Telomerase Peptide Vaccination in NSCLC: A Phase II Trial in Stage III Patients Vaccinated after Chemoradiotherapy and an 8-years Update on a Phase I/II Trial

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Key words: Telomerase, lung cancer, multimodal therapy, cancer vaccine, immune response, T helper cell, survival

Conflicts of interest:
PB, JAK, CK, SS, MN, GLH, SA: Nothing to declare
GG: Member of advisory board, Kael Co (current patent holder for GV1001)
MM: Previous employee of GemVax AS

Running title:
Telomerase peptide vaccination in NSCLC
Translational relevance

The present article reports the use of the telomerase peptide vaccine GV 1001 in NSCLC patients. We report a phase II trial investigating GV1001 as an adjuvant after curatively intended chemo-radiotherapy and an 8 year follow-up of a previous phase I/II GV1001 trial. A GV 1001-specific immune response was detected in 80% of patients vaccinated after chemo-radiation (phase II study), and in 54% after vaccine monotherapy (phase I/II trial). The treatment was well tolerated in both studies. Most patients developed progressive disease. Nearly all long time survivors belonged to the immune responders and developed durable immunity (up to 9 years). The phase I/II study update revealed that immune responders had increased overall survival (p<0.001). We also observed increased progression-free survival for immune responders in the phase II trial (p=0.20). These findings support the general concept of combining vaccination with chemo-radiotherapy and warrant a randomized trial evaluating GV1001 in NSCLC patients.
ABSTRACT

Purpose
We report two clinical trials in non-small cell cancer (NSCLC) patients evaluating immune response, toxicity and clinical outcome after vaccination with the telomerase peptide GV1001: A phase II trial (CTN-2006) in patients vaccinated after chemo-radiotherapy and an 8-years update on a previously reported phase I/II trial (CTN-2000).

Experimental design
CTN-2006: 23 inoperable stage III patients received radiotherapy (2 Gy x 30) and weekly docetaxel (20mg/m²), followed by GV1001 vaccination.
CTN-2000: 26 patients were vaccinated with two telomerase peptides (GV1001 and I540). The immune responses were evaluated by T cell proliferation and cytokine assays.

Results
CTN-2006 trial: A GV1001-specific immune response developed in 16/20 evaluable patients. Long term immuno-monitoring demonstrated persisting responses in 13 subjects. Serious adverse events were not observed. Immune responders recorded a median PFS of 371 days, compared to 182 days for non-responders (p=0.20).
CTN-2000 trial update: 13/24 evaluable subjects developed a GV1001 response. The immune responders achieved increased survival compared to non-responders (median 19 months versus 3.5 months; p<0.001). Follow-up of 4 long time survivors demonstrated that they all harboured durable GV1001-specific T cell memory responses and IFNγ^{high}/IL-10^{low}/IL-4^{low} cytokine profiles. Two patients are free of disease after 108 and 93 months, respectively.

Conclusions
Vaccination with GV1001 is well tolerated, immunizes the majority of NSCLC patients and establishes durable T cell memory. The considerable immune response rate and low toxicity in the phase II trial support the concept of combining chemo-radiotherapy with vaccination. The survival advantage observed for immune responders warrants a randomized trial.
INTRODUCTION

Lung cancer remains the leading cause of cancer death in both men and women worldwide. Non-small cell lung cancer (NSCLC) accounts for about 80% of cases, and most subjects present with inoperable stage III or stage IV disease. Metastatic disease (stage IV) carries a dismal prognosis, with a five year survival of 1% (1). The successful treatment of stage III patients depends on the control of both local disease and occult metastases (2). If the disease can be encompassed within an appropriate radiation volume, curatively intended radiotherapy (≥60 Gy) is the treatment of choice. However, the 5-year survival for stage III patients treated with radiotherapy alone is less than 10% (3). Several approaches to multimodality treatment have been investigated, including induction chemotherapy, concurrent chemo-radiotherapy and consolidation chemotherapy (4). However, progress has been limited. Most patients die from relapsed disease, and new treatment strategies are needed (5).

