injection and before the first GCV therapy.

blood transfusion), and hyponatremia in patient 1. Both patients died because of disease progression, but not as a consequence of gene therapy, 6 and 61 d after the last RVPC injection, respectively. Autopsy was not performed because the patients' relatives did not gave their consent upon request.

No evidence of RCR was found in any of the tumor biopsies or biological samples obtained from patients. PCR analysis demonstrated the presence of proviral and RVPC DNA and the expression of transgenes (*i.e.* positive results from *HSV-TK*, human *IL-2*, *neo*, *gag*, and murine *GAPDH* DNA and RNA amplification) in peripheral blood obtained after RVPC injection. In particular, levels of DNA and transcripts markedly increased during the second week of GCV administration and decreased thereafter (Fig. 1). No retroviral vector sequences were detected in urine or stool samples.

Transduction of tumor cells was demonstrated by the presence of retroviral vector and RVPC DNA sequences and the expression of *HSV-TK* and *IL-2* transcripts in fine needle aspiration biopsy samples obtained before GCV administration (Table 1). Activation of an immune-inflammatory response was suggested by a marked increase in *TNFα*, *IFNγ*, *IL-2*, *IL-1β*, and *IL-10* mRNA levels in tumor specimens obtained after RVPC injection compared with those from biopsies performed before gene therapy. The presence of proviral DNA sequences and the expression of therapeutic transgenes were limited to the site of RVPC injection, because tumor biopsies performed at distant sites gave negative results (Table 1). A systemic cytokine response was also achieved, as indicated by a marked increase in *TNFα*, *IL-2*, *IL-10*, and *IL-1β* mRNA levels in peripheral blood mononuclear cells (PBMCs) obtained in the days immediately after RVPC injection. Cytokine levels in PBMCs were more elevated after the second cycle of gene therapy than after the first cycle.

Evaluation of tumor response to treatment by MRI and CT scan showed tumor necrosis and shrinkage at the site of RVPC injection after GCV treatment in both patients. This effect was more evident after the second cycle of gene therapy (Fig. 2).

