Cancer Gene Therapy (2008) 15, 676–684
 0929-1903/08 $30.00
www.nature.com/cgt

ORIGINAL ARTICLE

Phase I trial of DNA-hsp65 immunotherapy for advanced squamous cell carcinoma of the head and neck

P Michaluart[1,2,4], KA Abdallah[1,3,4,11], FD Lima[1,3], R Smith[1,2], RA Moysés[1,2], V Coelho[1,3,4], GD Victora[1,3], A Socorro-Silva[1,3], EC Volsi[1,3], CR Zárate-Bladés[1,5], AR Ferraz[1,2], AK Barreto[1,3], MC Chammas[1,6], R Gomes[1,6], E Gebrim[1,6], L Arakawa-Sugueno[1,2], KP Fernandes[1,2], PA Lotufo[1,7], MR Cardoso[1,8], J Kalil[1,3,4] and CL Silva[1,5,9,10]

[1]HSP65 Clinical Trial Group, University of São Paulo, São Paulo, Brazil; [2]Department of Head and Neck Surgery, University of São Paulo Medical School, São Paulo, Brazil; [3]Department of Immunology, Heart Institute (InCor), University of São Paulo Medical School, São Paulo, Brazil; [4]Institute for Investigation in Immunology—Millennium Institute, University of São Paulo, São Paulo, Brazil; [5]Department of Biochemistry and Immunology, The Centre for Tuberculosis Research, School of Medicine of Ribeirão Preto, University of São Paulo, São Paulo, Brazil; [6]Department of Radiology, University of São Paulo Medical School, São Paulo, Brazil; [7]Department of Medicine, University of São Paulo Medical School, São Paulo, Brazil; [8]Department of Epidemiology, Faculty of Public Health, University of São Paulo, São Paulo, Brazil; [9]Millennium Institute for Tuberculosis Research, University of São Paulo, São Paulo, Brazil and [10]Farmacore Biotechnology Ltd., Ribeirão Preto, São Paulo, Brazil

Considering that mycobacterial heat-shock protein 65 (hsp65) gene transfer can elicit a profound antitumoral effect, this study aimed to establish the safety, maximum-tolerated dose (MTD) and preliminary efficacy of DNA-hsp65 immunotherapy in patients with advanced head and neck squamous cell carcinoma (HNSCC). For this purpose, 21 patients with unresectable and recurrent HNSCC were studied. Each patient received three ultrasound-guided injections at 21-day intervals of: 150, 600 or 400 μg of DNA-hsp65. Toxicity was graded according to CTCAE directions. Tumor volume was measured before and after treatment using computed tomography scan. The evaluation included tumor mass variation, delayed-type hypersensitivity response and spontaneous peripheral blood mononuclear cell proliferation before and after treatment. The MTD was 400 μg per dose. DNA-hsp65 immunotherapy was well tolerated with moderate pain, edema and infections as the most frequent adverse effects. None of the patients showed clinical or laboratory alterations compatible with autoimmune reactions. Partial response was observed in 4 out of 14 patients who completed treatment, 2 of which are still alive more than 3 years after the completion of the trial. Therefore, DNA-hsp65 immunotherapy is a feasible and safe approach at the dose of 400 μg per injection in patients with HNSCC refractory to standard treatment. Further studies in a larger number of patients are needed to confirm the efficacy of this novel strategy.
Cancer Gene Therapy (2008) **15,** 676–684; doi:10.1038/cgt.2008.35; published online 6 June 2008

Keywords: immunotherapy; DNA vaccine; head and neck cancer; phase I trial

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most frequent form of cancer worldwide.[1,2] It corresponds to 3% of all malignant cancers in the United States, but is much more prevalent in Brazil, where it accounts for 6% of all cancer deaths.[3] Although major advances in surgical, radiation and chemotherapeutic approaches have been made in the last 25 years, these efforts had no significant impact on the survival of patients with advanced HNSCC.[4,5] Therefore, novel therapeutic strategies aiming at improving survival and quality of life are needed. Different treatment modalities are currently being tested in clinical trials, for example electrochemotherapy,[6] intra-arterial chemotherapy,[7] cytokine peritumoral injection,[8,9] vaccines [10] and gene therapy.[11]

Immunotherapy—the concept of boosting the immune system to target and destroy cancer cells—has been a goal

Correspondence: Professor CL Silva, The Centre for Tuberculosis Research, Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto, University of São Paulo, Av Bandeirantes 3900, Ribeirão Preto, São Paulo CEP 14049-900, Brazil.
E-mail: clsilva@cpt.fmrp.usp.br
[11]Current address: Bristol Myers Squibb, Plainsboro, NJ 0853, USA
Received 5 January 2008; accepted 8 March 2008; published online 6 June 2008

in cancer treatment for over 100 years. However, limited success has been achieved with traditional immunotherapy, as cancer cells tend to evolve mechanisms to evade effector immune response. A wide array of gene therapy techniques are being used to overcome this limitation and the cancer vaccines based on heat-shock proteins (HSPs) are considered as a promising approach.[12–14] It has been demonstrated that purified preparations of Gp96, Hsp70, Hsp90 and certain other HSPs from normal or cancer cells are noncovalent associations between HSPs and peptides, known as—HSP–peptide complexes.[15–17] Likewise, the mycobacterial Hsp65 can also be associated with different peptides.[18] These HSP–peptide complexes are potent inducers of immunity [19,20] and have been employed as vaccine adjuvants targeted to different cancers and infections.[21] In addition, HSPs can induce a polyclonal immune response and activate NK cells.[22,23] Thus, HSP-based vaccines emerge as promising strategy for immunotherapy. Accordingly, vaccination with autologous tumor-derived HSP–peptide complexes has been shown to result in both prophylactic and therapeutic antitumoral activity in a variety of animal models of cancer and several advanced clinical studies using autologous tumor-derived HSPs are underway.[21] Recently, two ongoing clinical trials using HSP-based vaccines in cancer have been registered at the NIH clinical trials site (http://www.clinicaltrials.gov/), but no results have yet been published.

