Oncolytic immunotherapy of advanced solid tumors with a CD40L expressing replicating adenovirus: assessment of safety and immunological responses in patients

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Running Title: Oncolytic adenovirus CGTG-401 for immunotherapy of cancer

ABSTRACT

The immunosuppressive environment of advanced tumors is a primary obstacle to the efficacy of immunostimulatory and vaccine approaches. Here we report an approach to arm an oncolytic virus with CD40 ligand (CD40L) to stimulate beneficial immunological responses in patients. A double targeted chimeric adenovirus controlled by the hTERT promoter and expressing CD40L (CGTG-401) was constructed and 9 patients with progressing advanced solid tumors refractory to standard therapies were treated intratumorally. No serious adverse events resulting in patient hospitalization occurred. Moderate or no increases in neutralizing antibodies were seen, suggesting effective Th1 immunological effects. An assessment of the blood levels of virus indicated 17.5% of the samples (n=40) were positive at a low level early after treatment, but not thereafter. In contrast, high levels of virus, CD40L and RANTES were documented locally at the tumor. Peripheral blood mononuclear cells were analyzed by IFN-γ ELISPOT analysis and induction of both survivin-specific and adenovirus-specific T cells was seen. Anti-tumor T cell responses were even more pronounced when assessed by intracellular cytokine staining after stimulation with tumor-type specific peptide pools. Of the evaluable
patients, 83% displayed disease control at 3 months and in both cases where treatment was continued the effect was sustained for at least 8 months. Injected and non-injected lesions responded identically. Together, these findings support further clinical evaluation of CGTG-401

**Precis**

This study describes a modification to oncolytic viral strategies for cancer treatment that may improve their ability to elicit tumor-specific immunity by defeating tumoral immune suppression.

**Keywords:** CD40 ligand, oncolytic adenovirus, immune response, tumor-specific immunity, immunotherapy
INTRODUCTION

Metastatic tumors often become resistant to conventional therapies and therefore new approaches are needed. With regard to cancer immunotherapy, one of the major obstacles is the immune suppressive environment induced in tumors (1). In this situation, even though the immune system can recognize tumor antigens it is unable to eliminate all malignant cells. Advanced tumors may escape from immune detection due to loss of antigens (2), defects in antigen-presenting molecules (3), or by releasing local inhibitory cytokines (4). Furthermore, host related factors, such as stimulation of regulatory T cells (T-regs) (5), inappropriate T-cell signaling (6), and dominance of Th2-type cytokines may also hinder immune attack against tumors (7).

Oncolytic viruses have been shown to mediate beneficial immunological responses, including induction of anti-tumor T-cells and modulation of the tumor microenvironment from Th2 to Th1, which has been suggested to contribute to breakage of tolerance in tumors (8-10). Nevertheless, oncolysis per se is usually not enough for immunological eradication of advanced tumors. Therefore, it may be of relevance that it can be augmented by arming the virus with immune stimulatory molecules, such as CD40 ligand (CD40L). Preclinical studies have suggested that this approach can result in enhanced anti-tumor activity but human data has been lacking heretofore (11). Soluble CD40L (sCD40L) has been shown to mediate various antitumor activities in vitro and in vivo including direct growth inhibition and induction of apoptosis in different human tumor types (11-13). CD40L immunotherapy has also been shown to target the chemoresistant subpopulation of cancer cells (14) and has been suggested to sensitize chemoresistant tumors to
cytotoxic drugs (15, 16). Furthermore, a preclinical study suggested that CD40L gene therapy counteracted T-regs (17).

The expression of CD40 is particularly prominent in advanced human tumors (18). The utility of CD40L as an adenoviral transgene in clinical use has been previously tested in the context of non-replicating virus for the treatment of invasive bladder cancer (19) and chronic lymphocytic leukemia (CLL) (15). In isolated CLL B cells, adenovirally induced CD40L expression was shown to increase expression of co-stimulatory surface molecules and death-signaling receptor CD95 (20, 21). Furthermore, ex vivo analysis of dendritic cells (DC) isolated from healthy donors demonstrated that CD40L induces recruitment and activation of natural killer (NK) cells (22).

