Telomerase peptide vaccination combined with temozolomide: A clinical trial in stage IV melanoma patients

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TRANSLATIONAL RELEVANCE

Cancer vaccination and chemotherapy attack the tumor from different angels and may synergize to overcome tumor escape. Chemotherapy may facilitate immunization by provoking inflammation and eliminating regulatory T cells. The present trial evaluated combined therapy with temozolomide and the telomerase peptide vaccine GV1001 in advanced melanoma patients. The results demonstrated that 18/23 subjects developed a specific immune response, without notable toxicity. Patients developing long term immunological memory survived longer than those rapidly losing their responses. Survival was extended compared to matched controls from a benchmark meta-analysis (one year: 44% versus 24%). Five patients developed partial tumor regression, including one survivor that is negative on PET-CT scans after 5 years. The 78% immune response rate is considerable compared to most vaccine trials, including previous GV1001 studies without concomitant chemotherapy. The results warrant further studies of GV1001/temozolomide treatment and support the concept of combining cancer vaccination with chemotherapy.
ABSTRACT

Purpose: The study is a proof-of-principle trial evaluating toxicity, immune response and clinical response in melanoma patients after combined therapy with temozolomide and the telomerase peptide vaccine GV1001. Our previous GV1001 trials demonstrated immune responses in ~60% of lung- or pancreatic cancer patients.

Experimental Design: Twenty-five subjects with advanced stage IV melanoma (M1B or M1C) received concomitant temozolomide and GV1001. Temozolomide was administered 200 mg/m² orally for 5 days every fourth week, and GV1001 as eight injections over 11 weeks. Immune response was evaluated by DTH, T cell proliferation and cytokine assays. The immunological responders continued monthly vaccination.

Results: The treatment was well tolerated. A GV1001-specific immune response was demonstrated in 18/23 evaluated subjects (78%). Patients developing long term T cell memory survived longer than those rapidly losing their responses. The immune response exhibited several characteristics of possible clinical significance, including high IFNγ/IL-10 ratios, polyfunctional cytokine profiles and recognition of naturally processed antigens. Survival compared favourably to matched controls from a benchmark meta-analysis (one year: 44% versus 24%, two years: 16%
versus 6.6%). The clinical responses developed gradually over years, contrary to what is expected from chemotherapy. Five patients developed partial tumor regression and six more recorded stable disease. One patient has no remaining disease on FDG-PET scans after 5 years.

Conclusions: The immunological response rate is considerable compared to previous GV1001 trials without concomitant chemotherapy, while low toxicity is retained. The results warrant further studies of GV1001/temozolomide treatment and support the general concept of combining cancer vaccination with chemotherapy.
INTRODUCTION

Cancer chemotherapy had been to a large extent ineffective until the development of broad regimes combining different agents. The limited efficacy of monotherapy reflects tumor heterogeneity and genetic instability, favouring escape of resistant tumor subpopulations. Cancer vaccination offers a different angle of attack, as compared to cytostatics, and may therefore be particularly effective in combinatory regimes. However, most chemotherapeutic agents are immunosuppressive, and it was long assumed that vaccines should therefore not be combined with chemotherapy. More recent knowledge has challenged this conventional wisdom (1, 2). In particular, it is discussed whether chemotherapy may be utilized to counter tumor protection from regulatory T cells (Tregs), while prevailing the immunocompetence necessary for vaccine response (3-5).

Here we report a proof-of-principle trial (phase I/II) of combined therapy with temozolomide and telomerase peptide vaccination in 25 stage IV melanoma patients. The primary objectives were toxicity and immunological response. Tumor response was a secondary study objective. Current prognosis for metastatic melanoma is dismal, with median survival of 4-9 months in patients with visceral involvement (6, 7). Dacarbazine has been considered standard treatment, but the tumor response rate is only 6-15%, mostly partial responses, and there is no documented effect on survival (7). The oral drug temozolomide is metabolized to dacarbazine in vivo and carries a potential advantage in penetration of the blood-
brain barrier. In phase III trials, temozolomide and dacarbazine have produced similar response rates (8, 9).