The development of vaccines for NSCLC has received more attention in recent years. Three large phase III trials are currently underway, investigating different vaccine strategies (NCT 00480025, NCT 00409188, NCT 00676507). Herein, we report a phase II trial (CTN-2006) with the telomerase peptide GV1001 in stage III NSCLC patients. The study evaluated GV1001 vaccination shortly after chemo-radiotherapy. We also report an 8-year update on our first GV1001 NSCLC trial, the phase I/II study CTN-2000 (6).

The enzyme telomerase is expressed in most human cancers, including NSCLC (7, 8) and is considered as an attractive target for a universal cancer vaccine (9-14). Telomeric DNA confers stability to chromosomes (15). Normal somatic cells can undergo a limited number of cell divisions because the telomeres are shortened at each mitosis. Tumor cells bypass this biological clock by expressing telomerase, that synthesize new telomere units (9, 16). Peptide GV1001 consists of 16 amino acids derived from the active site of human telomerase reverse transcriptase (hTERT) (17-19). As reported from our previous GV1001 studies, the peptide is recognized on multiple HLA class II molecules encoded by both DP, DQ and DR subloci (6, 20-22). This promiscuous HLA-binding profile suggests that the GV1001 vaccine may be applicable to the general patient population and may elicit a broad T-helper response within each individual. Further, GV1001 includes nested HLA class I epitopes, facilitating recruitment of CD8+ cytotoxic T cells.
There is increased interest in combining cancer vaccines with conventional therapy. Previous concerns that chemotherapy would preclude immunization is making way for a recognition of possible synergistic effects (23-27). In many cases, the majority of tumor cells are eliminated by conventional therapy, but the tumor eventually relapses. Cancer vaccines work through different mechanisms and may be effective against cancer cells resistant to chemo- and radiotherapy. Moreover, chemo-radiation may enhance rather than preclude the immune response. Tissue damage may induce “danger signals” (28) that provide a pro-inflammatory microenvironment. There is also evidence suggesting that gene products induced by radiation make the tumor more susceptible to a T-cell attack (29). Furthermore, lung tumors harbor regulatory T-cells (Tregs) considered to inhibit the host immune response (30), and several studies suggest that chemotherapy may suppress Tregs and myeloid-derived suppressor cells (31-34). Docetaxel, applied in the present regime, has also been suggested to enhance the vaccine response through other mechanisms (35).

**MATERIALS AND METHODS**

Materials and methods for the previous CTN-2000 trial (phase I/II) were described in the original trial publication (6). The following methods description primarily refers to the CTN-2006 trial (Phase II). The T cell assay methods were identical in the two protocols.

**Patients and study protocol**

The primary objective of the phase II trial (CTN-2006) was immunological response. Toxicity and time to progression were secondary objectives. Twenty-three subjects (20 evaluable per protocol) with inoperable stage IIIA/B NSCLC were enrolled between November 2006 and July 2008 from three different centers in Norway. Twelve patients were enrolled at The Norwegian Radium Hospital, four at St Olav’s Hospital and seven at The Southern Hospital of Norway, Kristiansand. The trial was approved by the Norwegian Medicines Agency, the Regional Committee for Medical Research Ethics and the Hospital Review Board. The study was performed in compliance with the World Medical Association Declaration of Helsinki. Signed informed consent was obtained from all patients.
The study population had been treated with weekly docetaxel 20mg/m² and 3 D radiotherapy 2 Gy x 30 within the last 4 weeks. Subjects with metastatic disease were excluded based on a pre-study CT scan of the thorax/upper abdomen and a MRI scan of the brain. The eligibility criteria also included Eastern Oncology Group (ECOG) performance status 0-2, age ≥ 18 years, WBC ≥ 1.5 x 10⁹/L; platelets ≥ 100 x 10⁹/L, Hb ≥ 9g/dL; creatinine ≤ 140 μmol/L, albumin ≥ 2.5 g/L, bilirubin ≤ 20% above the upper limit of normal (ULN), ASAT ≤ 1.5 ULN and ALAT ≤ 1.5 ULN. Exclusion criteria included a history of other prior malignancy, except curatively treated basal cell or squamous cell skin carcinoma or cervical cancer stage IB, active infection requiring antibiotic therapy, serious adverse reactions to vaccines, known autoimmune disease, positive tests for hepatitis B, C or HIV or significant cardiac or other medical illness, such as severe congestive heart failure.