Our group and others have previously shown the induction of a robust inflammatory immune response by vaccination with DNA-hsp65 in different animal models of infections and cancer. The use of the DNA-hsp65 vaccine showed an important prophylactic and therapeutic effect *in vivo* against tuberculosis.[24,25] This vaccine also induced upregulation of MHC class I and class II molecules on macrophages.[26,27] Moreover, different groups have found that mycobacterial *hsp65* gene transfer can elicit a profound antitumoral effect.[28,29] The transfection of J774 histiocytic sarcoma cells with the *hsp65* gene resulted in reduced tumor development, and immunization with hsp65-transfected sarcoma cells in mice was protective against challenge with unmodified parental tumor cells.[28] Taken together, these data indicate the remarkable immunogenicity of Hsp65.

In contrast, a concern when dealing with HSPs-based vaccines is their potential to induce autoimmune disease, due to their high molecular conservation among species and crossreactivity with endogenous HSPs. However, preclinical data from our group and others using different animal models show no evidence of autoimmune disease when DNA-hsp65 was used for immunization.[24–26,30]

It is relevant to point that the immunogenicity of tumor-derived HSP–peptide complexes has been shown to be individually tumor specific and not tumor-type specific. This suggests that the relevant immunoprotective peptides are most likely derived from individual tumor-specific antigens rather than from shared tumor antigens.[19] This was the rationale behind the vaccination protocol used in the present study, in which each patient was vaccinated intratumorally with DNA-hsp65 favoring the formation of chaperone–peptide complexes between transfected Hsp65 and antigens of the patient's own tumor. Here, we have evaluated the feasibility, safety and preliminary efficacy of DNA-hsp65 immunotherapy in advanced HNSCC patients.

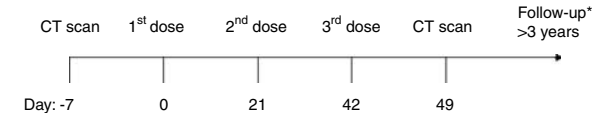

Figure 1 Dose escalation schedule: 7 days before the beginning of the treatment a computed tomography scan (CT scan) for tumor estimation was performed. Three intratumoral doses of 'naked' DNA-hsp65 were injected with ultrasonography guidance with 3 weeks intervals. Each dose was of 150 μg (Group A), 600 μg (Group B) and 400 μg (Group C). *Patients were followed up until death. Two patients are still alive >3 years after DNA therapy.

Patients and methods

Trial design

The study was designed as uncontrolled, nonrandomized, single-institution, open-label, dose escalation, phase I trial to test the safety and feasibility of DNA-hsp65 immunotherapy of patients with recurrent HNSCC. Three different doses of DNA were tested with six patients in each dose group (Figure 1).

Patient eligibility

Eligibility criteria included patients with unresectable locoregional HNSCC after standard therapies that necessarily included radiotherapy. Subjects were also required to have tumor lesions accessible for injection; histologically confirmed diagnosis of HNSCC; life expectancy $\geqslant$3 months; age $\geqslant$18 years; Karnofsky index >70%; no previous malignancies; adequate hematologic, renal and hepatic function; psychosocial status compatible with participation; and to have signed a term of free informed consent, according to Institutional and National Guidelines. Exclusion criteria included pregnancy, HIV infection, significant comorbidities (including coronary artery disease, symptomatic congestive heart failure, active alcohol abuse, diabetes and autoimmune diseases), other malignancies and chemotherapy, hormonal therapy, immunotherapy, biological therapy, surgery or radiotherapy within 4 weeks before treatment with DNA-hsp65. Between 2003 and 2006, 21 patients were enrolled into this phase I study at the Clinical Hospital of the School of Medicine of the University of São Paulo. The demographic and clinical features of patients are reported in Table 1. The protocol was approved by the Institutional Review Board (CAPPesq 183/2) and by the Brazilian National Ethics Committee (CONEP).

DNA-hsp65 immunotherapy

DNA-hsp65 was manufactured and formulated for intratumoral injection in GMP (Good manufacture procedures) conditions at The Centre for Tuberculosis

Table 1 Patient characteristics

Dose group	*Patient*	*Gender*	*Age*[a]	*Tumor location*	*Stage*[b]	*Previous treatment*	*BMI (kg/m²)*	*Karnosfky score*
A. 150 μg per dose	1	M	64	Pyriform sinus	IVb	CheT/RT	19.92	90
	2	M	59	Oral cavity	IVb	Surg/CheT/RT	22.23	90
	3	F	59	Tongue	IVa	Surg/RT	21.87	90
	4	F	49	Larynx	III	CheT/RT	17.74	80
	5	M	56	Oral cavity	III	Surg/RT	18.24	80
	6	M	64	Larynx	IVb	Surg/RT	17.55	80
B. 600 μg per dose	7	M	62	Oral cavity	IVa	Surg/RT	19.25	90
	8	F	73	Oral cavity	IVb	Surg/RT	17.12	90
	9	M	55	Oral cavity	IVa	Surg/RT	21.91	90
	10	M	65	Larynx	IVa	Surg/CheT/RT	22.97	90
	11	M	51	Oropharynx	IVa	Surg/CheT/RT	25.32	90
	12	M	45	Larynx	IVa	Surg/RT	16.92	90
C. 400 μg per dose	13	M	64	Oral cavity	IVa	CheT/RT	17.21	90
	14	M	54	Larynx	IV	Surg/RT	27.30	80
	15	M	64	Larynx	IVb	Surg/RT	18.56	90
	16	M	33	Oropharynx	IVa	Surg/CheT/RT	20.73	80
	17	M	66	Pyriform sinus	IVa	Surg/CheT/RT	19.62	80
	18	M	63	Oropharynx	IVb	Surg/RT	15.00	90
	19	M	49	Oropharynx	IV	Surg/RT	20.00	70
	20	M	67	Oral cavity	IV	Surg/RT	14.20	90
	21	M	50	Larynx	IVb	CheT/RT	16.26	90

Abbreviations: BMI, body mass index; CheT, chemotherapy; F, female; M, male; radiotherapy (RT); Surg, surgery.
[a]The median age was 57.7 years (±9.2 years). Range 33–73 years.
[b]Stage disease was according to AJCC 2002.