Telomerase represents a possible target for universal cancer vaccines (10, 11). The enzyme maintains telomere length in dividing cells and is considered essential for tumor growth. Telomerase activity has been demonstrated in all studied cancer forms, including stem-cell like tumor cells (12-14). Interesting results have been reported from phase I/II studies with three telomerase peptide vaccines (15-20). Peptide GV1001 represents a 16 amino acid sequence (EARPALLTSRLRFIPK) from the active site of human telomerase reverse transcriptase (hTERT) (21, 22). The peptide was selected based on computer algorithms predicting strong HLA class II binding properties and multiple nested HLA-class I binding motifs. The GV1001 vaccine may thus recruit both CD4+ T-helper cells and CD8+ cytotoxic T cells. We have previously conducted two phase I/II trials with GV1001, in pancreatic and lung cancer patients (19, 20). Both studies were conducted without chemotherapy. The results indicated no serious adverse effects, a GV1001-specific immune response in ~60% of patients and an association between immune response and survival. T cell clone studies demonstrated both T helper- and cytotoxic responses (19, 20, 23). In the present trial, we hypothesized that temozolomide may enhance the immune response by inducing antigen release or modulating tumor tolerance. The study is to our knowledge the first clinical trial evaluating combinatory therapy with temozolomide and a cancer vaccine.
PATIENTS AND METHODS

Patients

Twenty-five subjects with stage IV melanoma were enrolled between January 2004 and November 2005. Inclusion criteria: Histologically confirmed melanoma, non-resectable metastatic disease (AJCC stage IV), any HLA genotype, measurable tumor, age > 18 and < 75 years, Eastern Cooperative Oncology Group (ECOG) performance status 0-2, adequate haematological, renal and hepatic function. Exclusion criteria: Previous chemotherapy, clinical signs of brain metastases, severe cardiac disease, severe active infections, need for immunosuppressive medication. Screening for brain metastases was not performed.

The trial was approved by the Norwegian Medicines Agency, the Regional Committee for Medical Research Ethics and the Hospital Review Board. It was performed in compliance with the World Medical Association Declaration of Helsinki. Written informed consent was obtained from all patients.

Treatment

The treatment schedule is shown in Appendix Figure A1. Temozolomide was administered according to standard recommendations for malignant melanoma, 200 mg/m² orally on 5 consecutive days every 28 days. GV1001 was given as three injections during week 2 (Monday, Wednesday, Friday), 2 injections during week 3.
(Monday, Friday) and single injections at weeks 6, 7 and 11. Immune responders were given booster vaccines at 4 week intervals. GV1001 (300nmol peptide in 125 μl saline) and adjuvant GM-CSF (75 μg) was administered by intra-dermal injection in the right para-umbilical area.

The duration of treatment depended on the clinical and immunological response, according to the protocol: Patients with objective tumour response or stable disease at week 12 and immunological response received additional cycles with Temozolomide and GV1001, with response evaluation at every third cycle. Patients with objective response/stable disease without immunological response received additional cycles with Temozolomide only. Patients with progressive disease and immunological response discontinued temozolomide, but were offered booster vaccination with GV1001 every 4 weeks. All patients completing the first 7-week sequence of vaccines (23/25 patients) were considered evaluable for immunological response.

**Clinical evaluation**

Tumor response was assessed by CT scans and clinical examination at 12-week intervals, and classified according to the Response Evaluation Criteria in Solid Tumors (RECIST)(24). Tumor response and survival was evaluated by intention-to-treat analysis.
Blood screening and assessment of adverse drug reactions were performed at each visit. Adverse events were graded according to the National Cancer Institute common toxicity criteria (NCI-CTC) version 3.0 and considered related to treatment if the relationship was reported as probable or suspected.

**Immunological evaluation**

PBMCs were obtained at weeks 0, 5, 9 and 12 and at every third booster vaccination. Pre- and post-vaccination samples were analyzed in parallel for proliferation response to GV1001 peptide stimulation (³H-Thymidine assays). T cell responses were considered GV1001-specific when the stimulatory index (SI; response with antigen divided by response without antigen) was above 2. Specific immune responses were further characterised by cytokine assays and T cell clone experiments (HLA restriction, specificity for truncated peptides, response to recombinant hTERT protein). Materials and methods for immunological assays, DTH recording, flow cytometry and production of peptides and recombinant hTERT protein are described in Appendix 1.

**Statistics**

The study reports overall survival and progression free survival, calculated from start of study treatment. Overall survival for the intention-to-treat population, calculated using the Kaplan-Meier method, was compared with predicted survival based on a meta-analysis of clinical trials including 2100 patients (6). The meta-analysis was conducted by Korn and co-workers for providing survival benchmarks.
for small scale trials. We performed the calculation as recommended (6). Briefly, prognostic factors (sex, ECOG status, metastatic category) from our patients were inserted into a mathematical formula generating predicted survival. The calculation was based on trials where brain metastases were not excluded, because we did not perform screening for brain involvement (see Discussion). A 95% confidence interval was generated for survival of the study group (n=25), while the predicted survival curve was regarded as a fixed estimate (Fig 3). Survival after one year for the study group was compared with predicted survival by use of Student’s t-test.

