

# A Phase I Study of the Optimized Cryptic Peptide TERT<sub>572Y</sub> in Patients with Advanced Malignancies

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## Key Words

Cancer vaccines · Cytotoxic T lymphocytes · TERT<sub>572</sub> cryptic peptide

## Abstract

**Objective:** It was the aim of this study to evaluate the safety of the optimized cryptic peptide TERT<sub>572Y</sub> in pretreated patients with advanced cancer. **Methods:** Nineteen patients with progressive and chemotherapy-refractory tumors received escalated doses (2–6 mg) of 2 subcutaneous injections of the optimized TERT<sub>572Y</sub> peptide followed by 4 subcutaneous injections of the native TERT<sub>572</sub> peptide every 3 weeks. Both TERT peptides were coinjected with adjuvant Montanide ISA51. Toxicity was evaluated every 3 weeks and peptide-specific CD8+ cells were detected by flow cytometry using TERT<sub>572Y</sub> tetramers. **Results:** Fourteen out of 19 patients completed the vaccination program. No grade III/IV toxicity was observed. Grade I anemia was observed in 4 patients and local skin reaction at the injection site in 11 patients. Other nonhematologic toxicities were mild, and no late toxicity was observed after a median postvaccination follow-up period of 10.7 months. There was no dose-limiting toxicity. Peripheral blood TERT<sub>572Y</sub>-specific CD8+ lympho-

cytes were detected in 13 out of 14 evaluable patients after 2 injections with the optimized TERT<sub>572Y</sub> peptide. There was no complete or partial response, but 4 patients (21%) with persistent TERT<sub>572Y</sub>-specific CD8+ experienced stable disease for a median of 10.5 months. **Conclusion:** TERT<sub>572Y</sub> peptide vaccine is well tolerated and effective in eliciting specific TERT<sub>572Y</sub> CD8+ lymphocytes in pretreated cancer patients, demonstrating that cryptic peptides could be used in cancer immunotherapy.

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## Introduction

Cancer immunotherapy is intended to stimulate cytotoxic T lymphocytes (CTL) recognizing peptides derived from tumor antigens and presented at the tumor cell surface by human leukocyte antigen (HLA) class I molecules. CTL-targeted peptides can be dominant or cryptic [1]. Dominant peptides have high HLA affinity and are frequently presented by tumor cells. In contrast, cryptic peptides have low HLA affinity and are rarely presented by tumor cells. All cancer vaccines tested so far have targeted dominant peptides, with relatively little success [2–

6]. Studies using mouse models showed that this lack of efficacy is due to tolerance to tumor antigens, and especially to their dominant peptides [7–12].

To circumvent this tolerance, we recently proposed vaccination with cryptic peptides. In humanized mice, we found that tolerance of cryptic peptides was weak or absent, and that cryptic peptides efficiently induced antitumoral immunity *in vivo*, providing their immunogenicity had been optimized [8, 13, 14]. We have previously described a peptide sequence modification that optimizes immunogenicity of almost all low-affinity HLA-A\*0201-restricted peptides tested [13].

TERT<sub>572Y</sub> is an HLA-A\*0201-associated optimized cryptic peptide derived from telomerase reverse transcriptase (TERT), an antigen overexpressed by 85% of human tumors [15]. TERT<sub>572Y</sub> is present in both human and murine TERT and was able to induce antitumoral immunity in HLA-A\*0201 transgenic mice; however, no autoimmunity against normal TERT-expressing tissues was observed [8]. *In vitro*, TERT<sub>572Y</sub> stimulated antitumor CTL from both healthy donors and prostate cancer patients. CTL killed TERT-expressing tumor cells but not TERT-expressing normal cells [14, 16].

The present phase I vaccination study was designed to evaluate the safety and the risk of inducing autoimmune reactions against TERT-expressing normal cells and tissues such as hematopoietic precursors, gut, thymus and liver as well as to determine the maximum tolerated dose of the peptide. The vaccination protocol consisted of two sequential administrations of escalated doses of the optimized cryptic TERT peptide (TERT<sub>572Y</sub>) followed by four administrations of the corresponding native peptide. The rationale for this strategy was to select among T cells recruited by the optimized TERT<sub>572Y</sub> those with the highest specificity for the native TERT<sub>572</sub> presented by tumor cells. The immunogenicity of the optimized cryptic peptide was investigated by monitoring the peripheral blood TERT<sub>572Y</sub>-specific CD8+ cells.