Study design
The strategy behind the study design was to pave the way for a phase III trial in stage III NSCLC patients, evaluating the vaccine within a multimodal treatment regime. The dosage of GV1001 was based on data from our previous dose-escalation trials in NSCLC and pancreatic cancer. The chemo-radiotherapy represented institutional standard treatment in 2006 for inoperable stage III NSCLC. Our decision to include 20 evaluable patients was based on the main study objectives; to demonstrate that combined treatment with chemo-radiotherapy and GV1001 is feasible and may yield immunization, to provide safety data and to obtain an estimate for PFS and immune response rate. In a given sample size, the number of subjects with immune response and serious adverse events (SAE) will follow a binomial distribution. Statistical calculations based on n=20 and a binomial distribution demonstrated: The probability of detecting ≥5 immune responders was 99.8%, assuming a true response rate of 54% as observed in the phase I/II study. The probability of detecting ≥1 SAE was 87.8%, assuming a true SAE frequency of 10%.

Treatment
In the CTN-2006 trial, vaccination with GV 1001 started within 4 days to 4 weeks following the last radiotherapy treatment. Immunization was given in week 1 (Monday, Wednesday and Friday) and once in week 2, 3, 4, 6, 8 and 10. A boost immunization was given in week 14, 18, 22, at month 6 and at month 9. GV1001 (300 nmol peptide in 0.20 ml saline) was injected
intradermally (i.d.) in the lower abdomen. GM-CSF (75 μg Leukine; Bayer, Oslo, Norway) was injected at the same site 10-15 minutes prior to GV1001.

**Peptides**

The vaccine peptide GV1001 corresponds to the 16 amino acid residue 611–626 (EARPALLTSRLRFIPK) of hTERT. GV1001 was supplied by Pharmexa (Horsholm, Denmark). Manufacturing was in compliance with GMP. RAS-peptide 508 (KRAS 52-70, Q61H; Norsk Hydro, Norway) served as a negative control in T cell assays.

**T cell cultures and assays**

Peripheral blood mononuclear cells (PBMCs) were obtained prior to start of therapy, at weeks 6, 10 and at every vaccination thereafter. The PBMCs were isolated and frozen as previously described (36). Thawed PBMCs were stimulated once *in vitro* with the vaccine peptide prior to T cell assays, as described earlier (6, 20, 37). At this initial stimulation, the PBMCs were cultured with GV1001 (25 μM) for 7-10 days, with addition of IL-2 (10U/ml) from day 3.

T cell proliferation assays (3H Thymidine) were performed essentially as previously described (36). Pre- and post-vaccination samples were analyzed in parallel for response to peptide stimulation. The T cells were seeded at 50 000 cells/well, in 96-well-plates. Irradiated autologous PBMCs were used as antigen presenting cells (APCs). The T cells were stimulated with/without GV1001 or an irrelevant peptide (K-RAS 508). Stimulation with Staphylococcal enterotoxin C (SEC) was used as positive control and as a measure of immunocompetence. Proliferation was assessed at day 3, after overnight incubation with 3H-Thymidine. All patients responded to SEC. T cell cultures were tested in triplicates. SEM was usually below 10%. Proliferation counts after stimulation with the irrelevant peptide were generally not significantly different from controls without peptide. T cell responses were considered antigen-specific when the stimulatory index (SI; response with antigen divided by response without antigen) was above 2.

Bioplex cytokine analyses were performed on supernatants harvested 48 hours after T cell stimulation, according to the manufacturer’s protocol (Bio-Rad Laboratories, Hercules, Ca, USA). Supernatants were analyzed in duplicates/triplicates, each parallel kept separate through T cell stimulation and Bioplex assays.
Delayed-Type Hypersensitivity

Delayed-type hypersensitivity (DTH) skin test was performed at baseline, at week 2, 3, 4, 6, 10 and at the time of later vaccinations. For DTH testing, 60 nmol GV1001 in 0.10 ml saline was injected i.d. at a separate site from the vaccine, without GM-CSF. The patients registered the DTH skin reaction 48 hours after administration. A positive DTH test was defined as an erythema/induration with average diameter ≥ 5 mm.