RESULTS

Patient characteristics

Twenty-five patients with advanced stage IV melanoma were enrolled (Table 1). The majority (16/25) had M1C disease (visceral metastases or elevated LDH); the remaining nine patients had M1B disease (pulmonary metastases). Metastatic sites, age, sex and treatment details are listed in Appendix Table A1.

Toxicity

The combined therapy with temozolomide and GV1001 was well tolerated. One patient developed transient neutropenia (CTC grade IV) and thrombocytopenia (grade III), considered related to temozolomide. Otherwise, no treatment related
grade III-IV toxicity was observed. Milder side effects were recorded in all patients, most commonly representing nausea, vomiting, fatigue or local skin reactions at the injection site. Allergic reactions (grade II) were observed in two subjects. Finally, there was no evidence of long term toxicity in follow-up samples from patients with extended survival, in spite of durable GV1001-specific responses and repeated booster vaccination (maximum observation 5 years, see below).

**Induction of immune responses**

T cell proliferation assays were performed on pre- and post vaccination PBMCs from 23/25 patients, i.e. all patients where the relevant PBMCs were obtained. A GV1001- specific T cell response was demonstrated in 18/23 (78%) of patients after vaccination (Fig. 1A). Six subjects had a GV1001-response prior to vaccination (Fig. 1A), suggesting spontaneous priming against naturally expressed GV1001 or cross-reacting epitopes. The stimulatory index increased substantially after vaccination even in these six patients (2.1→79 ; 3.2→35 ; 4.6→29 ; 7.5→24 ; 11→116 ; 26→122). Among those developing de novo responses, 40% developed a response by week 5 and 90% by week 9.

The DTH-reactions were negative during standard study treatment in all subjects where recordings were obtained, including 11 immune responders in T cell assays. Interestingly, three patients turned positive after omission of temozolomide and continuous booster vaccination. In our previous GV1001 trials without temozolomide (19, 20), the DTH reactions were positive in a substantial number of
patients (26/62). These observations may point to a modulating effect of temozolomide on the GV1001 response. DTH-reactions have been associated with Th-1 profiles, but cytokine analyses demonstrated that the present DTH negativity did not reflect low levels Th1 cytokines (see below).

**Development of long term T cell memory**

Most cancer vaccine trials have for practical reasons been limited to short term vaccination and immuno-monitoring, while clinical efficacy probably requires long term T cell responses (25, 26). Here, immune responders were offered continued monthly vaccination. Long term follow-up revealed that 10/12 patients continuing vaccination developed durable GV1001-specific T cell activity. Strikingly, the 10 patients with durable T cell responses at month 6 all survived longer than the two patients rapidly losing their responses (Table 1). Moreover, the survivors exhibited retained responses in follow-up samples obtained at later time points, ranging from week 36 to week 258 (Fig. 1B, Table 1 and data not shown).

**Tumor response**

All 25 patients enrolled had progressive disease at study entry and belonged to the most advanced categories within stage IV (64% M1C; 36 % M1B; 0% M1A). Interestingly, five subjects developed partial tumor regression (PR). Their tumor volumes decreased by 65-96% (Fig 2A). An additional six patients experienced disease stabilization, while 14 subjects had continued progressive disease (PD). All five patients with PR developed GV1001-specific T cell responses, and the
immune responses prevailed throughout their tumor regression periods (Fig 1B). Interestingly, the clinical responses developed far more gradually than expected from chemotherapy, with only one reaching PR at week 12, three more within week 36 and the fifth at week 48.

Patient 19 (P19) had disseminated MIC melanoma at study entry, including multiple visceral metastases. After start of vaccination, no new lesions appeared. The total tumor diameter started gradually decreasing, reached PR at week 48 and has continued to regress over a period exceeding 5 years (Fig 2B). Remarkably, the patient has no symptoms of tumor disease, and all residual CT-lesions are negative on FDG-PET scans. As melanomas are highly PET-sensitive, the patient may have a complete response. She currently receives booster vaccines at 3-month intervals and retains a strong GV1001-specific immune response (Fig 1B).