## Patients and Methods

### Patients

Patients with chemotherapy-resistant malignant solid tumors were eligible for the study. Other eligibility criteria were progressive disease for which there was no other therapeutic option of proven benefit and an expected survival of at least 6 months. Patients had to be HLA-A\*0201 positive, aged 18–75 years, with a performance status (WHO) <2 and adequate bone marrow (absolute neutrophil count  $\geq 1,500/\text{mm}^3$ , absolute lymphocyte count  $\geq 1,300/\text{mm}^3$ , platelets  $>100,000/\text{mm}^3$ , hemoglobin  $>10$  g/dl), renal (creatinine  $<1.5$  mg/dl) and liver (bilirubin  $<1.5$  times the

upper normal value) function. Patients were excluded if they had received chemotherapy, radiotherapy, hormone therapy, immunotherapy or corticosteroids within 1 month before enrolment, or if they had a known immunodeficiency or autoimmune disease. The protocol was approved by the Ethics and Scientific Committees of the University Hospital of Heraklion and the National Drug Administration of Greece. All patients gave written informed consent in order to participate in the study.

### Peptide Vaccine Preparation

The vaccine consisted of optimized TERT<sub>572Y</sub> (YLFFYRKSV) and native TERT<sub>572</sub> (RLFFYRKSV) peptides emulsified in Montanide ISA51 (Seppic Inc., France). The vaccine peptides were synthesized at the Faculty of Pharmacy, University of Patras, Greece, by means of solid-phase Fmoc/Bu chemistry. Quality assurance studies included confirmation of identity, sterility and purity ( $>95\%$  for both peptides). No decrease in purity or concentration was observed after more than 2 years of storage at  $-80^\circ\text{C}$ . Each peptide was prepared as a lyophilized powder for reconstitution and dilution in sterile water.

### Vaccination Protocol

Patients received a total of 6 subcutaneous vaccinations administered every 3 weeks. Peptides in 0.5 ml aqueous solution were emulsified with 0.5 ml Montanide ISA51 immediately before being injected. The optimized TERT<sub>572Y</sub> peptide was used for the first 2 vaccinations and the native TERT<sub>572</sub> peptide for the remaining 4 vaccinations. Five dose levels of the peptides were studied; dose levels included 2, 3, 4, 5 and 6 mg of both peptides. Three patients were entered at each dose level. An additional 3 patients were planned to be enrolled at the dose level where a dose-limiting event was observed. Each patient received the same peptide dose for all 6 vaccinations. No other treatment with possible antitumor activity, *i.e.* chemotherapy, radiotherapy, hormone therapy or administration of corticosteroids, was allowed during the course of vaccination.

### Patient Evaluation

Before entering the study, all patients were assessed by complete medical history, physical examination and complete blood cell count with differential, serum chemistry and baseline measurements of relevant tumor markers. Moreover, measurable disease was determined by standard imaging procedures (chest X-ray, ultrasound, computed tomography scans of the thorax and abdomen, magnetic resonance imaging, if indicated, and whole body bone scans). Toxicity during the vaccination protocol was evaluated by repeating the complete blood cell count weekly and by performing medical history, physical examination and serum chemistry every 3 weeks before each subsequent injection during the vaccination period and every month thereafter during the follow-up. Toxicity was assessed and scored using the National Cancer Institute common toxicity criteria [17]. Dose-limiting toxicity (DLT) was assessed during the entire vaccination protocol and was defined as the occurrence of any of the following: grade 4 hematologic toxicity, grade 3–4 neutropenia with fever  $>38.2^\circ\text{C}$ , grade 3–4 nonhematologic toxicity, and any treatment delay because of toxicity. Dose escalation was discontinued and the DLT dose level was reached if at least 50% of the patients treated at that level develop a DLT. The maximum tolerated dose level was defined as the first level below the DLT dose level.