Research, School of Medicine of Ribeirão Preto, University of São Paulo. The preparations were sterile, lacked detectable contamination with bacterial RNA and genomic DNA, and had protein and endotoxin levels in compliance with US and European pharmacopeias.[31] The product was supplied in ready-to-use 2-ml glass vials containing naked DNA-hsp65 as lyophilized powder, diluted with sterile water to obtain the desired concentration for intratumoral injection. A fixed volume of 1.5 ml was injected in one or two areas of the tumor depending on its' volume. On the basis of toxicological studies in rodents, the dose of 150 μg was selected as the starting dose for this trial. All patients followed the same treatment course and received intratumoral injections with their group dose (Group A 150 μg, Group B 600 μg and Group C 400 μg)—on days 0, 21 and 42 (Figure 1). Enrollment proceeded to the next dose level after all patients in the previous dose group were treated for ⩾30 days and safety data were reviewed. If no dose-limiting toxicity event occurred, the next dose level was opened. Intratumoral injection was guided by ultrasonography to avoid injection into vessels and necrotic areas. To prevent infections, injections were performed through the neck, whenever possible, even in the case of ulcerated tumors of the mouth.

Toxicity evaluation

Toxicity was graded according to version 3.0 of the US National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE). Patients were evaluated for side effects related to the injections, including a complete clinical evaluation, electrocardiogram, chest X-ray, echocardiogram and standard blood tests including differential blood counts, serum chemistry, urinalysis, and cardiac, hepatic and renal functions. Clinical and laboratory examinations were carried out throughout the study to assess possible autoimmune reactions (rheumatic factor and antimicrosomal, antithyroglobulin and anti-nucleoprotein antibodies).

Measurement of tumor volume and potential predictors of immunostimulation

A computed tomography scan was performed less than 7 days before the first injection of DNA-hsp65 and at day 49 of treatment, when the last dose was given. All scans were performed using a Philips Medical Systems multi-slice machine, model IDT10 or IDT16 with 1.5-mm thick slices. Tumor volume was calculated using the Shaded Surface Display 3-D software (SSD-3D). The selection of tumor borders was performed manually on sequential axial images 2 mm thick. Volume was then automatically calculated by the software in mm^3 (Figure 2). All scans and reconstructions were recorded. Response to treatment was considered in terms of variation in tumor volume according to the Response Evaluation Criteria in Solid Tumors (RECIST):[32] no evidence of disease was considered as a complete response; partial response applies to a greater than 30% decrease in tumor volume; stable disease corresponds to less than 30% decrease and less than 20% increase in tumor volume; and progression corresponds to greater than 20% increase in tumor volume.

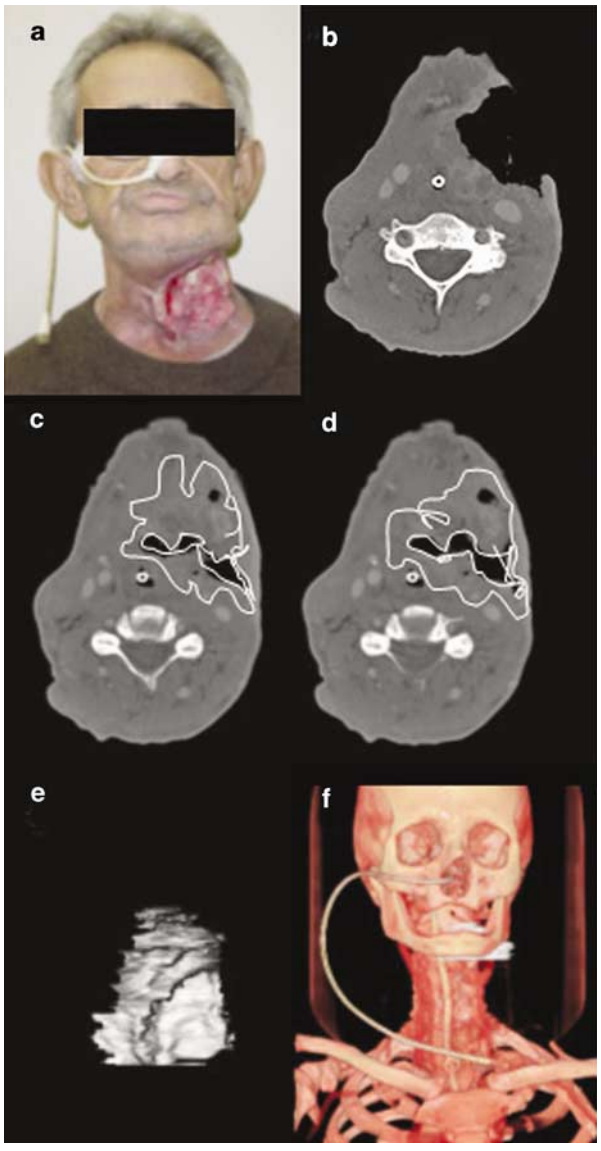

Figure 2 Example of tumor volume measurement. (**a**) Picture of a patient from Group A. (**b**) Axial computed tomography scan (CT scan) image showing tumor and the ulcerated area. (**c**, **d**) Sequential axial images of 2 mm thickness with the tumor limits drawn. (**e**) Tumor volume reconstruction. (**f**) Image reconstruction showing tumor relations.