Patient 11 (P11) had multiple metastases to lung and mammary glands at study entry. CT-evaluations demonstrated a 29% regression in total tumor diameter at week 12, improving to 74% at week 36. All five metastases regressed partly or completely (Fig 2A and Appendix Fig A3). We recorded PD at week 84 due to a new subcutaneous lesion, but continued vaccination and temozolomide. The patient stayed in good general health for 3 years, before developing brain metastases. Subjects P13, P16 and P22 also responded with gradual tumor regression, accompanied by durable hTERT-specific T cell responses (Figs 1B,
2A). CT scans demonstrated regression of 6/8, 4/4 and 7/7 pre-study lesions, respectively (Fig 2A and Appendix Figs A2, A3).

**Progression-free and overall survival**

Table 1 lists progression-free and overall survival (PFS and OS), calculated from start of study treatment. By intention-to-treat analysis (n=25), median OS was 10.4 months (range 2.2-66 months) and mean OS 16.3 months (M1B 20 months; M1C 14 months). Immune responders had increased median OS and PFS compared to non-responders (median OS 396 days versus 250 days; median PFS 112 days versus 84 days). These differences did not reach statistical significance (log rank test; p>0.05), reflecting the low number of subjects. Advanced melanoma patients usually progress further within weeks or few months (6, 8). It is therefore of interest that the five PR patients exhibited extended PFS ranging from 8 to >62 months, as well as OS ranging from 19 to >62 months.

Trial subjects may differ from other patients, e.g. due to selection processes determining which individuals are referred to trial units. Korn et al have conducted a meta-analysis of 2100 trial patients and suggested that survival in phase I/II melanoma studies is compared with their data set (6). They also outlined a method for calculating predicted survival, correcting for prognostic factors. Applying this method, we calculated a survival advantage for the present study treatment, bordering 95% statistical significance (Fig. 3; intention-to-treat analysis). The
apparent advantage was relatively stable at different time points (one year: 44% versus 24%, two years: 16% versus 6.6%, three years: 12% versus 3.7%). Moreover, one may note that the “control group” was not based on untreated subjects, but on trial patients receiving potentially active therapy. Korn et al suggested declaring a treatment worthy of further study if the one-year survival was better than predicted, with a \( p \) value less than 0.10. This criterion was met both by intention-to-treat and per protocol analysis (\( p = 0.059 \) and \( p = 0.032 \), respectively). The interpretation of these findings is still complex, as discussed below.

**T cell clones from clinical responders**

We generated eight GV1001-specific T cell clones from two clinical responders (P11 and P22). The clones were HLA class II DR restricted (Fig. 4A and data not shown). Taken together with our data from other studies ((19, 23) and unpublished), we find that GV1001 is recognised on a series of DP, DR and DQ molecules. The HLA-promiscuousity of GV1001 suggests that the vaccine is applicable to the general patient population, without a need for HLA-typing.

A diverse response in terms of HLA-restriction and fine-specificity may reduce the risk of tumor escape (25). Here, we determined the fine-specificity of T cell clones by experiments with truncated peptides spanning the GV1001 aa-sequence. The four analyzed clones from subject P22 recognised three different core motifs from the N-terminal part of GV1001 (Fig. 4B). One of the motifs was shared by a P11
clone (Fig. 4D). In previous studies, we have observed that multiple motifs from the central and C-terminal parts of peptide GV1001 may also be recognised (21).

Vaccine-specific T cell clones may be of sparse relevance in vivo if they fail to recognize naturally processed antigens. Here, we observed recognition of naturally processed hTERT epitopes (Fig. 4C,D). Most clones responded more vigorously to hTERT protein than to pure GV1001 peptide (Fig 4C and data not shown). We also noted strong responses against low concentrations of truncated peptides (Fig. 4C,D). These observations suggest that the clones may have higher affinity for naturally processed epitopes than for the vaccine peptide. The data also point to a simple mechanism for epitope spreading, as the cross-reacting natural epitopes may stretch beyond the GV1001-sequence.

**Cytokine profiles**

The cytokine profiles for 14/18 immunological responders were investigated in Bioplex assays by use of panels measuring up to 27 cytokines. The results demonstrated GV1001-specific secretion of multiple cytokines in all 14 patients (Fig 5 and Appendix Table A2). According to the Th1/Th2-paradigm, a Th1-like pattern is desirable for cancer eradication (27). However, we have previously observed that human responses frequently do not to follow a Th1/Th2-delineation (23, 28, 29). The present responses comprised high levels of key Th1-cytokines IFNγ and TNFα, but also of hallmark Th2-cytokines IL-5 and IL-13. This applied
both to the T cell bulk cultures (Fig 5) and to the T cell clones [(23) and data not shown]. Collectively, our present and previous results indicate that cytokine profiling should not rely on a Th1/Th2-dichotomy. Of note, we detected only low levels of IL-4 and IL-10 (Fig 5). The responses would therefore easily be designated “Th1” with commonly used panels measuring only IFNγ, TNFα, IL4 and/or IL-10. Interestingly, the high IFNγ/IL-10 ratios may reflect a favourable balance between immunity and tolerance, as IL-10 is considered to promote regulatory T cells and “tolerogenic” dendritic cells.