**Table 1.** Patient characteristics

Pa-tient	Age	Sex	Primary tumor	Sta-ge	PS	Previous treatment	Dose of vaccine, mg	Injec-tions	Clinical response	Survival months
1	73	M	colorectal	IV	1	7 lines CT	2	4	PD	6.1
2	75	F	breast	IV	1	5 lines CT	2	6	PD	27.6
3	64	M	melanoma	IV	1	1 line CT	2	6	PD	8.8+
4	60	M	NSCLC	IV	1	2 lines CT	3	6	PD	22.8+
5	71	M	NSCLC	IV	1	6 lines CT	3	4	PD	5.7
6	73	F	cervix	IV	1	2 lines CT	3	6	PD	5.3
7	53	M	head and neck	IV	1	4 lines CT	4	6	PD	15.1
8	57	F	colorectal	IV	1	5 lines CT	4	6	PD	12.3
9	66	M	renal	IV	1	1 line IT	4	6	SD	11.1+
10	73	F	colorectal	IV	1	2 lines CT	5	6	PD	4.6
11	49	M	NSCLC	IIIb	0	3 lines CT	5	6	SD	17.5+
12	45	F	breast	IV	1	2 lines CT	5	6	SD	11.2+
13	51	M	renal	IV	1	1 line CT, 1 line IT	6	6	SD	6.9+
14	61	M	unknown origin	IV	1	2 lines CT	6	4	PD	8
15	70	F	colorectal	IV	0	2 lines CT	6	6	PD	10.7+
16	69	M	prostate	IV	1	2 lines CT 1 line HT	6	6	PD	17+
17	69	F	ovarian	IV	1	8 lines CT	6	6	PD	13.7+
18	51	F	ovarian	IV	1	4 lines CT	6	5	PD	6.4
19	48	M	esophagus	IV	1	1 line CT	6	4	PD	4.4+

PS = Performance status; NSCLC = non-small cell lung cancer; CT = chemotherapy; IT = immunotherapy (interleukin 2, interferon- $\alpha$ ); HT = hormone therapy; PD = progressive disease; SD = stable disease.

Response to treatment was evaluated by repeating the baseline imaging studies and relevant tumor marker measurements after every 2 vaccinations or sooner, if clinically indicated. Response to treatment was scored as complete response, partial response, stable disease and progressive disease using the standard WHO criteria [18]. Radiological responses were confirmed by an independent panel of radiologists. Complete response and partial response had to be maintained for a minimum of 4 weeks. The duration of response was measured from the first documentation of response to disease progression. Time to progression was determined by the interval between the initiation of therapy to the first date that disease progression was objectively documented. Overall survival was measured from the date of study entry to the date of death. The follow-up time was measured from the day of first treatment administration to last contact or death. Immune responses were examined before the first injection, and after the second and sixth injections. Peripheral blood mononuclear cells (PBMC) were collected at each time point and frozen.

#### Peptides

Class I restricted peptides used for laboratory studies included TERT<sub>572Y</sub> (YLFFYRKS<sub>V</sub>), TERT<sub>572</sub> (RLFFYRKS<sub>V</sub>) and FluM58 (GILGFVFTL), all produced by Epytop (Nimes, France).

#### In vitro Stimulation of PBMC

Thawed PBMC ( $3 \times 10^5$  cells/well in 200  $\mu$ l) were incubated in the presence of 10  $\mu$ M TERT<sub>572Y</sub> peptide in complete medium

(RPMI 1640 supplemented with 8% human AB serum) in 96-well round-bottom plates. Interleukin 2 was added at a final concentration of 10 U/ml after 48 and 96 h. Cells were incubated at 37°C in 5% CO<sub>2</sub> air. On day 9 of culture, cells from 6 wells were pooled and analyzed for the presence of TERT<sub>572Y</sub>-specific CD8 cells by TERT<sub>572Y</sub> tetramer staining.

#### TERT<sub>572Y</sub> Tetramer Staining

Cells were incubated with phycoerythrin-conjugated TERT<sub>572Y</sub> tetramer (Proimmune Ltd., Oxford, UK) for 30 min at room temperature, and then with APC-conjugated anti-CD8 (BD Pharmingen, Mississauga, Canada) and FITC-conjugated anti-CD3 (BD Pharmingen) monoclonal antibodies for 30 min at 4°C. Stained cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences, Mountain View, Calif., USA).