Replication deficient viral vectors may not be an optimal approach for treatment of advanced tumors because of insufficient tumor penetration but replication competent oncolytic viruses could be useful in this regard (23). They present the additional utility that oncolysis releases tumor epitopes for sampling by APC and the danger signal resulting from non-apoptotic cell death can also help in immunity versus tolerance. Therefore, we constructed a double targeted chimeric oncolytic adenovirus Ad5/3-hTERT-CD40L (CGTG-401). The fiber knob was replaced by the serotype 3 knob domain for enhanced delivery to tumor cells and the telomerase (hTERT) promoter was placed to control E1A for cancer cell restricted replication. The human CD40L (hCD40L) transgene was inserted in the E3 region to replace gp19k and 6.7k which also enhances tumor selectivity and immunological activity since gp19K is no longer able to down-regulate HLA-I (24). Insertion of a transgene
under the E3 promoter leads to replication-associated transgene expression starting about 8 hours after infection (24).

9 patients with refractory solid tumors were treated intratumorally with CGTG-401. In this paper we report safety, immunological parameters and preliminary evidence of antitumor efficacy.
MATERIALS AND METHODS

Construction of the virus

Construction and preclinical testing of Ad5/3-hTERT-CD40L is described in (25). Clinical grade virus was produced by Oncos Therapeutics, Ltd (Helsinki, Finland).

Treatment protocol

9 cancer patients (3 females and 6 males) with advanced solid tumors were treated intratumorally (or intraperitoneally for patients with peritoneal disease) with Ad5/3-hTERT-CD40L (Table 1) in a Finnish Medicines Agency regulated Advanced Therapy Access Program (ISRCTN10141600). Prior therapies are described in Supplementary Table 1. The starting dose of 3x10e10 VP was chosen based on previous experience with similar viruses (10, 26). Time-lapse dose escalation was utilized to maximize patient safety but minimize delays in enrolling new patients at potentially more effective higher doses. Dose could be escalated when sufficient time (typically 2 weeks) had lapsed (and relevant safety information collected) from the treatment of the first patient at that dose. Intra-patient dose escalation was allowed. Metronomic low dose cyclophosphamide (50mg/d, starting one day after virus and continued until progression) was used in all consenting patients (Table 1) to reduce regulatory T-cells (26, 27), which have been proposed counterproductive for induction of antitumor immune responses (28). Side effects were graded according to CTCAE v3.0 (Supplementary Table 2). A serial treatment of 3 injections was planned for all patients, and in case of evidence of benefit, treatment could be
continued further. In two patients, the serial treatment consisted of several viruses (29).

Patients were imaged with computed tomography (CT) and/or positron emission tomography CT (PET-CT) before and after treatment and RECIST1.1 (30) (Response Evaluation Criteria in Solid Tumors) criteria and PET criteria, respectively, were used to evaluate antitumor efficacy. Both criteria take into account the body-wide tumor burden thus including injected and non-injected lesions. RECIST1.1 criteria are: PR (>30% reduction in the sum of tumor diameters), SD (no response/progression), PD (>20% increase). For tumor markers, the same percentages were used. The following PET criteria were used (modified from (31)): PMR (≥30% decrease in summed SUVmax, up to 5 lesions counted, max. 2/organ), MMR (29% to 10% decrease in summed SUVmax), SMD (-9% to +29% change in summed SUVmax), PMD (≥30% increase in summed SUVmax or ≥ 2cm PET positive clinically relevant new lesions (except local lymph nodes whose signal might indicate immune reaction).

**Neutralizing antibodies**

Neutralizing antibody titers (NAb) were determined as described (32).

**Assessment of viral particles in the serum**

Total DNA was extracted and viral loads were determined as described (32). PCR amplification was based on primers and probe targeting the E3 region flanking the
CD40L transgene (forward primer 5′- CCGAGCTCAGCTACTCCATC -3′, reverse primer 5′- GCAAAAAGTGCTGACCCAAT -3′ and probe onco 5′FAM-CCTGCCGGAACGTACGATG -3′MGBNFQ). The limit of linear quantification for the assay was 500 VP/ml of serum and therefore samples definitely positive for virus but outside this range are reported as <500 VP/ml. Negative samples showed no amplification.