The analyses further demonstrated GV1001-specific secretion of a broad range of pro-inflammatory cytokines and chemokines (Appendix Table A2), including IL-1β, IL-6, IL-8, GM-CSF and MIP-1β. This polyfunctional cytokine profile was observed in all evaluated patients, both in T cell bulk cultures and T cell clones [Appendix Table A2, (23) and data not shown]. We also noted that the GV1001-specific responses included IL-17. A subset of activated T cell clones may thus belong to the Th17-lineage (30, 31).

Finally, we asked whether temozolomide had affected the cytokine profile of the GV1001 response and compared the cytokine patterns to data from patients in our other GV1001 trials (Appendix Figure A4, (20, 23) and data not shown). The results suggested no substantial difference in the range of cytokines secreted or the ratio between key cytokines.
DISCUSSION

The concept of combining cancer vaccination with chemotherapy is attractive, but also challenging. Due to the long-standing assumption that chemotherapy would preclude immunization, there is sparse experience on how these modalities interact. Here, we combined temozolomide with an hTERT peptide vaccine. The lack of increased toxicity is of particular interest as temozolomide is bone marrow toxic and telomerase is expressed by haematological stem cells. There is also no evidence of long term toxicity in subjects receiving up to 5 years booster vaccination, in spite of durable immune responses. Interestingly, the immunological response rate of 78% is higher than in any previous GV1001 trial (19-22, 32). Follow-up studies of 12 immune responders revealed that 10 patients with durable GV1001 responses all survived longer than the two subjects rapidly losing their T cell activity. The clinical evaluation demonstrated objective tumor responses in 5 subjects. Overall survival was extended compared to matched controls from a benchmark meta-analysis.

The immune response rate of 78% indicates that temozolomide did at least not preclude immunisation. Most subjects had normal lymphocyte counts at time of vaccination (data not shown). This may be related to the temozolomide-free intervals in the dosage regime for melanoma. Temozolomide is known to affect T cell counts, but mostly if administered continuously (33-36). The considerable
immune response rate may, moreover, reflect a beneficial effect of temozolomide. Several studies in mice have reported enhanced immunisation after combining vaccination with temozolomide, or that temozolomide suppresses Treg function (4, 36-40). Chemotherapy may also suppress myeloid-derived suppressor cells (5).

The immunological evaluation per protocol did not include Treg analyses, but we made an attempt to investigate the influence of temozolomide on Treg counts. Flow cytometry analyses demonstrated that CD4⁺CD25high cells with a Treg phenotype were present in peripheral blood after temozolomide treatment and after the subsequent vaccine injections (data not shown). We did not obtain sufficient pre-treatment samples to determine whether temozolomide had still affected Treg frequency and can therefore not make any conclusions about the effect of temozolomide on T cell populations. In our ongoing melanoma vaccine trial (NCT00961844), this issue is addressed. Interestingly, preliminary data suggest that temozolomide induces decreased Treg counts (unpublished). The cytokine data reported above indicated that temozolomide had not substantially altered the cytokine profiles compared to previous GV1001 trials. Nevertheless, temozolomide is known to induce apoptosis of melanoma cells and is likely to induce inflammation and cross-priming of tumor-associated antigens. We hypothesize that this process may synergize with CD4+ T helper cells recruited through GV1001-vaccination. T helper cells may in particular engage APCs presenting antigens from apoptotic tumor and induce epitope spreading (27). In
ongoing studies of long term survivors after GV1001 vaccination, we have detected responses against telomerase antigens not included in the vaccine (unpublished).

While most peptide vaccines have represented short CTL epitopes, the use of longer peptides recruiting T helper cells may yield advantages in vivo (1, 25, 27). A T helper response may more effectively synergize with apoptosis and inflammation induced by radiation or chemotherapy, as depicted above. The durable memory responses reported herein for GV1001 point to another possible advantage; several studies have suggested that T-helper activity is necessary for the development of CTL memory (41, 42). Long peptides may, moreover, be processed by endogenous APCs and recruit T cell clones with diverse specificities, as observed for GV1001. Several long peptides, including GV1001, have furthermore been shown to elicit cytotoxic responses against nested short epitopes (1, 19, 43-45).