Delayed-type hypersensitivity (DTH) skin tests were also performed for the evaluation of cellular immune response status before and after treatment. DTH was carried out by subcutaneous injection of 0.1/2 UT of purified protein derivative (PPD) RT23SSI (Stateus Serum Institute, Copenhagen, Denmark), and tricophytin and candidine (FDA Allergenic, São Paulo, Brazil). In addition, both cellular and humoral immunity to Hsp65 were evaluated before and following treatment (Victora *et al*.,[40] manuscript submitted for publication). In the present paper, we present data on the spontaneous proliferation of peripheral blood mononuclear cells (PBMCs) in the course of treatment. For this analysis, 10–40 ml of heparinized blood were obtained from patients whenever possible, before each dose of vaccine and at 3, 6 and 12 months after the initial dose. PBMCs were isolated by Ficoll–Hypaque gradient centrifugation, 2×10^5 PBMCs were cultured in quadruplicate for 5 days in round-bottom 96-well plates. At the end of the fifth day, cells were incubated for 18 h with 2.5 μCi ml^{-1} ^{3}H-thymidine (Amersham Biosciences, Little Chalfont, UK). ^{3}H-Thymidine incorporation was measured in counts per minute (c.p.m.) using an automated betaplate reader (Wallac, Turku, Finland), and the median of the quadruplicate was considered. For spontaneous proliferation c.p.m. before and after vaccination were compared. For antigen-driven proliferation, stimulation indexes (SIs) were calculated by dividing the c.p.m. of antigen-driven proliferation by that of spontaneous proliferation. Samples were considered as positive when $SI > 2$.

Results

A total of 21 patients were enrolled in the study (Table 1). Median age was 57.7 years (range: 33–73 years). Two of these patients had stage III disease and the remaining 19 had stage IV disease on the first presentation. Seventeen patients had previously undergone surgery, 9 had received at least one prior chemotherapy regimen, and all patients had been treated with radiotherapy (Table 1).

The most frequent adverse events were increased pain, edema and infections (Table 2). Although all of these events may have been natural consequences of disease progression, we adopted a systematic approach considering them related to the treatment. According to this evaluation, the great majority of related adverse events were of minor intensity. Nonetheless, in Group B, two patients showed related edema (grades 3 and 4), and two other patients showed grade 4, possibly and probably related pain, the former accompanied by grade 4, possibly related edema. We, therefore, decided to downscale the original 1200 μg per dose intended for Group C to 400 μg per dose.

None of the patients showed either clinical or laboratory alterations compatible with autoimmune disease. However, on day 103, Patient no. 11, who had stable disease on day 49, presented an asymptomatic pericardial effusion diagnosed by echocardiogram, together with clinical progression of disease. We considered this pericardial effusion as a possibly related event (Tables 2 and 3). This patient responded to nutritional and clinical treatment but died on day 228 due to progression of disease with skull base involvement. Autopsy showed no evidence that the effusion was of autoimmune origin.

Only five patients were responsive to DTH tests prior to treatment and this was not correlated to clinical response (Table 3). Two patients (Patient nos. 4 and 10) who tested negative before the beginning of the treatment became positive for PPD after vaccination. All but two

Table 2 Adverse events after three doses of DNA-hsp65 immunotherapy

Dose group	*Patient*	*Pain*		*Edema*		*Asthenia*		*Infections*		*Other*	
		Gr	*Cause*	*Gr*	*Cause*	*Gr*	*Cause*	*Type/Gr*	*Cause*	*Type/Gr*	*Cause*
A. 150 μg per dose	1	1	R	—	—	—	—	—	—	—	—
	2	2	R	2	R	—	—	Cellulitis/2	PB	—	—
	3	3	PS	1	R	2	R	Periodontitis/1 Mucositis/1 Cellulitis/1	PS PS PS	—	—
	4	4	PS	3	R	—	—	Acute sinusitis/1	PS	Aphonia/3	PS
	5	3	PS	—	—	3	PB	Cellulitis/1 Cervical abscess/2	PS PS	—	—
	6	3	PS	—	—	1	R	—	—	—	—
B. 600 μg per dose	7	4	PS	2	R	3	PS	Cellulitis/1 Acute sinusitis/2	PB PS	—	—
	8	4	PB	4	PS	—	—	Cellulitis/2	PS	—	—
	9	3	R	4	R	3	PS	Cellulitis/1 Acute sinusitis/2	PB PS	—	—
	10	—	—	—	—	—	—	—	—	—	—
	11	2	R	3	R	—	—	—	—	Asymptomatic pericardial effusion[a]	PS
	12	1	R	—	—	—	—		—	—	—
C. 400 μg per dose	13	—	—	3	PS	—	—	Cellulitis/3 Pneumonia/3	PS PS	—	—
	14	—	—	—	—	—	—	—	—	—	—
	15	2	R	—	—	1	R	—	—	—	—
	16	4	PS	—	—	2	PS	Acute sinusitis/1	PS	—	—
	17	—	—	—	—	—	—	Pneumonia/3	PS	—	—
	18	1	R	—	—	—	—	—	—	—	—
	19	4	PS	—	—	3	PS	—	—	—	—
	20	2	R	1	R	—	—	—	—	—	—
	21	3	R	4	PB	—	—	Pneumonia/2	PS	Retroauricular lymph node enlargement	R

Abbreviations: Gr, grade of the adverse event; hsp, heat-shock protein; PB, probably related and PS, possibly related to the treatment; R, related.
[a]The asymptomatic pericardial effusion was evidenced in Patient no. 11, on day 103 after the beginning of the therapy.

patients showed at least one sign of immunostimulation—including DTH, pain, edema and spontaneous PBMC proliferation—following vaccination (Table 3).