## Results

### Patient Characteristics, Vaccination and Clinical Responses

The characteristics of the 19 patients enrolled in the trial are shown in table 1. All but 1 patient (no. 11) had stage IV cancer with multiple metastases mainly in the bones, liver and lung. They all had active and progressive

**Table 2.** Toxicity

Pa-tient	Level mg	Toxicity		
		grade I	grade II	grade II/IV
1	1	no	no	no
2	2	local skin	no	no
3	2	anemia, local skin	no	no
4	3	local skin	no	no
5	3	no	fatigue, nausea	no
6	3	anemia, local skin, fatigue, anorexia	no	no
7	4	no	no	no
8	4	thrombopenia, local skin	no	no
9	4	local skin	no	no
10	5	no	anorexia, fatigue, nausea	no
11	5	thrombocytopenia	no	no
12	5	anemia, local skin	no	no
13	6	local skin	no	no
14	6	local skin	no	no
15	6	no	no	no
16	6	anemia	no	no
17	6	anemia, local skin	no	no
18	6	local skin, anemia	anorexia, fatigue	no
19	6	no	no	no

disease and had received several treatments, mainly chemotherapy, before entering the vaccination protocol. Three patients were enrolled at dose levels 2, 3, 4 and 5 mg, and 7 patients at 6 mg of the peptides at the dose level. Five patients were withdrawn from the protocol after the fourth (patients nos. 1, 5, 14 and 19) or fifth (patient no. 18) vaccine injection because of rapid disease progression. All 5 patients subsequently died within 6 months of disease progression. The remaining 14 patients completed the vaccination protocol. The disease stabilized in 4 patients (nos. 9 and 11–13; 29%) and continued to progress in 10 patients. The latter 10 patients subsequently received chemotherapy, and 6 of them are still alive. One (patient no. 11) of the 4 patients whose disease stabilized for 9 months subsequently progressed, while the other 3 patients still have stable disease (after 12 months for patients nos. 9 and 12, and after 9 months for patient no. 13) with no additional therapy after the end of vaccination.

Overall, after a median follow-up of 10.7 months (range 4.4–27.6), 9 patients (47.4%) have died, all due to disease progression. The median time to tumor progression was 4.2 months (range 2.3–11.2) and the median overall survival was 15.2 months (range 4.4–27.6).

### Toxicity and Adverse Events

No DLT was observed throughout the entire study, and therefore, the maximum tolerated dose level has not been reached (table 2). Thirteen patients developed grade I toxicity, which consisted of local skin reaction (11 patients), anemia (6 patients), thrombocytopenia (2 patients), fatigue (1 patient) and anorexia (1 patient). Three patients developed grade II toxicity consisting of fatigue (3 patients), nausea (2 patients) and anorexia (2 patients). The observed grade I anemia and thrombocytopenia were transient and resolved in all but 1 case, despite continuing the vaccinations. In 1 patient (no. 17), anemia persisted and was attributed to progressive disease. Except for local skin reaction, other toxicities were most likely related to the disease rather than to the vaccination. Specifically, no significant hematologic, renal, gastrointestinal or hepatic toxicity was observed. Patients were monitored for toxicity for a median of 10.7 months (range 4.4–27.6). Even after completing or discontinuing the vaccination program, patients were followed monthly for the occurrence of any delayed toxicity. However, no signs or findings of delayed toxicity or symptoms and clinical findings suggesting autoimmune reactions were observed.