The present GV1001-responders developed polyfunctional cytokine profiles with mixed Th1/Th2-patterns and a high IFNγ/IL-10 ratio (Fig 5 and Appendix Table A2). The broad and pro-inflammatory cytokine profile may mobilize the adaptive and the innate immune system. Interestingly, polyfunctional cytokine profiles have been associated with protective immunity in patients surviving HIV or Leishmania (46-48). Polyfunctional cytokine patterns may be particularly important in a cancer vaccine setting, where there is a need to overcome established tumor tolerance and transform the inflammatory milieu.
Six patients in the present trial harboured T cell responses against GV1001 prior to vaccination, and others have reported spontaneous GV1001-specific activity in subjects with chronic lymphocytic leukaemia (49). Further, we demonstrate that GV1001-specific T cell clones recognize endogenous APCs pulsed with hTERT protein. Both the pre-vaccination reactivity and the hTERT protein responses suggest that GV1001-associated epitopes are naturally processed and immunogenic in cancer patients. The present study did not include screening of tumors for telomerase expression and hence did not address whether quantitative differences in expression level between patients may have influenced the vaccine response.

We report objective tumor regression in 5 patients and temporary disease stabilisation in an additional 6 subjects. Several observations suggest that the regressions are unlikely to be caused only by temozolomide. First, all clinical responders belonged to the immunological response group and developed long term GV1001-specific memory. Second, the response rate is relatively high compared to the ~13% rate reported in phase III temozolomide trials (8, 50). Third, chemotherapy responses rarely occur in patients like P19 with widespread visceral metastases outside of lung. Fourth, P19 developed most of her tumor regression after omitting temozolomide at month 8 and continuing vaccination (Fig. 2B). Fifth, responses to temozolomide are frequently short-lived, while 4/5 responders here exhibited PFS>1 year. Finally, responses to chemotherapy usually materialize
shortly after start of treatment. By contrast, 4/5 responses in the present trial fell short of PR criteria at month 3, and all responses gradually improved over months or years. This pattern is in accordance with vaccine responses, depending on mobilizing the immune system and overcoming tumor tolerance. Taken together, these observations suggest a role of GV1001 in the development of clinical responses. There is, however, a need to perform a randomized study before making any conclusions on clinical efficacy. The slow development of tumor responses shows that a conventional 3-month evaluation based on RECIST may be misleading in cancer vaccine trials and supports the concept of developing adjusted response criteria, as suggested by others (26).

The group of study patients survived longer than expected, compared to predicted survival as calculated from Korn’s meta-analysis. This finding should be interpreted with utmost caution, but is in line with possible clinical benefits suggested by the tumor regression and PFS data. Subjects with clinical signs of brain metastases were excluded from our trial. Based on the high frequency of asymptomatic brain involvement among patients with disseminated melanoma, it is still likely that a considerable fraction carried brain metastases. Because screening for cerebral involvement was not performed, we calculated predicted survival based on Korn’s data from studies not excluding brain metastases. Of note, patients with clinical signs of brain metastasis are rarely enrolled in trials, and the calculation takes into account the generally good performance status in our patients. Survival in our trial was calculated from the start of study treatment, while Korn’s meta-analysis gives
survival from a possibly earlier point ("time of registration"). The latter difference suggests that our survival advantage may be underestimated. In all, these issues illustrate that any comparison between different trials carries considerable uncertainty. The apparent survival benefit of GV1001/temozolomide is interesting, but need to be reproduced with a randomized control group.

We conclude that combined therapy with temozolomide and GV1001 is well tolerated in advanced melanoma patients, and that standard temozolomide dosage for melanoma does not preclude development of vaccine responses. The immunological responses exhibit several features of possible clinical significance, including durable T cell memory, recognition of naturally processed antigens, high IFNγ/IL-10 ratios and polyfunctional cytokine profiles. Collectively, present and previous trials show that peptide GV1001 may recruit a diverse spectrum of T cell clones with different HLA-restrictions and fine-specificity. Regarding clinical outcome, we observed objective tumor responses in five long term immune responders. Overall survival was extended compared to predicted survival from a meta-analysis. There have also been signs of a possible clinical activity in our previous GV1001-studies (19-21). However, both clinical and immunological response rates are higher in the present trial, while the low toxicity from GV1001 monotherapy is retained. In our opinion, the results warrant further studies of combinatory therapy with GV1001 and temozolomide. Our findings also support the general concept of combining cancer vaccination with chemotherapy.
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FIGURE LEGENDS