Clinical evaluation

Adverse drug reactions and ECOG performance status were assessed at each visit. Blood screening and a general physical examination were performed at start of vaccination (week 1), week 2, 3, 4, 6, 8, 10 and all later vaccinations. CT scans were performed before start of vaccination, at week 14 and every third month thereafter.

Progression-free-survival was defined as main clinical end point in the CTN-2006 protocol, because overall survival was likely to be influenced by standard treatment after progression. Radiation fibrosis is difficult to reliably distinguish from tumor. Thus, at start of vaccination, the patients had residual CT lesions after chemo-radiotherapy that may, or may not, include viable tumor tissue. The terms complete response, partial response and stable disease were therefore not applicable. Progressive disease was defined as new or progressing lesions, identified by CT scans, bronchoscopy and/or biopsy.

Statistics

Overall survival (OS) and progression-free survival (PFS) were both calculated from start of vaccination. Kaplan-Meier/log rank analysis was applied for comparing immune responders versus non-immune-responders, with regard to OS (CTN-2000 trial) or PFS (CTN-2006 trial). To assess whether the immune response represented an independent prognostic factor, Cox-regression with enter analysis was performed. Disease stage represented the most important identifiable prognostic factor, apart from immune response. For Cox-regression analysis of the CTN-2000 trial, the single stage IIB patient was grouped together with the stage III subjects and compared with the stage IV group. For CTN-2006, the subjects were categorized as stage IIIA or stage IIIB.
RESULTS

PHASE II TRIAL IN STAGE III NSCLC (CTN-2006)

Patient characteristics and adherence to treatment (CTN-2006)
Patient characteristics and treatment details are listed in Supplemental Table S1 (online only).
At the end of 2007, Bayer withdrew liquid GM-CSF from the market. This led to a sudden shortage until the lyophilized product was supplied. Two patients at Radiumhospitalet therefore received one of their GV1001 injections without adjuvant GM-CSF. At St Olav’s hospital, two other patients missed the combined vaccination twice each (week 8 and 10). The study monitoring panel decided that patients missing GM-CSF at two vaccinations (#203 and #204) were to be replaced and excluded from the per protocol analysis. Patient #110 was also not evaluable per protocol, because she was withdrawn at week 8 due to a lung abscess and progression of disease.

Safety (CTN-2006)
The safety population includes all patients who received at least one vaccination (n=23). A total of 323 vaccine doses were administered (8-21 doses per patient). Seven serious adverse events were reported, in six patients. All seven events were regarded as related to underlying disease, not to study therapy. One event, a bronchiolar fistula, was initially reported as probably related to a study drug. However, a bronchoscopy with biopsy demonstrated that the fistula was due to tumor relapse.

Immune response (CTN-2006)
A GV1001-specific T cell response was demonstrated in 16 patients after vaccination, compared to no patients in pre-vaccination samples (Fig. 1A and Supplemental Figure S1). A positive DTH response was observed in one patient only. Three subjects were not evaluable according to the protocol, as described above. The immunological response rate was 70% by intention to treat (ITT) analysis, and 80% per protocol.

To achieve a sustainable clinical effect, development of T cell memory is likely to be required. We therefore provided booster vaccines and monitored the long term development
of immune responses. Follow-up samples were obtained from 15/16 immune responders. The results demonstrated a durable GV1001-specific T cell response in 13/15 subjects, with a maximum observation period of 91 weeks (Table 1 and Fig 1B-C).

Comparing the different centers, we recorded a GV1001-specific T-cell response in 9/12 patients from Radiumhospitalet Oslo (9/11 per protocol), 1/4 patients (1/2 per protocol) from St Olav’s Hospital Trondheim and 6/7 from Kristiansand. The samples from Trondheim and Kristiansand were sent to Oslo for T cell analyses. Some of the first samples were stored overnight prior to PBMC isolation. These samples gave only negative test results. We therefore decided to isolate subsequent samples from the same patients immediately upon arrival. Interestingly, most of the previously negative patients then tested positive. Moreover, later samples from some patients were stored over night and again tested negative (Fig. 1D-F). We also tested samples isolated the same day or stored over night in parallel and observed distinctly stronger responses in freshly isolated samples (Fig. 1G). These observations illustrate the complexity of managing a multi-center trial with T cell analyses, and suggest that T cell data may be misleading if analyses are performed on samples not optimally handled.