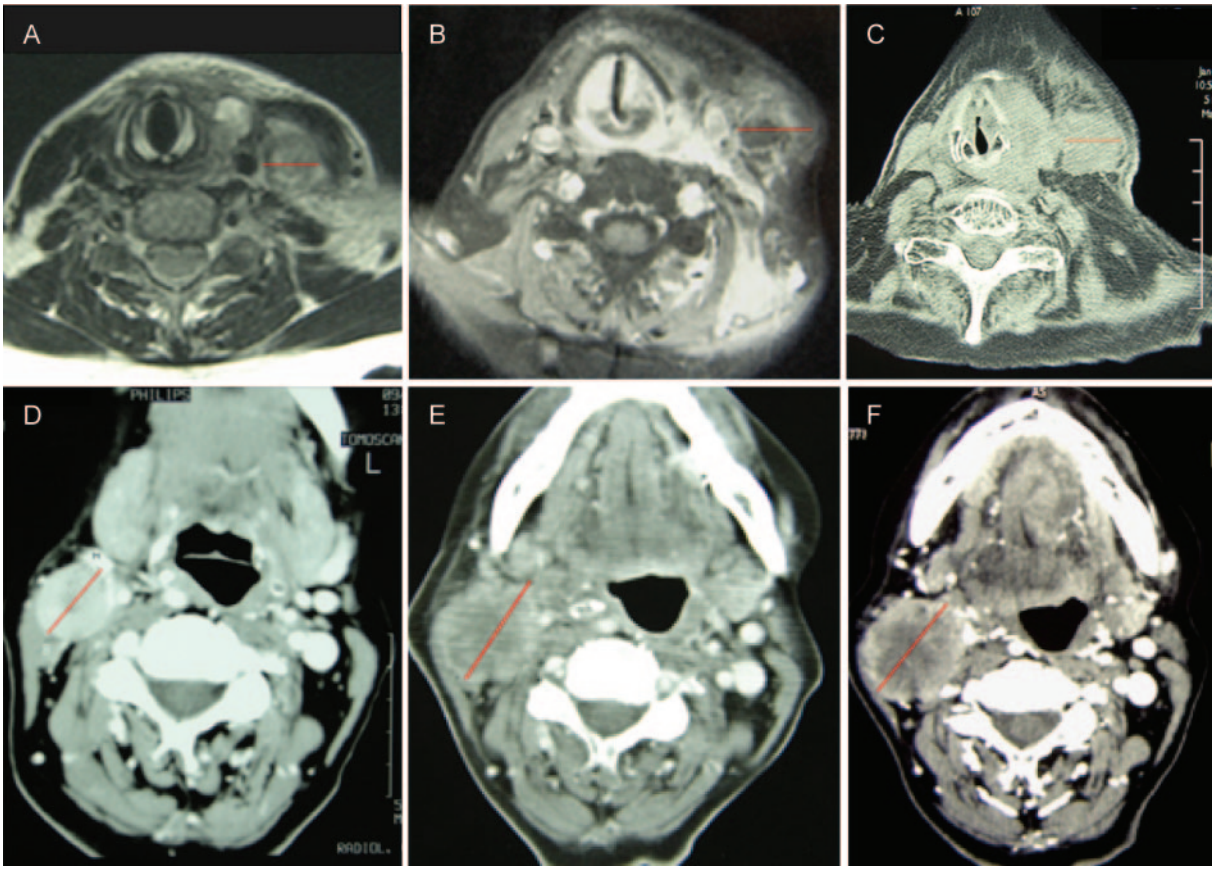

FIG. 2. Radiological evaluation of patients undergoing gene therapy. Patient 1 (A–C): In a neck MRI performed before gene therapy, the tumor appeared as a solid mass, which extended mainly in the left posterior cervical region, displaced the larynx and the trachea, and infiltrated C2–C4 cervical vertebrae. A nodule 2 cm in diameter, which was chosen for RVPC injection, is indicated (A). After the first cycle of gene therapy, an MRI scan demonstrated unchanged size, but more inhomogeneous intensity, of the tumor mass injected with RVPCs than in the previous MRI scan (B). A neck CT performed after the second cycle of gene therapy showed a slight reduction of the tumor mass to 1.5 cm in diameter (C). All images represent C6 cervical vertebrae level. Patient 2 (D and E): A neck CT scan performed before gene therapy showed the presence of 4.5-cm tumor mass surrounding and infiltrating the right carotid artery and cervical lymph node metastases of 1.5 and 2.5 in diameter, the largest of which had a marked contrast enhancement. The first cycle of gene therapy consisted of injection of RVPCs into the right cervical mass, 2.5 cm in diameter (D). A neck CT performed after the first cycle of gene therapy showed an increase in tumor mass to 4 cm (E). After the second cycle of gene therapy, a neck CT scan showed a tumor mass of 3.5 cm in diameter with signs of necrosis (F). However, the size and number of lung metastases increased.

Discussion

This is the first report of gene therapy in patients with thyroid cancer. In two patients with end-stage ATC, we demonstrated the feasibility and safety of direct intratumor injection of murine packaging cells producing a Moloney-derived retroviral vector that simultaneously expressed human *IL-2* and the suicide gene *HSV-TK*.

The tumor masses could be easily injected with RVPCs by the percutaneous route, even though, because of the large tumor bulks of our patients, only a small proportion of the tumor could be treated. The procedure was not associated with significant side effects, notwithstanding the high risk of tumor hemorrhage due to the rich tumor vasculature that characterizes ATC. Side effects, which included anemia and transient impairment of renal and liver function, were attributed to GCV administration in concomitance with steroids.

No RCRs were detected in the biological fluids recovered from the patients, confirming the safety of this type of vector. However, some concern derives from our finding of vector DNA and RNA sequences in PBMCs of both patients. Because RVPC sequences were also detected in the whole blood fraction, it is conceivable that the positive PCR signal not only derived from circulating lymphomonocytes that had been transduced while passing through the thyroid, but also from tumor cells and packaging cells killed by GCV and shedding into the bloodstream. If true, quantitative analysis of vector DNA in the whole peripheral blood fraction could therefore represent a useful tool to indirectly monitor the extent of tumor destruction by gene therapy.

Transduction of tumor cells was demonstrated by PCR amplification in fine needle aspiration biopsies performed just before GCV administration. The risk of tumor hemorrhage discouraged us to perform a tumor biopsy, so an *in situ* analysis of transduction efficiency could not be conducted.

Evidence of an immune-inflammatory response to RVPC administration was obtained indirectly by analysis of cytokine expression. A limitation of our results is the lack of data on the stimulation of specific CTLs against tumor antigens or RVPCs. Our study, however, showed in the tumor samples a marked increase in the expression of T helper type 1 cytokines, which could have been activated by the presence of xenoantigens (RVPCs, *HSV-TK*, and *neo*) and by local production of IL-2. A systemic activation of the immune system was also observed, as demonstrated by a peak of T helper type 1 cytokine transcript levels after RVPC injection. However, this systemic immune response was not able to control the disease in patient 2, who showed stabilization of the injected tumor lesion, but progression of distant metastases. This lack of efficacy of immuno-gene therapy could probably be accounted for by the loss of expression of thyroid- or tumor-specific antigens that characterizes undifferentiated thyroid carcinomas and ATC, as well as to the advanced stage of the disease. Immunotherapy could be more useful as an adjuvant treatment for differentiated thyroid carcinomas, which typically express tumor- or tissue-specific antigens and have a slow growth rate, but a tendency for metastatic spread. Quite interestingly, our patients did not show side effects related to the immune-inflammatory response, notwithstanding systemic overexpression of cytokines. Support therapy with steroids probably relieved this potential toxicity.

In conclusion, our study represents the first report of gene therapy for thyroid cancer. It demonstrates in two patients the safety and feasibility of retroviral vector-mediated delivery of a suicide and a cytokine gene. Treatment led to transduction of tumor cells and activation of a local and systemic immune-inflammatory response. Radiological examination demonstrated tumor necrosis and shrinkage at the site of RVPC injection. Our pilot study represents proof of the feasibility of this combined gene therapy approach. An additional improvement may derive from the use of vectors with higher titer, enhanced transduction efficiency of thyroid tumor cells, and vectors endowed with thyroid tumor-restricted oncolytic activity.

Acknowledgments

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