Figure 1
GV1001-specific T cell responses. PBMCs were obtained prior to start of therapy, at weeks 5, 9 and 12 and at every third booster vaccination. The PBMCs were stimulated once in vitro and tested for proliferation against irradiated PBMCs +/- peptide GV1001.
(A) Pre- and post vaccination T cell response from all evaluated patients. Columns represent mean stimulatory index (SI), i.e. response with antigen (GV1001) divided by response without antigen. Responses with SI>2 were considered GV1001-specific.
(B) Long term T cell memory in clinical responders. Columns represent mean cpm of triplicates. Tumor response periods are indicated with horizontal bars below charts. The assays demonstrate durable GV1001-specific T cell responses in 5 patients with partial tumor response (PR) and one with stable disease (SD).

Figure 2
Tumor response.
(A) Partial tumor regression in five patients. Bar chart (top): Columns represent total volume of all measurable lesions at study entry and minimum volume after vaccination. Box diagrams (bottom): Response of individual tumor lesions for each patient. Location of lesion: LH Liver hilus LN Lymph Node Ma Mammary glands Med Mediastnum Mu muscle P Pulmonal SC Subcutaneous V Visceral
(B) Tumor response in patient P19. Top diagram: Total diameter of all measurable lesions (corresponding to RECIST) at time points for CT evaluation. P19 stopped temozolomide at month 8, but still receive booster vaccines at 3-month intervals. Middle diagram: Response of individual lesions at different time points.
(m=months). All residual CT lesions are negative on FDG-PET scans, suggesting that no viable tumor tissue may remain. PET-scans have not been repeated after month 42. Bottom: Abdominal CT scans at study entry and after 61 months, showing regression of three metastatic lesions (CR=complete regression; PR=partial regression). The month 61 scan was taken without contrast due to allergy.

**Figure 3**

**Survival in study patients compared to benchmark meta-analysis.** Red line: Overall survival for the study patients, calculated from start of vaccination. Dashed red lines represent the 95% confidence interval (CI). Blue line: Predicted survival for our patients (fixed estimate), calculated from Korn’s meta-analysis of melanoma trials were brain metastases were not excluded (6). The calculation corrects for prognostic factors (performance status, site of metastases etc) and was performed as recommended by Korn et al. The analysis suggests a difference in favour of the present study treatment, reaching a borderline statistical significance.

**Figure 4**

**GV1001-specific T cell clones from clinical responders.** T cell clone (T) proliferation after stimulation with irradiated EBV-transformed cells (EB) +/- peptide GV1001 or hTERT protein. Columns represent mean cpm of triplicate wells.

(A) Patient 22. HLA-restriction was determined by blockage with mAbs against DP, DQ or DP-molecules.

(B) Patient 22. Fine specificity analysis by stimulation with truncated peptides covering the GV1001 sequence (aa sequences given in right text box). The four P22 clones recognize three different core sequences, annotated in text boxes bellow chart.

(C) Patient 22. Recognition of naturally processed antigens. T cell clones were stimulated with EBV-transformed cells incubated with recombinant hTERT
protein. The assay indicates response to naturally processed protein in clones # 5, 10, 18 (not #45).

(D) Patient 19. Fine specificity and recognition of naturally processed antigens. T cell clones were stimulated with truncated peptides or EBV-transformed cells incubated with recombinant hTERT protein. The core sequence recognized is highlighted in red below chart.

Figure 5
Secretion of Th1/Th2-cytokines in post-vaccination T cell cultures.
Cytokine assays were performed in 14/18 immune responders. i.e. all subjects were sufficient samples were obtained. T cells were stimulated with irradiated PBMCs +/- peptide GV1001. Supernatants were analyzed in duplicates or triplicates by Bioplex cytokine assays. Columns represent mean concentration (pg/ml). Data for non-Th1/Th2 cytokines are shown in Appendix Table A2.
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the vaccine peptide and kill autologous tumor cells carrying this mutation. Int J Cancer 1997;72:784-90.