Assessment of tumor and adenovirus specific immunity by interferon gamma ELISPOT

Peripheral blood mononuclear cells (PBMCs) were isolated by Percoll gradient. Cells were frozen in CTL-CryoABCTM serum-free media (Cellular Technology Ltd. Cleveland, Ohio). For adenovirus ELISPOT, cells were stimulated with the HAdV-5 Penton peptide pool (ProImmune, Oxford, UK). For survivin, BIRC5 PONAB peptide was used (ProImmune). No pre-stimulation or clonal expansion of PBMCs was done in this assay and thus the results indicate the actual frequency of these cells in blood.

Analysis of intracellular cytokines of PBMCs

PBMCs were pulse-stimulated upon thawing either with a hAd5 mixture of hexon peptides or with a mixture of 3-7 TAA PeptMixes (CEA + SURVIVIN + MAGE-A1 + MAGE-3 + MAGE-4 + SSX2 + NY-ESO-1; AFP + SURVIVIN + PSA) chosen by cancer type in concentration of 1μg/mL (all peptides from JPT Technologies, Berlin, Germany). After stimulation, cells were fed with CTL growth medium RPMI 1640
(HyClone, Logan, UT) + Click’s Medium (EHAA; Irvine Scientific, Santa Ana, CA) 1:1, supplemented with 5 % Human AB Serum (Valley Biomedical) and 2 mmol/L L-glutamine (GlutaMAX TM-I; Invitrogen, Carlsbad, CA) containing either IL-4 and IL-7 (hAd5-pulsed cells), or IL-12 and IL-7 (TAA-pulsed cells; R&D Systems, Minneapolis, MN) in concentrations of 1000 U/mL for IL-4, and 10 ng/mL for IL-7 and IL-12. After 10 days in culture, the cells were re-stimulated with hAd5 or TAA peptide mixes as previously, with CD28 and CD49 (0.1 ug/ml; BD, Franklin Lakes, NJ, USA) added for co-stimulation, and surface stained with monoclonal antibodies to CD3 and CD8 (Becton Dickinson, Franklin Lakes, NJ) in saturating amounts (5 μl). Cells were stained for cytokines with 20 μl FITC–anti-IFN-γ or Pe-anti-TNF-α-antibody (BD Biosciences) and analyzed with a FACSCalibur equipped with Cell Quest software (BD, San Diego, CA).

Assessment of effect of virus produced CD40L on the activity of donor PBMCs ex vivo

A549 (ATCC) monolayers (5×10^6 cells/T25 flask) were infected with 1000 VP/cell of Ad5/3-hTERT-E1A-hCD40L and Ad5/3-hTERT-E1A or not infected (mock). Supernatant was collected 48h following infection and filtered with 0.02μm filters (Whatman 6809-1002, Maidstone, England). PBMCs of healthy donors were isolated and frozen as described above and thawed for the experiment. Supernatant from A549 cells was added to PBMCs. The PBMCs were maintained in RPMI 10% fetal bovine serum and 1% of L-glutamine and penicillin/streptomycin and 50ul samples were collected 24, 48, 72 and 96 hours after adding the filtered supernatant. FACSAarray analysis was performed according to the manufacturer’s protocol (BD
Cytometric Bead Array Mouse Flex Sets, BD, San Diego, CA) to assess the level of IL-8 produced by the stimulated PBMCs.