### Peptide-Specific CD8+ Cells

Peptide-specific CD8+ cells were detected in peripheral blood by triple staining of PBMC with TERT<sub>572Y</sub> tetramer, anti-CD8 and anti-CD3 monoclonal antibodies, both ex vivo and after 9 days of stimulation in vitro with TERT<sub>572Y</sub> peptide. In a preliminary study, TERT<sub>572Y</sub> tetramer labeled less than 0.11% of CD8 cells in 7 HLA-A\*0201 healthy donors (mean 0.035 ± 0.035%, range 0.0–0.11; data not shown). Therefore, the positivity cutoff for peripheral blood TERT<sub>572Y</sub>-specific CD8+ cells was set at 0.14%, which represents the mean value plus 3 standard deviations. The frequency of TERT<sub>572Y</sub>-specific CD8+ cells was investigated in 14 patients. Table 3 shows that the administration of the optimized peptide induced TERT<sub>572Y</sub>-specific T cells in 13 (92.8%) out of 14 evaluable patients. Only 1 patient (no. 2) failed to respond to the vaccine. TERT<sub>572Y</sub>-specific cells were detected ex vivo in 4 (28.6%) out of the 14 investigated patients. In 1 patient (no. 11), TERT<sub>572Y</sub>-specific CD8+ cells could be detected ex vivo even before vaccination, and their frequency was increased after in vitro stimulation. Representative results from patient no. 11 are presented in figure 1. Moreover, in 2 additional patients (nos. 1 and 8), in vitro stimulation of the prevaccination peripheral blood mononuclear cells revealed the presence of TERT<sub>572Y</sub>-specific

**Table 3.** Percentage of tetramer TERT<sub>572Y</sub>-positive CD8 cells among PBMC of vaccinated patients

Patient	Before vaccination		After 2 injections with the optimized cryptic peptide TERT <sub>572Y</sub>		After completion of the entire vaccination program	
	unstimulated	stimulated	unstimulated	stimulated	unstimulated	stimulated
1	0.14	<i>0.29</i>	<i>0.69</i>	<i>1.25</i>	ED	ED
2	0.02	0	0	0.11	0	0.1
3	0	0	0.11	<i>1.14</i>	NT	NT
4	NT	NT	0.05	<i>4.00</i>	0.02	<i>0.48</i>
5	0.01	0	0.06	<i>0.36</i>	ED	ED
6	0	0.01	0	<i>4.20</i>	NT	NT
7	0.02	0.14	0.01	<i>0.42</i>	0.12	<i>0.36</i>
8	0.05	<i>0.33</i>	<i>0.33</i>	<i>0.98</i>	NT	NT
11	<i>0.2</i>	<i>1.00</i>	<i>0.7</i>	<i>1.30</i>	0.05	<i>0.52</i>
12	0.04	0.11	0.10	<i>0.98</i>	0.12	<i>1.16</i>
13	0	0	0	<i>0.88</i>	<i>0.32</i>	<i>0.48</i>
15	0	0.04	0.05	<i>0.45</i>	NT	NT
18	0	0	0.06	<i>0.62</i>	ED	ED
19	0	0.03	0	<i>0.73</i>	ED	ED

Figures in italics indicate percentages above background. ED = Early discontinuation; NT = not tested.

CD8+ cells. In all 3 patients, the frequency of TERT<sub>572Y</sub>-specific CD8+ cells was increased after the administration of 2 injections of the optimized peptide, both ex vivo and after in vitro stimulation. In patient no. 13, TERT<sub>572Y</sub>-specific T cells were not detected in prevaccination blood samples, neither ex vivo nor after in vitro stimulation, but they appeared after the sixth vaccination.