Proliferation of PBMCs was analyzed in 11 vaccinated patients; however, only 8 patients could be evaluated before and after vaccination. Sample losses were substantial, mainly due to the poor health status of patients, which prevented the collection of the amount of blood originally intended, and to the lack of viability of some cell samples. At least a twofold increase in spontaneous proliferation was observed after vaccination in four out of eight patients evaluated before and after vaccination (Patient nos. 1, 6, 11 and 21) (Table 3 and Figure 3). Spontaneous proliferation decreased after vaccination in one patient (Patient no. 20) (Table 3).

Computed tomography scan with tumor volume measurement was performed in all patients before the first injection. Six patients died before the second scan, none of which due to causes considered as related to DNA-hsp65 vaccination. The most frequent causes of death were disease progression and fatal bleeding. Patient no. 20 was not examined due to technical difficulties (Table 3).

Of the 14 patients whose tumor volume was measured before and after the treatment, 4 showed partial regression (Patient nos. 1, 3, 4 and 10), 1 showed stable disease (Patient no. 11) and 9 showed disease progression (Table 3). Patient nos. 4 and 10, who became positive for PPD after vaccination, had a more favorable clinical outcome and presented a decrease of tumor size and to date, are still alive—Patient no. 4 with 3 years and 6 months and Patient no. 10 with 3 years and 5 months of survival (Table 3).

Discussion

It is thought that cancer cells survive because they are not recognized by the immune system or fail to elicit an adequate immune response. Therefore, several

Table 3 Tumor volume determination, immunostimulatory response and survival following vaccination with DNA-hsp65

Group	Patient	Tumor volume (mm^3)			DTH[a]		Spontaneous PBMC Proliferation (c.p.m.)			Survival days[b]	Cause of death
		Pre	Post	Pre/post	Pre	Post	Pre	Post	Post 2[c]		
A. 150 μg per dose	1	52.87	25.00	−52.71%	Negative	Negative	554	2215	2138	389	Disease progression and parasitic infestation with bleeding
	2	19.47	52.11	+167.64%	Negative	Negative	ND	ND	ND	274	Disease progression
	3	84.36	48.12	−42.95%	Negative	Negative	ND	ND	ND	100	Disease progression
	4	4.88	1.16	−76.22%	Negative	PPD (18 mm)	541	ND	547	Alive	—
	5	87.05	172.40	+98.04%	Negative	Negative	ND	ND	1805	148	Disease progression
	6	146.42	ND	ND	Negative	Negative	131	ND	325	37	Fatal bleeding after tumor manipulation
B. 600 μg per dose	7	81.53	140.85	+72.75%	Negative	Negative	ND	ND	ND	105	Disease progression and fatal bleeding
	8[d]	8.47	ND	ND	Negative	Death	1123	ND	245	38	Pulmonary carcinomatosis and acute respiratory failure
	9[d]	8.26	14.37	+173.90%	Tricoph (30 mm)	Negative	ND	ND	ND	69	Disease progression and fatal bleeding
	10	5.63	3.45	−38.72%	Negative	PPD (10 mm)	314	511	487	Alive	—
	11	178.62	175.03	−2.00%	Negative	Negative	308	1032	673	228	Disease progression and fatal bleeding
	12	59.56	ND	ND	PPD (20 mm)	Death	ND	ND	ND	22	Upper digestive bleeding
C. 400 μg per dose	13	70.49	222.06	+215.00%	Negative	Negative	ND	ND	ND	186	Disease progression
	14	171.69	ND	ND	Negative	Death	ND	ND	ND	49	Disease progression
	15	70.78	ND	ND	PPD (15 mm)	Death	ND	ND	ND	41	Disease progression and fatal bleeding
	16	22.34	59.77	+167.50%	Negative	Negative	ND	685	1640	181	Disease progression
	17	85.24	ND	ND	PPD (10 mm)	Death	ND	ND	ND	21	Disease progression and fatal bleeding
	18	8.07	9.95	+23.20%	Negative	Negative	ND	2633	ND	171	Disease progression
	19	96.23	264.54	+175.00%	Negative	Negative	797	ND	862	100	Disease progression
	20	81.88	ND	ND	Negative	Negative	717	ND	680	118	Disease progression
	21	13.23	79.44	+500.40%	PPD (18 mm)	Negative	277	918	ND	127	Disease progression

Abbreviations: DTH, delayed-type hypersensitivity; hsp, heat-shock protein; ND, not done; PBMC, peripheral blood mononuclear cell; PPD, tuberculin-purified protein derivative; Pre, pre-treatment; Post, post-treatment on day 49 of the schedule.

Patients who showed parameters of immunostimulation are indicated in gray lines. All patients showed pain and edema.

Patient nos. 4 and 10 are still alive—Patient no. 4 with 3 years and 6 months and Patient no. 10 with 3 years and 5 months of survival.

The tumor volume post treatment for Patient no. 20 was not determined due to technical problems.

[a]DTH of all patients were tested against PPD (positive >5 mm), candidin and tricophytin (Tricoph) antigens, but only the immunostimulatory antigen for each patient is indicated.

[b]The day of death was determined after the first dose of DNA-hsp65.

[c]Post 2 indicates that PBMC proliferation was evaluated at a different day than day 49: Patient no. 1, at 1 year; Patient no. 4, at 6 months; Patient no. 5, after second dose; Patient no. 6, after first dose; Patient no. 8, after first dose; Patient no. 10, at 1 year; Patient no. 11, at 6 months, Patient no. 16, at 3 months; Patient no. 19, after second dose and Patient no. 21, after first dose.