### Table 1: Disease stage, immune response and clinical outcome

<table>
<thead>
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<th>Patient</th>
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<th>T cell response</th>
<th>SI after vacc</th>
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<td>POS</td>
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<td>PD</td>
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<td>583</td>
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<td>POS</td>
<td>35</td>
<td>POS (&gt;9m)</td>
<td>SD</td>
<td>168</td>
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</tr>
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<td>P7</td>
<td>IV; M1B</td>
<td>POS</td>
<td>26</td>
<td>POS (&gt;9m)</td>
<td>PD</td>
<td>-</td>
<td>317</td>
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<td>POS</td>
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<td>NEG (&lt;6m)</td>
<td>SD</td>
<td>140</td>
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<td>P9</td>
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<td>POS</td>
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<td>NEG (&lt;6m)</td>
<td>PD</td>
<td>-</td>
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<td>PD</td>
<td>-</td>
<td>194</td>
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<td>POS (&gt;25m)</td>
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<td>PD</td>
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<td>POS</td>
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<td>POS (&gt;60m)</td>
<td>PR</td>
<td>1905+</td>
<td>1905+ (alive)</td>
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<tr>
<td>P20</td>
<td>IV; M1C</td>
<td>POS</td>
<td>24</td>
<td>POS (&gt;9m)</td>
<td>SD</td>
<td>168</td>
<td>590</td>
</tr>
<tr>
<td>P21</td>
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<td>POS</td>
<td>29</td>
<td>POS (&gt;13m)</td>
<td>PD</td>
<td>-</td>
<td>413</td>
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<tr>
<td>P22</td>
<td>IV; M1C</td>
<td>POS</td>
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<td>POS (&gt;14m)</td>
<td>PR</td>
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<td>594</td>
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<td>IV; M1B</td>
<td>NEG</td>
<td>1.1</td>
<td>NA</td>
<td>SD</td>
<td>168</td>
<td>312</td>
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<tr>
<td>P24</td>
<td>IV; M1C</td>
<td>POS</td>
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<td>NA</td>
<td>PD</td>
<td>-</td>
<td>418</td>
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<td>NEG</td>
<td>1.1</td>
<td>NA</td>
<td>SD</td>
<td>154</td>
<td>169</td>
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</table>

| M1A | 18/23 | 10/12 | 5 PR | Mean: 497 |
| M1B | 6 SD  | 6 SD  |       |
| M1C | 14 PD | 14 PD |       |

**Notes:**

- **AJCC stage and metastasis category (within stage IV) at study entry**
- **T cell response**
  - POS: Positive, i.e. T cell proliferation specific for GV1001 (SI>2). NEG: Negative (no specific response). NE: Not evaluable, i.e. first round of vaccination (7 injections) not completed and post vaccination samples not available.
- **SI after vacc**
  - Stimulatory index (response with GV1001 divided by response without GV1001) after vaccination
- **Long term T cell memory**
  - Long term T cell response. POS: GV1001-specific response ≥ 6 months (last available and positive sample is indicated in brackets, m=month) NEG: Turning negative within 6 months NA: Not applicable (because no initial T cell response or no available long term samples).
- **Tumor response (RECIST)**
  - Best response during vaccination (compared to baseline, evaluated at 12 week intervals). CR=Complete response; PR = Partial response; SD = Stable disease; PD = Progressive disease
- **PFS**
  - Progression free survival from start of study treatment. CT scan at study entry was used as reference.
- **Survival**
  - From start of study treatment
- **Patient #17** was included in a different phase I study and developed clinical response.
- **Patient #19** has some residual lesions on CT, but these lesions are negative on FDG-PET.
Figure 1

A

B
Figure 2

A

Total tumor volume (% of baseline)

P11 P13 P16 P19 P22

Individual target lesions

CR: Complete regression
PR: Partial regression
Stable

B

Total tumor diameter (mm)

Months from start therapy

PET negative

Clinical response

Study entry 3m 9m 12m 15m 31m 42m 61m

Subcutaneous PR CR CR CR CR

Liver hilus PR PR PR PR PR

Pelvic PR PR PR PR PR

Visceral PR PR PR PR CR CR

Visceral PR PR PR PR PR PR

Study entry

Month 61

Tumor

Tumor

Kidney

CR

PR

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Figure 3
Figure 4

A

P22 clones: HLA restriction

B

P22 clones: Fine-specificities

C

P22 clones: hTERT protein reactivity

D

P11 clone #72: Fine specificity and hTERT protein reactivity

Core motif: EARPALLTSRLRFPK
Figure 5

- PBMC+T
- PBMC+T+GV1001

Graphs showing cytokine levels for different samples labeled P2 to P24.
Telomerase peptide vaccination combined with temozolomide: A clinical trial in stage IV melanoma patients

Jon Amund Kyte, Gustav Gaudernack, Svein Dueland, et al.

Clin Cancer Res  Published OnlineFirst May 17, 2011.

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Supplementary Material  Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2011/05/17/1078-0432.CCR-11-0184.DC1

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