**Clinical response (CTN 2006)**

Table 1 lists progression-free survival (PFS), overall survival (OS) and site of relapse. PFS was the clinical end point per protocol and was assessed by CT scans at three month intervals. To date, tumor progression has been recorded for 17/23 patients in the ITT population (median PFS 357 days). Five out of six patients without evidence of relapse are immune responders. Considering all included patients, immune responders recorded increased PFS compared to non-responders, with a median of 371 days versus 182 days (p=0.20; Fig. 2A). Cox-regression analysis suggested that the trend associating a positive immune response with extended PFS remained unchanged after correction for other variables, most importantly the disease stage (Hazard ratio 1.9, p=0.21).

**UPDATE ON PHASE I/II TRIAL (CTN-2000)**

Our first GV1001 trial, CTN-2000, evaluated vaccination of 26 advanced NSCLC patients (mostly stage IV) without concomitant chemo- or radiotherapy. As previously published, we
detected immune responses against GV 1001 in 11 out of 24 evaluable patients during the primary regimen and an additional two patients following booster vaccines (54%) (6). Here, we report survival and long term immunological and clinical follow-up.

Patient characteristics, clinical and immunological responses for CTN-2000 are listed in Table 2 and Supplemental Table S2 (online only). The Kaplan Meier analysis demonstrated that overall survival was significantly increased in immune responders compared to non-responders (p<0.001; log rank test), with a median survival of 19 months versus 3.5 months (Fig. 2B). The analysis was primarily performed on all patients evaluable for immunological response (24/26). To assess the robustness of the observed difference, we also compared survival for immune responders versus non-responders within the stage IV patients (n=19) and within the subjects completing the 10-week vaccination schedule (n=17). Both analyses demonstrated a statistical significant survival advantage for immune responders (p<0.005; log rank test). To further investigate whether the immune response represented an independent prognostic factor, we performed Cox-regression analyses. Disease stage represented the most important identifiable factor, apart from immune response. After correction for disease stage, the analysis indicated a Hazard Ratio of 11 between immune responders and non-responders, with p=0.001.

Two patients from CTN-2000 are still alive, 9 years after start of vaccination. Patient 710 had stage IIIA NSCLC, with an inoperable pulmonary lesion involving mediastinal glands. After GV1001 vaccination, he developed a complete response that has been sustained for nine years (Supplemental Fig. S2, online only). He has in total received 39 vaccine injections over a period of 9 years without any side-effects. Another patient (#727), with stage IIIB NSCLC at study entry, has received 43 vaccines and has no evidence of disease after 93 months. These patients are still receiving GV 1001 every 6th and 3rd month, respectively. Telomerase-based vaccines carry a putative risk of bone marrow toxicity. We have therefore closely monitored hematological counts and also performed regular hematopoietic progenitor cell assays on bone marrow samples. The results do not suggest any hematological toxicity (data not shown).

Long term immuno-monitoring was performed on four long term survivors, including patients 710 and 727. All four subjects exhibited strong and durable GV1001-T cell responses (Fig. 3). In patients 712 and 725, T-cell assays were performed on blood samples taken 22 and 43
months after last vaccination. GV1001-specific T-cells were still present, suggesting that the vaccination had induced durable T cell memory (Fig. 3B, C). Patient # 712 had bilateral lung metastases (stage IV) at start of vaccination, but experienced disease stabilization after vaccination and survived for six years.

A Th-1 like cytokine profile is considered desirable for cancer eradication (38). In three long term survivors (patients 710, 712 and 725), we investigated the cytokine patterns by use of Bioplex assays. The responses in all three subjects exhibited high levels of key Th1 effector cytokines IFNγ and TNFα, and low levels of IL4 and IL-10 (Fig. 4). Secretion of the Th2-like cytokines IL-5 and IL-13 was also detected. The implications of these findings are discussed below.