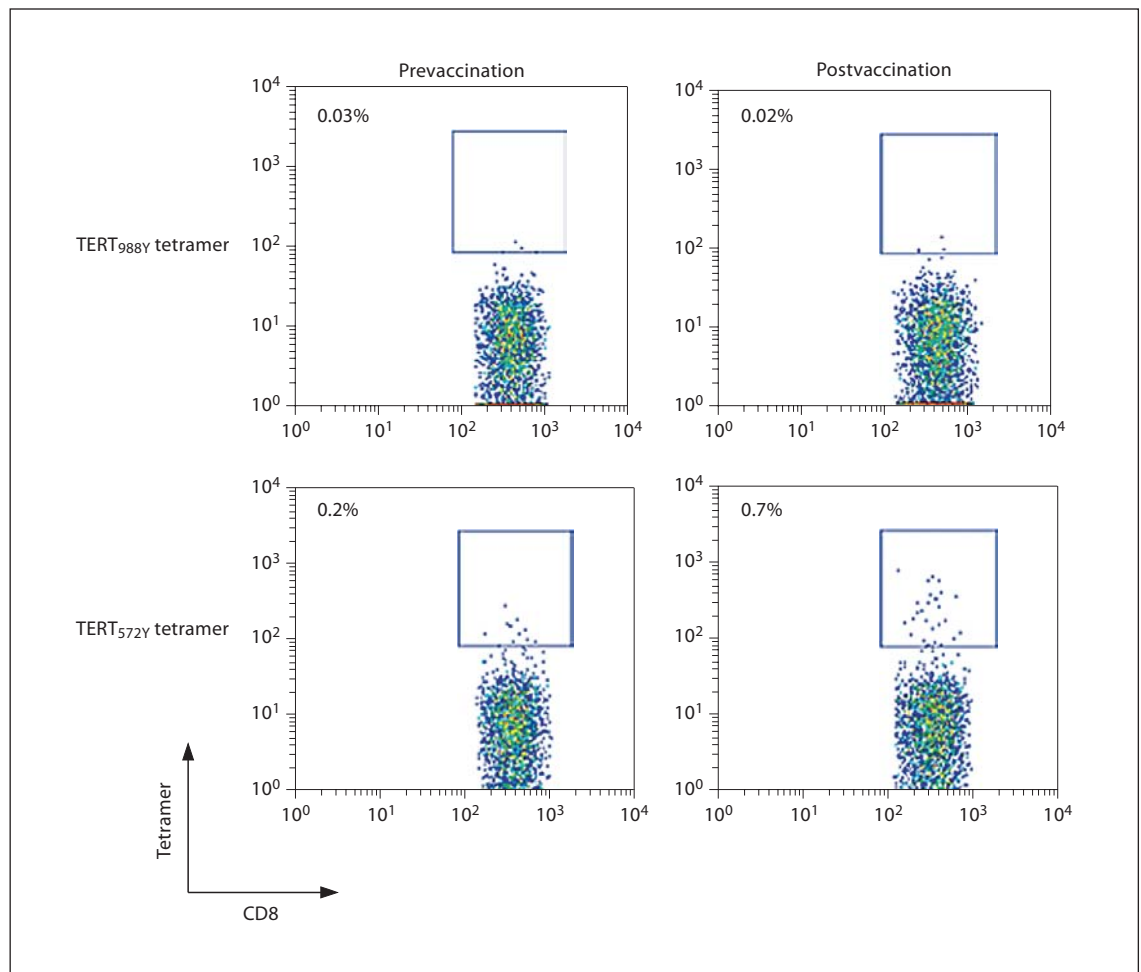
TERT<sub>572Y</sub>-specific cells were also detected in 9 patients (nos. 3–7, 12, 15, 18 and 19; 64.3%) after in vitro stimulation, 3 weeks after the second injection with the optimized TERT<sub>572Y</sub> peptide. TERT<sub>572Y</sub>-specific CD8+ cells were still detected after the sixth vaccination in 5 out of 6 patients tested (nos. 4, 7 and 11–13). In 2 patients (nos. 13 and 11) with stable disease, 1.85 and 1.1% TERT<sub>572Y</sub>-specific CD8+ cells were measured 3 and 14 months after the end of the vaccination protocol, after in vitro stimulation, respectively.

## Discussion

The aims of the present phase I vaccination study were to determine the maximum tolerated dose and to evaluate the toxicity of the optimized cryptic peptide TERT<sub>572Y</sub>, presented by HLA-A\*0201, when used as antigen in vaccination protocols in patients with advanced solid tumors. The TERT<sub>572Y</sub> peptide is derived from TERT, a uni-

versal tumor antigen overexpressed by 85% of tumors, by substituting its first amino acid by a tyrosine, thus increasing its immunogenicity [13]. Our results showed that TERT<sub>572Y</sub> vaccination of patients with advanced cancer is well tolerated and did not appear to induce autoimmune reactivity against TERT-expressing normal tissues even after a median postvaccination follow-up period of 10.7 months. These results offer the first human in vivo confirmation that optimized cryptic peptides could be safely used for tumor immunotherapy.