_Cytokines in the serum, ascites, and pleural effusion_

Cytokine analysis was performed with BD Cytometric Bead Array (CBA) Human Soluble Protein Flex Set (BD, San Diego, CA). For Th1 to Th2 ratios, data is presented normalized to baseline. Since the normal concentration range between cytokines varies significantly, each cytokine was compared to its value at baseline to obtain a ratio (below 1 is decrease, above 1 is increase) and then these ratios were multiplied with the ratios of the two other cytokines and then presented relative to the result obtained after performing the same calculation for Th2.
RESULTS

Safety of the treatment

Treatment with Ad5/3-hTERT-CD40L was generally well tolerated and safe. All patients experienced some grade 1-2 adverse events the most common ones being chills, fever, and fatigue. One patient experienced grade 3 fatigue and one patient experienced grade 3 fever after treatment. In addition, patients I244 and C239 experienced transient grade 3 liver enzyme increases on days 1 and 29 after the treatment, respectively, and patient T181 experienced transient grade 3 lymphocytopenia on day 1. No serious adverse events resulting in patient hospitalization were observed. All adverse events are listed in Supplementary Table 2.

Serum levels for interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-alpha) were measured to further assess the safety of the treatment. IL-6 and TNF-alpha have been suggested as sensitive markers for acute adenoviral toxicity (33) but no significant increases were seen in these cytokines after treatment (Supplementary Figure 1). Furthermore, no post-treatment increases in the serum levels of interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-12 (IL-12), interferon-γ (IFN-γ) (all presented in Supplementary Figure 1) were seen.

Systemic CD40L and RANTES levels
Systemic levels for soluble CD40L (sCD40L) and RANTES (a down-stream molecule whose expression is determined in part by CD40L) were assessed before and after virus treatment but no significant increases were seen in any patients, suggesting that immunological effects were restricted to the tumor site as predicted by virus design (Supplementary Figure 2). Patient R8 had malignant ascites which allowed measurement of sCD40L and RANTES levels at the tumor. 4- and 13-fold increases in sCD40L and RANTES levels were seen locally after treatment, whereas no concomitant increases in serum levels were seen (Figures 1A-B).

Viral particles in the serum and ascites

Serum samples were collected before treatment and on days 1, 3, and 21 after each round. Detectable but low (<500 VP/ml) viral titers were measured in 6 out of 47 (13%) post-treatment samples (Table 1). As expected, all baseline samples were negative for virus. Interestingly, and in contrast to reports on oncolytic adenoviruses not armed with CD40L (32, 34, 35), viral titers over 500 VP/ml were not detected in the serum of any patients. Ascites containing malignant cells was obtained from patient R8 before and 28 days after virus treatment and VP-titers for both compartments were assessed. As expected, no virus was present before treatment in either serum or ascites. However, 28 days after treatment viral titers of 6727 and 4827 VP/ml were detected in the supernatant and cell compartments of her ascites, respectively, while no virus was seen in the systemic circulation, suggesting tumor specific virus replication (Figures 1C-D).
Induction of neutralizing antibodies

6 out of 8 evaluable patients showed moderate increase in their anti-Ad5/3 neutralizing antibody (NAb) titer following treatment, whereas two patients showed no change (Table 2). In contrast to previous reports (10, 26, 32, 35), and in line with the expected mechanism of action to CD40L (Th2 to Th1 switch), the NAb titer did not increase to maximum in any patient.

Tumor and adenovirus-specific PBMCs by IFN-gamma ELISPOT

4 out of 8 evaluable patients (C239, P251, I244, C220) showed an increase in systemic adenovirus-specific PBMCs suggesting induction of anti-adenoviral immunity. Two patients (T181, R73) showed a decrease (Figure 2A), which could be due to trafficking of the cells to the tumor, where viral concentrations are highest as reported for other T-cell stimulating therapies (36). Patient N235 showed a minor increase in adenovirus-specific PBMCs 5 weeks after the first virus injection but no circulating adenovirus-specific PBMCs were seen after 9 weeks from the first treatment. R8 showed constantly low or undetectable levels of circulating adenovirus-specific PBMCs.