DISCUSSION

We report a phase II trial and an update on a phase I/II trial that both investigated vaccination of NSCLC patients with the telomerase peptide GV1001. Taken together, the two clinical studies included 49 patients, and no treatment related serious adverse effects were observed. The phase II study demonstrated an 80% immune response rate per protocol. This response rate is high compared to most cancer vaccine trials, including those investigating telomerase-based approaches (17, 18). Furthermore, both studies demonstrated the generation of durable GV1001-specific T cell memory responses. The phase I/II study update revealed that immune responders had increased survival compared to non-responders, and that the four subjects with most extended survival all harbored sustained T cell memory activity. The multimodal approach, the availability of long term data and the association between immune response and clinical outcome are of particular interest within this report. Below, we particularly focus the discussion on these issues.

There are a number of theoretical arguments suggesting that cancer vaccines may be most effective if applied in combinatory regimes, but sparse data from clinical studies on how the different modalities interact (24, 26, 39). Interestingly, the frequency of immune responders in the CTN-2006 trial was superior to our two GV1001 trials investigating vaccination as monotherapy, where we observed a response rate of about 60% (6, 20). Furthermore, we
observed a similar high response rate of 78% in a recent trial combining GV1001 vaccination with temozolomide in melanoma patients (22). These findings suggest that the chemo-radiation (NSCLC) or chemotherapy (melanoma) applied in these trials did at least not impair immunization and may have contributed favourably to the immune response. Long HLA II-matched peptides like GV1001 may be particularly suited for combined protocols, compared to the short HLA-class I matched epitopes that are used in most peptide vaccine studies. The long peptides recruit CD4+ T-helper cells that are known to interact extensively with other immune cells (38, 40, 41). In tumor tissue pre-treated with chemo- or radiotherapy, we hypothesize that GV1001-specific T-helper cells may engage APCs presenting antigens from apoptotic tumor cells and induce epitope spreading. We address this issue in ongoing studies on long term survivors from GV1001 trials and have identified responses against hTERT epitopes outside GV1001 (unpublished). In two new phase I/II trials in patients with NSCLC or prostate cancer, we plan to combine conventional therapy and peptide vaccination using a mixture of three of these novel hTERT peptides.

There is limited knowledge on the long term development of cancer vaccine responses and on how to design booster vaccine schedules. Some subjects in the present GV1001 trials tested negative in the first post-vaccination immuno-assays, but developed detectable T cell responses after several months of booster injections ((6) and data not shown). Likewise, we have observed in previous studies with GV1001 and other vaccines that T-cell responses appear to be enhanced by booster vaccination (21, 42). These findings suggest that repeated vaccination over an extended period of time yields a higher immune response rate and more durable responses. The question of how long to continue booster vaccination, remains to be clarified. Here, we reported that patients immunized for 6-12 months had persistent GV1001-responses in samples obtained up to 43 months after last vaccine. The latter observation suggests that GV1001-vaccination may provide durable T cell memory. In trials with mutated RAS peptides, we have also observed long term T cell responses without booster vaccination (21, 43). These findings suggest that, after establishing a robust response, it may be sufficient to provide booster injections less frequent than applied for patients #710 and #725.

The cytokine analyses of responses in long term survivors demonstrated high levels of key Th1 effector cytokines INFγ and TNFα, and low levels of IL4 and IL-10. This cytokine pattern may suggest a favorable balance between immunity and tolerance, in particular as IL-10 is secreted by regulatory T cells (44). If these responses were analyzed only for IFNγ, IL-4...
and/or IL-10, as is common in vaccine trials, they may easily be designated as “Th1”. One may therefore note, that we detected considerable levels of the key Th2 cytokines IL-5 and IL-13 (Fig. 4). The latter observation is in line with our finding in other studies, that cytokine profiles in cancer vaccine trials frequently do not follow a Th1/Th2 delineation (21, 22, 42). It has been suggested that Th2 cytokines may arise in response to powerful immuno-activation. In the present long term survivors, the wide range of Th1/Th2 cytokines may also point to a polyfunctional response. Several studies, in particular of infectious diseases, have suggested that polyfunctional cytokine profiles are associated with protective immunity (45-47).