[d]Patients who showed a decrease of immunostimulation.

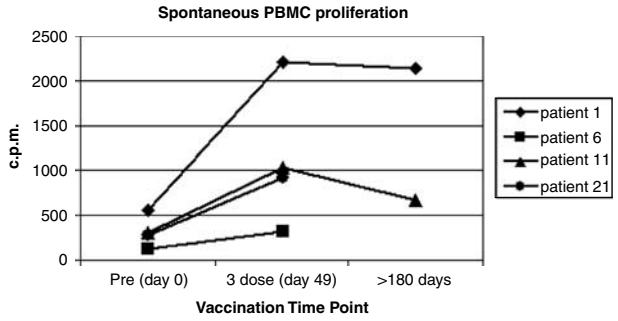

Figure 3 Patients with increased spontaneous PBMC proliferation following DNA-hsp65 vaccination. 2×10^5 PBMCs were cultured for 5 days in DMEM 10% inactivated human serum medium with human inactivated serum. After 5 days, the cells were pulsed with 0.5 μCi per well ^{3}H-thymidine. Abbreviations: Pre, samples collected prior to immunization.

immunotherapeutic approaches for cancer involve adding a molecular 'danger signal' to previously unrecognized or poorly recognized tumor antigens, thereby enhancing presentation of antigens to the immune system.[33] HSPs may well be used in such scenario because they are known to augment innate and antigen-specific effectors functions.[21] Here, we report the use of DNA-hsp65 vaccine in HNSCC patients. This is the first clinical trial using the *hsp65* gene from mycobacterial origin, previously shown by different groups to be efficient against tuberculosis and in preventing and reducing tumor growth in preclinical models.[24–26,28,30,34,35] We have had the care to create GMP conditions in our laboratory exclusively to produce DNA-hsp65 vaccine for clinical use. The vaccine injections were ultrasound guided, which guaranteed the injection in solid parts of the tumor, avoiding necrotic areas and vessels. This could give our protocol two additional advantages. First, it enhanced the possibility of transfection of tumor cells with DNA-hsp65, thus increasing the repertoire of peptide-complexed antigens. This approach was considered to be particularly promising for tumors in which dominant antigens are poorly characterized as is the case of HNSCC. Second, considering that Hsp65 is an immunodominant antigen in mycobacteria,[36] the expression of this foreign protein by tumor cells in the context of HLA class I molecules could lead to breakdown of the tolerogenic environment found in cancer patients.

As with any new type of therapy, there are important safety concerns. Newer and safer gene delivery agents have been created and thousands of cancer patients worldwide either have participated or are currently enrolled in gene therapy trials, with remarkably few treatment side effects.[37] There are often localized swelling and inflammation at the site of the injection.[38] However, when compared with the side effects of conventional chemotherapeutic treatments, these side effects are minimal. In our study, the most important adverse events, namely pain and edema, were locoregional and could be the result of local inflammation induced by the intratumoral naked DNA injection or correspond to the immune response elicited against the tumor. Interestingly, toxicity seemed to be dose dependent, but clinical response was not.

Tumor volume is an important parameter for assessing therapeutic results. The traditional assessment of tumor size, measuring the two greatest diameters of the tumor, is not accurate for the cases in the present study, which have recurrent, often ulcerated lesions with irregular limits. Although more complex, the method used here allowed a more reliable measurement of tumor volume. Despite the fact that two patients with initial tumor volumes smaller than 10 mm^3 showed disease progression, the two patients who best responded to treatment, and who are still alive 3 years after completion of the trial, had small tumors to start with. This is particularly important considering the type of tumor and the type of immunotherapy tested in the present trial, which consisted of naked DNA encoding a single gene. This contrasts with other therapeutic trials reported in the literature, in which mixed plasmid constructions at higher doses and with adenoviral or cytokine boosters were tested.[39] Another concern is that patients in this trial may not be immunocompetent enough to respond to the DNA-hsp65 stimulus. Interestingly, all four patients who showed reductions in tumor volume were DTH-negative prior to vaccination. However, these patients showed at least one sign of immunostimulation—including DTH, pain, edema and spontaneous PBMC proliferation after immunizations. In contrast, none of the five patients initially DTH responders, showed favorable clinical outcome.

Immunological evaluation of patients in this clinical trial included humoral response to mycobacterial Hsp65 and human Hsp60, antigen-specific proliferation and both IFN-γ and IL-10 production by ELISPOT. These data are presented in a separate paper (Victora *et al*.,[40] manuscript submitted for publication). It is relevant to point out in the present report that almost all patients (19 out of 21) showed signs of immune stimulation and 4 patients also showed a decrease in tumor size, suggesting that the vaccine was able to interfere with the immunological status of patients. Moreover, in four patients, spontaneous PBMC proliferation increased after vaccination, and in two patients a progressive increase was detected during the course of treatment.

In summary, considering the type of tumor and the therapy tested, our data indicate that vaccination with DNA-hsp65 is a feasible and safe approach. Moreover, the clinical benefit observed in a limited number of patients who responded to the vaccine and the urgent need for new therapeutic resources for advanced HNSCC patients warrant further confirmatory studies designed to evaluate clinical and immunological outcomes in a larger number of patients.

Acknowledgements

We would like to thank Roger Chammas for his useful advice during the planning of the trial, Dr José Maciel Rodrigues Jr and Dr Karla Lima for their help in vaccine

production. This work was supported by grants from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and FINEP (Financiadora de Estudos e Projetos).