With regard to survivin-specific PBMCs, 4 out of 8 evaluable patients (P251, N235, C220, I244) showed an increased number of cells after the treatment (Figure 2B). In patients R73 and R8 a decrease in the number of tumor-specific T-cells was seen, perhaps suggesting trafficking to the tumor as proposed (36). Patients T181 and C239 had undetectable levels of circulating survivin-specific PBMCs.
Survivin is a useful prototype target for estimating tumor specific immunity since it is expressed by most tumors (37). However, it is probably not the most immunogenic epitope (38) and therefore anti-survivin T cells could underestimate induction of anti-tumor immunity. *Ex vivo* expansion of anti-tumor cells, followed by testing of reactivity against a pool of tumor specific epitopes, could provide an alternative view on anti-tumor immunity to complement ELISPOT, which was performed without *ex vivo* expansion of T-cells in order to obtain data on actual proportions of these cells in blood. Therefore tumor-specific CD8+ and CD4+ T-cells were measured with intracellular cytokine analysis when sufficient cell numbers were available. Patients T181 and C239 showed an increase in tumor-specific CD8+ T-cells in all post-treatment measurements (Figure 3A). With regard to CD4+ cells, T181 and P251 showed an increase in comparison to baseline (Figure 3B). Furthermore, the number of adenovirus recognizing CD4+ T-cells were studied for a patient C239 and a transient increase was seen three weeks after virus administration (Figure 3C), confirming ELISPOT data.

**Induction of Th1 type immune response**

Patient serum samples were analyzed for Th1 type cytokines IFN-γ, TNF-α, and IL-2 and Th2 cytokines IL-4, IL-5, and IL-10 and the ratio between Th1 and Th2 type cytokines was assessed (Supplementary Figure 3A). 4 patients (P251, N235, T181, C239) showed dominance of type 1 immune response one or two months after the
treatment, whereas two patients (I244, R8) showed a dominance of type 2 immune response both one and two month after the treatment.

Furthermore, malignant ascites and pleural effusion were available for patients R8 and T181, respectively. IFN-γ, IL-2, and IL-10 levels in these samples were assessed before and after the treatment (Supplementary Figure 3B). For patient T181, IFN-γ level in pleural effusion increased from 9 pg/ml to 35 pg/ml and IL-2 level increased from undetectable up to 34 pg/ml after 7 treatment rounds. At the same time, Th2 type cytokine IL-10 was undetectable after 7 treatment rounds while the value was 60 pg/ml before treatment. For patient R8, IFN-γ in ascites increased 10-fold (from 18 to 188 pg/ml) within 4 weeks after treatment while IL-2 levels where under the detection limit at both time points. Interestingly, also IL-10 level increased from 0 to 300 pg/ml within the same time period.

CD40L induced ex vivo activation of PBMCs from healthy individuals

IL-8 production by donor PBMCs was assessed by stimulating cells with supernatant collected from A549 lung cancer cells infected in vitro with CGTG-401 or control virus not coding CD40L (Supplementary Figure 4). Control virus infection induced some IL-8 production after 12, 24, 48, and 72 hours but more was seen after stimulation with filtered supernatant from CGTG-401 infected cells. IL-8 production is a marker of CD40L induced activation of human PBMCs.

Antitumor efficacy
The anti-tumor effects of oncolytic viruses and immunotherapy are difficult to quantitate with imaging because of inflammatory swelling of tumors and recruitment of metabolically active inflammatory cells. Thus, both CT and PETCT based imaging are likely to result in false positives causing underestimation of treatment benefits. 6 out of 9 of our patients were evaluable for radiological response by using CT imaging before and after the treatment (Table 2). 3 patients (T181, I244, R8) showed stable disease (SD) whereas 3 patients (C239, C229, P251) showed progressive disease (PD).

3 patients (C239, C229, I244) were imaged with PET-CT. Patient C239 showed 25% reduction in the metabolic activity of the overall tumor load (Fig. 4C) and was scored as a minor metabolic response (MMR) while patients C229 and I244 were scored as stable metabolic disease. Overall, 5/6 patients (83%) showed evidence of antitumor activity in either CT or PET-CT. Efficacy was seen regardless of concomitant low-dose cyclophosphamide (Table 1), which is theoretically an attractive combination with CGTG-401 since it may be able to reduce the activity of regulatory T-cells, potentially counter-productive for anti-tumor immunity (27).