In both previous GV1001 trials without chemotherapy (6, 20), the frequency of immune responders was similar as assessed by DTH-recordings or T cell assays. By contrast, most subjects were DTH negative in the GV1001 trial with concomitant temozolomide (22) and in the present study, where vaccination followed shortly after chemo-radiotherapy. In the GV1001/temozolomide trial, the DTH-reactions were negative during standard study treatment in all patients. Interestingly, three subjects turned positive after omission of temozolomide and continuous booster vaccination. These observations may point to a modulating effect of chemotherapy on the GV1001 response. DTH-reactions have been associated with Th-1 profiles. However, our cytokine analyses indicate that the present DTH negativity does not reflect low levels of Th1 cytokines ((22) and unpublished).

Telomerase is expressed by normal stem cells. It is therefore notable that stem-cell related toxicity did not materialize in the CTN-2006 trial, where GV1001 vaccination was initiated shortly after heavy chemo-radiotherapy. Regarding the long term safety, we have monitored 19 study subjects for more than two years without detecting toxicity (Table 1 and Table 2). Moreover, the data from patients #710 and #725 suggested that booster immunization exceeding eight years was well tolerated. Continued monitoring of their bone marrow samples and peripheral blood counts has not revealed any toxicity. In melanoma and colon cancer patients, we have also observed unchanged hematological counts after several years of GV1001 vaccination (21, 22).

A majority of patients in the two NSCLC trials experienced progressive disease in spite of their immune response. On the other hand, we observed durable tumor responses in some subjects and found that nearly all long term survivors belonged to the immune responders. The survival data from CTN-2000 indicate a substantial difference between immune
responders and non-responders, with a median survival of 19 months versus 3.5 months (p<0.001). Some patients stopped study treatment before week 10 due to disease progression. This issue may represent a confounding factor, as these subjects may have had too short time to develop an immune response. However, even after excluding these subjects, a statistical significant survival advantage for immune responders was observed. This finding is in line with an overall survival difference (p<0.001) observed in our GV1001 trial in pancreatic cancer patients (20). A similar advantage for immune responders, though not reaching statistical significance, was observed for OS and PFS in our recent melanoma GV1001 study (22) and for PFS in the CTN-2006 trial reported here. Considering the different patient populations in the two present NSCLC trials, it is not appropriate to conduct a statistical analysis of survival in the two studies taken together. It is still interesting, that 12/13 patients surviving more than 1000 days to date are immune responders. Of note, these uncontrolled trials were not designed to determine the cause of any association between immune response and clinical outcome. To answer the question of clinical efficacy, a large randomized trial is needed.

We conclude that GV1001 vaccination immunizes a high proportion of NSCLC patients. The high immunological response rate is encouraging and indicates that the vaccine may be useful for the general patient population without prior HLA typing. Moreover, GV1001 vaccination induces long term T cell memory against telomerase antigens, while not compromising bone marrow function. The particular high immune response rate and low toxicity observed in the phase II trial support the concept of combining vaccination with chemo- or radiotherapy. This is of interest for the clinical development of both GV1001 and other cancer vaccines. The phase I/II trial update further demonstrated a strong correlation between immune response and survival. Taken together, the findings warrant a randomized GV1001 trial in NSCLC patients.

ACKNOWLEDGEMENTS

Pharmexa supplied GV1001 free of charge. The authors thank S Trachsel, KT Lande and V Roness for valuable technical assistance. We also thank doctors and nurses at the study hospitals for excellent clinical follow-up and patient care. Special thanks to S Dueland, OT Brustugun, K Dolven-Jacobsen, K Øwre, I Sve and C Hoelstad.
FIGURE LEGENDS

Figure 1

**GV1001-specific T cell responses (CTN-2006).** PBMCs were obtained prior to start of therapy, at weeks 6, 10 and at every vaccination thereafter. The PBMCs were stimulated once in vitro and tested for proliferation against irradiated PBMCs +/- peptide GV1001. Columns represent mean cpm or mean stimulatory index (SI; response with GV1001 divided by response without GV1001). If the recorded cpm or SI exceeds the upper limit of the respective chart, the exact cpm or SI is annotated at the top end of columns. 

A, the diagram shows pre- and post vaccination T cell responses from all evaluable patients. For each subject, the time point with highest SI is displayed. Responses with SI>2 were considered GV1001-specific.