Author contributions

Conception and design: Celio L Silva, Pedro Michaluart, Kald Abdallah, Verônica Coelho.
Administrative support: Celio L Silva and Pedro Michaluart.
Provision of study materials or patients: Celio L Silva, Pedro Michaluart, Kald Abdallah, Raquel A Moysés, Fanny D Lima, Rodney B Smith and Maria Cristina Chammas.
Collection and assembly of data: Pedro Michaluart, Kald Abdallah, Fanny D Lima, Rodney B Smith, Raquel A Moysés, Verônica Coelho, Gabriel D Victora, Jorge Kalil, Alberto R Ferraz, Ana K Barreto, Maria Cristina Chammas, Regina LE Gomes, Eloisa Gebrim, Lica Arakawa-Sugueno, Kariane P Fernandes, Paulo A Lotufo and Maria Regina Cardoso.
Data analysis and interpretation: Pedro Michaluart, Kald Abdallah, Fanny D Lima, Rodney B Smith, Raquel A Moysés, Verônica Coelho, Gabriel D Victora, Adalberto Socorro-Silva, Evelyn C Volsi, Jorge Kalil, Carlos R Zárate-Bladés, Alberto R Ferraz, Ana K Barreto, Regina LE Gomes, Eloisa Gebrim, Lica Arakawa-Sugueno, Kariane P Fernandes, Paulo A Lotufo, Maria Regina Cardoso and Celio L Silva.
Manuscript writing: Celio L Silva, Pedro Michaluart, Verônica Coelho, Carlos R Zárate-Bladés, Gabriel D Victora, Fanny D Lima, Rodney B Smith and Maria Regina Cardoso.
Finnal approval of manuscript: Pedro Michaluart, Kald Abdallah, Fanny D Lima, Rodney B Smith, Raquel A Moysés, Verônica Coelho, Gabriel D Victora, Adalberto Socorro-Silva, Evelyn C Volsi, Jorge Kalil, Carlos R Zárate-Bladés, Alberto R Ferraz, Ana K Barreto, Maria Cristina Chammas, Regina LE Gomes, Eloisa Gebrim, Lica Arakawa-Sugueno, Kariane P Fernandes, Paulo A Lotufo, Maria Regina Cardoso and Celio L Silva.

References

1 Vokes EE, Weichselbaum RR, Lippman SM, Hong WK. Head and neck cancer. *N Engl J Med* 1993; **328**: 184–194.
2 Ku TK, Nguyen DC, Karaman M, Gill P, Hacia JG, Crowe GL. Loss of p53 expression correlates with metastatic phenotype and transcriptional profile in a new mouse model of head and neck cancer. *Mol Cancer Res* 2007; **5**: 351–362.
3 Amar A, Franzi SA, Rapoport A. Evolution of patients with squamous cell carcinoma of upper aerodigestive tract. *Sao Paulo Med J* 2003; **121**: 155–158.
4 Winquist E, Oliver T, Gilbert R. Postoperative chemo-radiotherapy for advanced squamous cell carcinoma of the head and neck: a systematic review with meta-analysis. *Head Neck* 2007; **29**: 38–46.
5 Siu LL, Soulieres D, Chen EX, Pond GR, Chin SF, Francis P *et al*. Phase I/II trial of erlotinib and cisplatin in patients with recurrent or metastatic squamous cell carcinoma of the head and neck: a Princess Margaret Hospital phase II consortium and National Cancer Institute of Canada Clinical Trials Group Study. *J Clin Oncol* 2007; **25**: 2178–2183.
6 Sersa G, Miklavcic D, Cemazar M, Rudolf Z, Pucihar G, Snoj M. Electrochemotherapy in treatment of tumours. *Eur J Surg Oncol* 2007; **2**: 232–240.
7 Robbins KT, Storniolo AM, Kerber C, Vicario D, Seagren S, Shea M *et al*. Phase I study of highly selective supradose cisplatin infusions for advanced head and neck cancer. *J Clin Oncol* 1994; **12**: 2113–2120.
8 Whiteside TL, Letessier E, Hirabayashi H, Vitolo D, Bryant J, Barnes L *et al*. Evidence for local and systemic activation of immune cells by peritumoral injections of interleukin 2 in patients with advanced squamous cell carcinoma of the head and neck. *Cancer Res* 1993; **53**: 5654–5662.
9 Shin DM, Glisson BS, Khuri FR, Clifford JL, Clayman G, Benner SE *et al*. Phase II and biologic study of interferon alfa, retinoic acid, and cisplatin in advanced squamous skin cancer. *J Clin Oncol* 2002; **20**: 364–370.
10 Herold-Mende C, Karcher J, Dyckhoff G, Schirrmacher V. Antitumor immunization of head and neck squamous cell carcinoma patients with a virus-modified autologous tumor cell vaccine. *Adv Otorhinolaryngol* 2005; **62**: 173–183.
11 Nemunaitis J, Khuri F, Ganly I, Arseneau J, Posner M, Vokes E *et al*. Phase II trial of intratumoral administration of ONYX-015, a replication-selective adenovirus, in patients with refractory head and neck cancer. *J Clin Oncol* 2001; **19**: 289–298.
12 Castelli C, Rivoltini L, Rini F, Belli F, Testori A, Maio M *et al*. Heat shock proteins: biological functions and clinical application as personalized vaccines for human cancer. *Cancer Immunol Immunother* 2004; **53**: 227–233.
13 Binder RJ. Heat shock protein vaccines: from bench to bedside. *Int Rev Immunol* 2006; **25**: 353–375.
14 Prohaszka Z. Chaperones as part of immune networks. *Adv Exp Med Biol* 2007; **594**: 159–166.
15 Menoret A, Peng P, Srivastava PK. Association of peptides with heat shock protein gp96 occurs *in vivo* and not after cell lysis. *Biochem Biophys Res Commun* 1999; **262**: 813–818.
16 Paz P, Brouwenstijn N, Perry R, Shastri N. Discrete proteolytic intermediates in the MHC class I antigen processing pathway and MHC I-dependent peptide trimming in the ER. *Immunity* 1999; **11**: 241–251.
17 Kunisawa J, Shastri N. The group II chaperonin TRiC protects proteolytic intermediates from degradation in the MHC class I antigen processing pathway. *Mol Cell* 2003; **12**: 565–576.
18 Portaro FC, Hayashi MA, De Arauz LJ, Palma MS, Assakura MT, Silva CL *et al*. The *Mycobacterium leprae* hsp65 displays proteolytic activity. Mutagenesis studies indicate that the *M.leprae* hsp65 proteolytic activity is catalytically related to the HslVU protease. *Biochemistry* 2002; **41**: 7400–7406.
19 Srivastava P. Roles of heat-shock proteins in innate and adaptive immunity. *Nat Rev Immunol* 2002; **2**: 185–194.
20 Binder RJ, Srivastava PK. Peptides chaperoned by heat-shock proteins are a necessary and sufficient source of antigen in the cross-priming of CD8+ T cells. *Nat Immunol* 2005; **6**: 593–599.
21 Segal BH, Wang XY, Dennis CG, Youn R, Repasky EA, Manjili MH *et al*. Heat shock proteins as vaccine adjuvants in infections and cancer. *Drug Discov Today* 2006; **11**: 534–540.
22 Chen X, Tao Q, Yu H, Zhang L, Kao X. Tumor cell membrane-bound heat shock protein 70 elicits antitumor immunity. *Immunol Lett* 2002; **84**: 81–87.