Tumor antigens are nonmutated self-proteins expressed by normal tissues, including the thymus, and are involved in tolerance induction. Tolerance, the process by which CTL, mainly those with high avidity, are purged from the T cell repertoire, is a major barrier hindering the development of effective antitumor T cell responses. However, tolerance mainly shapes the T cell repertoire specific for dominant rather than cryptic peptides [1, 7]. Using a humanized mouse model, our group recently showed that vaccination with two optimized cryptic peptides derived from murine TERT (TERT<sub>572Y</sub> and TERT<sub>988Y</sub>) recruited high-avidity CTL capable of eliciting potent antitumoral immunity [8]. Conversely, vaccination with the native peptides did not stimulate any immune response [14]. In the present clinical study, more than 90% of the evaluated patients developed TERT<sub>572Y</sub>-specific CD8+ lymphocytes. However, no functional



**Fig. 1.** Expansion of TERT<sub>572Y</sub>-specific CD8 cells in vaccinated patients: TERT<sub>572Y</sub>-specific CD8 cells in fresh PBMC from patient No. 11 were detected before and after vaccination by using TERT<sub>572Y</sub> tetramers, but not with the irrelevant TERT<sub>988Y</sub> tetramers (control).

studies of TERT<sub>572Y</sub> CD8<sup>+</sup> cells were performed in the present trial, and therefore, it is yet unclear whether these peptide-specific CD8<sup>+</sup> cells are capable of killing TERT-overexpressing tumor cells. In contrast, only 50% of patients treated with the dominant peptide TERT<sub>540</sub> emulsified in Montanide responded to the vaccine [19]; it is interesting to note that the natural processing of the dominant TERT<sub>540</sub> described initially [3, 20, 21] could not be confirmed in more recent studies [19, 22], possibly suggesting that TERT<sub>540</sub> does not belong to the immunological self. Given this ambiguity regarding the presentation of the dominant TERT<sub>540</sub> peptide, a direct randomized comparison with the cryptic peptide could produce results which would be very difficult to interpret.

In the present study, 2 injections of the optimized cryptic peptide induced the development of TERT<sub>572Y</sub>-specific CD8<sup>+</sup> in more than 90% of the vaccinated patients. It is interesting to note that in 3 patients, pre-existing TERT<sub>572Y</sub>-specific T cells were detected either ex vivo or after in vitro stimulation. The reasons for the presence of these cells in nonimmunized patients are not obvious; we cannot exclude that in some cancer patients, some proteins from apoptotic tumor cells could induce an in vivo stimulation of the immune system leading to the development of specific T cells. On the other hand, the functional properties of these pre-existing TERT<sub>572Y</sub>-specific CD8<sup>+</sup> lymphocytes remain to be demonstrated. Unfortunately, the present study was not designed to evaluate the functional capacity of TERT<sub>572Y</sub>-specific

CD8+ cells. However, it is interesting to note that the frequency of these pre-existing TERT<sub>572Y</sub>-specific CD8+ lymphocytes was enhanced after the administration of 2 injections of the optimized cryptic peptide, suggesting a 'boosting' effect of the optimized peptide in already existing specific CD8+ lymphocytes. These findings are consistent with an important inter-patient variation of in vivo induction of TERT peptide-specific CD8+ clones in cancer patients. This hypothesis is further supported by the observation that in some patients, the vaccination with the optimized peptide did not allow the ex vivo detection of TERT<sub>572Y</sub>-specific CD8+ cells but these cells were revealed only after the in vitro stimulation of patients' T lymphocytes. Taking into account the limitations of our study concerning the functionality of the detected TERT<sub>572Y</sub>-specific CD8+ cells, our immunophenotypic data indicate that vaccination with optimized TERT<sub>572Y</sub> peptide may result in a higher CD8+ cell response rate than that obtained in the roughly 50 clinical studies of tumor vaccination reported so far [6, 23, 24]. It is also noteworthy that almost all previous clinical studies showing high immune response rates involved patients with minimal disease and excellent performance status [3, 25–27], although Scheibenbogen et al. [28] demonstrated that immune reactivity in melanoma patients correlated with disease remission; in contrast, in the present study, all patients had end-stage disease. The induction of TERT<sub>572Y</sub>-specific CD8+ clones was dose-independent as already shown for other peptides used in vaccination protocols [27]. In addition, the optimized TERT<sub>572Y</sub> peptide induced an early response of CD8-specific clones (after 2 administrations); this rapid induction of TERT-specific CD8+ cells may be of clinical relevance, especially for patients with rapidly progressive malignancies. In addition, the vaccination protocol used led to a sustained detection of TERT<sub>572Y</sub>-specific CD8+ lymphocytes which could also be of clinical relevance. However, appropriately designed studies are needed in order to address these specific questions.