B-C, long term T cell memory. The diagrams shows development of T cell responses as recorded from follow-up samples.

D-F, Samples stored overnight prior to PBMC isolation (*) mostly tested negative, even in subjects where freshly isolated samples tested positive.

G, PBMC samples isolated upon arrival or stored over night were tested in parallel.

Figure 2

**Survival.** Progression-free survival (CTN-2006) or overall survival (CTN-2000) was assessed by Kaplan-Meier analysis. Immune responders were compared to non-responders. Progression-free-survival was defined as clinical end point in the CTN-2006 protocol, because standard treatment after progression was likely to influence overall survival (stage III patients).

A, CTN-2006: Progression-free survival (PFS) for study patients with/without a GV1001-specific T cell response. Observed PFS is extended for the immune responders, but the study is underpowered to demonstrate a statistical significant difference (log rank test; p= 0.20).

B, CTN-2000: Survival for study patients with/without a GV1001-specific immune response. The analysis demonstrated a statistically significant survival advantage for immune responders (log rank test; p< 0.001).
Figure 3

Durable T cell memory responses in long term survivors (CTN-2000). PBMCs obtained at different time points were stimulated once in vitro and tested for proliferation against irradiated PBMCs +/- peptide. The assays show durable GV1001-specific responses throughout the clinical response periods in all four patients. Columns represent mean cpm of triplicates.

Figure 4

Cytokine patterns in clinical responders (CTN-2000). Post-vaccination PBMCs were stimulated once in vitro and tested for proliferation against irradiated PBMCs +/- peptide GV1001. Supernatants were analyzed in duplicates by Bioplex cytokine assays. Columns represent mean concentration (pg/ml).
REFERENCES

Figure 1

A

CTN-2006

Before vaccination

After vaccination

B

Patient #105

Week

C

Patient #107

Week

D

Patient #205

Week

E

Patient #302

Week

F

Patient #307

Week

G

Patient #107: Week 18 samples

PBMC + T

PBMC + T + GV1001

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Figure 2

A

CTN 2006

Immune responders
Non-responders
Censored (no progression to date)
Censored (no progression to date)

p=0.20

Progression-free proportion

Days

B

CTN-2000

Immune responders
Non-responders
Censored (alive)

p<0.001

Survival proportion

Days
Figure 3

A

Patient #710

Stimulatory Index

Week


B

Patient #712

CPM

Week

1 10 32 51 235

Last vaccine

C

Patient #725

CPM

Week

1 10 32 48 49 66 67 163

Last vaccine

D

Patient #727

Stimulatory Index

Week

2003 2004 2005 2006 2007 2008 2009 2010
Figure 4

Patient 710

Patient 712

Patient 725
Table 1: CTN-2006

<table>
<thead>
<tr>
<th>Patient</th>
<th>Evaluable per protocol</th>
<th>T cell response</th>
<th>Long term T cell response</th>
<th>Site of relapse</th>
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1 Not evaluable per protocol (included in Intention to treat population)
2 Long term T cell response. POS: GV100/1-specific response after week 20 (last available and positive sample is indicated in brackets, w=week). NEG: Turning negative within week 20. NT: Not tested after week 15
3 No evidence of relapse at time of reporting (Dec 1st 2010)
4 Alive at time of reporting (Dec 1st 2010)

| Median: 357 | Median: 877 | Mean: 519 | Mean: 833 |

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Table 2: CTN-2000

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1. 12/20 patients did not complete the vaccination period (9 vaccines) prior to first clinical evaluation at week 12, due to early disease progression. Four patients have received additional booster vaccines.
3. GV1001 specific immune response, as demonstrated in T cell assays (n=10) and/or DTH recordings (n=0).
4. Patient 710 has a complete response, durable to date (106 months observation).
5. Patient 727 has residual fibrosis, with no detectable disease (63 months observation).
Telomerase Peptide Vaccination in NSCLC: A Phase II Trial in Stage III Patients Vaccinated after Chemo-radiotherapy and an 8-years Update on a Phase I/II Trial


Clin Cancer Res Published OnlineFirst September 14, 2011.