23 Michaelsson J, Teixeira de Matos C, Achour A, Lanier LL, Kärre K, Söderström K. A signal peptide derived from hsp60 binds HLA-E and interferes with CD94/NKG2A recognition. *J Exp Med* 2002; **196**: 1403–1414.
24 Lowrie DB, Silva CL, Colston MJ, Ragno S, Tascon RE. Protection against tuberculosis by a plasmid DNA vaccine. *Vaccine* 1997; **15**: 834–838.
25 Lowrie DB, Tascon RE, Bonato VL, Lima VM, Faccioli LH, Stavropoulos E *et al*. Therapy of tuberculosis in mice by DNA vaccination. *Nature* 1999; **400**: 269–271.
26 Bonato VL, Goncalves ED, Soares EG, Santos Júnior RR, Sartori A, Coelho-Castelo AA *et al*. Immune regulatory effect of pHSP65 DNA therapy in pulmonary tuberculosis: activation of CD8+ cells, interferon-gamma recovery and reduction of lung injury. *Immunology* 2004; **113**: 130–138.
27 Silva CL, Bonato VL, Coelho-Castelo AA, De Souza AO, Santos SA, Lima KM *et al*. Immunotherapy with plasmid DNA encoding mycobacterial hsp65 in association with chemotherapy is a more rapid and efficient form of treatment for tuberculosis in mice. *Gene Therapy* 2005; **12**: 281–287.
28 Lukacs KV, Lowrie DB, Stokes RW, Colston MJ. Tumor cells transfected with a bacterial heat-shock gene lose tumorigenicity and induce protection against tumors. *J Exp Med* 1993; **178**: 343–348.
29 Yi H, Rong Y, Yankai Z, Wentao L, Hongxia Z, Jie W *et al*. Improved efficacy of DNA vaccination against breast cancer by boosting with the repeat beta-hCG C-terminal peptide carried by mycobacterial heat-shock protein HSP65. *Vaccine* 2006; **24**: 2575–2584.
30 Nuermberger E, Tyagi S, Williams KN, Rosenthal I, Bishai WR, Grosset JH. Rifapentine, moxifloxacin, or DNA vaccine improves treatment of latent tuberculosis in a mouse model. *Am J Respir Crit Care Med* 2005; **172**: 1452–1456.
31 Tuomela M, Stanescu I, Krohn K. Validation overview of bio-analytical methods. *Gene Therapy* 2005; **12**(Suppl 1): S131–S138.
32 Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L *et al*. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000; **92**: 205–216.
33 Todryk SM, Melcher AA, Dalgleish AG, Vile RG. Heat shock proteins refine the danger theory. *Immunology* 2000; **99**: 334–337.
34 Lima KM, dos Santos SA, Santos RR, Brandão IT, Rodrigues Jr JM, Silva CL. Efficacy of DNA-hsp65 vaccination for tuberculosis varies with method of DNA introduction *in vivo*. *Vaccine* 2003; **22**: 49–56.
35 Lowrie DB. DNA vaccines for therapy of tuberculosis: where are we now? *Vaccine* 2006; **24**: 1983–1989.
36 Kaufmann SH, Vath U, Thole JE, Van Embden JD, Emmrich F. Enumeration of T cells reactive with Mycobacterium tuberculosis organisms and specific for the recombinant mycobacterial 64-kDa protein. *Eur J Immunol* 1987; **17**: 351–357.
37 Cross D, Burmester JK. Gene therapy for cancer treatment: past, present and future. *Clin Med Res* 2006; **4**: 218–227.
38 Vattemi E, Claudio PP. Adenoviral gene therapy in head and neck cancer. *Drug News Perspect* 2006; **19**: 329–337.
39 Mincheff M, Tchakarov S, Zoubak S, Loukinov D, Botev C, Altankova I *et al*. Naked DNA and adenoviral immunizations for immunotherapy of prostate cancer: a phase I/II clinical trial. *Eur Urol* 2000; **38**: 208–217.
40 Victora G, Socorro-Silva A, Volsi E, Abdallah K, Lima F, Michaluart P *et al*. Immune response to vaccination with DNA-hsp65 in head and neck cancer patients. Submitted.