The hallmark of antitumoral immunity in vivo is autoimmunity. Autoimmunity is acceptable when it targets nonessential normal cells and tissues such as melanocytes, but may hamper vaccine development when it targets essential cells such as hematopoietic precursors. Although TERT is expressed by hematopoietic stem cells, and gut, thymus and activated B and T cells [29, 30], none of our patients who lived long enough after the completion of the vaccination protocol showed signs of autoimmunity, despite the fact that in some of them in whom blood samples were obtained, TERT<sub>572Y</sub>-specific CD8+

lymphocytes were detected even 12 months after vaccination. This confirms our previous results obtained in HLA-A\*0201 transgenic HHD mice vaccinated with TERT<sub>572Y</sub> peptide, which is also part of murine TERT [8]. Vaccinated HHD mice developed antitumor immunity without signs of autoimmunity. Moreover, TERT<sub>572Y</sub>-specific CTL killed tumor cells but not activated B cells; a possible explanation for this preferential destruction of tumor but not normal cells by TERT<sub>572Y</sub>-specific CD8+ lymphocytes could be the significantly lower expression of TERT on normal compared with tumor cells which limits the presentation of low-affinity peptides such as TERT<sub>572</sub>.

The vaccination protocol was extremely well tolerated since the observed toxicity was essentially minimal, with the exception of transient skin reactions caused by the Montanide adjuvant. Anemia and thrombocytopenia were mild and transient in all but 1 case despite continuing the vaccinations. There was no correlation between hematologic toxicity and development of TERT<sub>572Y</sub>-specific CD8+ lymphocytes. Given the limitations of the small number of patients enrolled in this trial and the relatively short follow-up due to the advanced disease, we could safely conclude that this vaccination program is free of any major acute and short-term toxicity. However, in a limited number of patients who were followed-up for more than 6 months after the completion of the vaccination protocol, no clinical or laboratory findings were observed which could be attributed to the vaccination. However, a more accurate assessment of the long-term toxicity profile of the vaccination protocol will have to be evaluated in patients with better prognosis who are more likely to live longer than patients with heavily pretreated end-stage disease.

For ethical reasons, this study involved patients with end-stage cancer, who are not the best candidates for tumor immunotherapy. It is now generally accepted that immunotherapy is best administered to patients with minimal residual disease, and the goal should be to prevent relapse rather than to cure advanced cancer. The inability of vaccines to eradicate actively growing tumors has been clearly shown in animal models [31]. Although response and survival data were not the aims of the present phase I study, an interesting observation was that long-lasting disease stabilization was obtained in 4 patients who presented disease progression before they were enrolled to the present vaccination study; TERT<sub>572Y</sub>-specific CD8+ cells were detected in the blood of these patients even months after completion of the vaccination program. Two of these patients (nos. 9 and 13), both with

renal cell carcinoma, had previously responded to recombinant interleukin 2 or interferon- $\alpha$ , confirming the sensitivity of this cancer to immunotherapy. In contrast, none of the 11 patients with renal cancer who were vaccinated with the dominant TERT<sub>540</sub> peptide had an objective clinical response, even when they developed a peptide-specific immune response [19].

In conclusion, the findings of the present phase I trial demonstrate for the first time that vaccination of cancer patients with the optimized cryptic peptide TERT<sub>572Y</sub> is safe and induces the generation of specific CD8+ clones;

however, additional studies are needed in order to demonstrate that these CD8 clones can destroy tumor cells expressing the native TERT peptide, but also to further evaluate the efficacy and long-term toxicity of the TERT<sub>572Y</sub> peptide in patients with less advanced disease.

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