Three patients had injected and non-injected lesions which could be reliably assessed separately. Patients C239 and P251 had injected and non-injected liver lesions and patient T181 had injected and non-injected lesions in his pleura (Supplementary Table 5). In all cases, the non-injected lesions behaved similarly to injected lesions suggesting systemic effects.

Tumor markers, measured from blood, were followed for 7 patients during treatments. For patient R73, tumor marker Ca15-3 normalized during treatment and therefore she was scored as a complete marker response (mCR). Patient T181
showed a 56% reduction in serum thyroglobulin and he was scored as partial response (mPR). Patients C229 and R8 showed 8% and 7% reduction in CEA and Ca15-3, respectively, and were scored as mSD, whereas three patients (C239, P251, N235) showed constant increase in their tumor markers and were scored as mPD.

Overall survival (OS; Table 2) and progression free survival (PFS) are shown in Figure 4. Cancer patients treated with an unarmed oncolytic adenovirus with an identical capsid (Ad5/3-D24-Cox2L) were used as controls. Survival data were plotted into a Kaplan-Meier curve and cohorts were compared with log-rank test. Median OS for CGTG-401 and Ad5/3-D24-Cox2L treated patients was 304 and 106 days, respectively, in this non-randomized comparison (p=0.01). Although the controls were treated at the same institution and by the same team, and have similar baseline characteristics (Suppl. Table 4), many caveats and biases apply to such comparisons.
DISCUSSION

Treatments were generally well tolerated, and no serious adverse events resulting in patient hospitalization were observed. Only two patients experienced symptomatic grade 3 adverse events. Patient C220 experienced grade 3 fatigue 1 month after the treatment and died one month later suggesting that increased fatigue might be related to the terminal phase of the disease rather than virus treatment. Patient I244 reported grade 3 fever shortly after the third virus injection but recovered completely by day 2. We assume the fever was treatment related. He was one of the three patients who showed measurable but low (<500 VP/ml) viral loads in his serum on day 1 after three treatment rounds. However, two other patients (C239, P251) showing detectable levels of virus in the serum on day 1 experienced no fever concomitantly.

Systemic cytokine levels were followed but values remained low throughout the study. Increases in systemic IL-6 and TNF-alpha levels are known to be markers for acute adenoviral toxicity (33), but none of the patients showed significant changes in these cytokines supporting good safety of the approach (Supplementary Figure 1).

In our approach, the telomerase promoter controls adenoviral E1A which limits viral replication and transgene expression to malignant cells, as transgene expression is under the replication-activated E3 promoter. In accord with preclinical data, malignant ascites of patient R8 showed a 4-fold increase in CD40L concentration between days 0 and 28 whereas systemic levels remained unchanged. Similarly with sCD40L, levels of a down-stream molecule (RANTES) increased 14-fold in ascites, but no increase in serum levels was seen. Liver toxicity became dose limiting when patients with advanced solid tumors were treated with subcutaneous recombinant
CD40L (39). In this regard, low systemic but high local sCD40L levels suggest that CGTG-401 can increase the therapeutic window of CD40L mediated therapy.

As further evidence of tumor specific replication, low viral titers were seen in serum throughout the study, whereas 10-fold higher titers were detected locally in malignant ascites (Fig. 1). One possible explanation for low systemic virus titers would be increased immunogenicity of the virus due to the immunostimulatory actions of CD40L, which might expedite virus clearance. Oncolytic adenoviruses armed with another immunostimulatory transgene, granulocyte macrophage colony-stimulating factor (GM-CSF), were reported to result in lower shedding into blood in comparison to unarmed viruses in a previous human study (34). In this study, 31 treatment rounds with CGTG-401 were analyzed for virus presence in blood on day 1 after treatment and only 16% of samples were positive. In our previous studies with viruses featuring an identical capsid but no tumor specific promoter, seroprevalences of 50% and 83% were seen for unarmed Ad5/3-D24-Cox2L and armed Ad5/3-D24-GMCSF viruses at the same time point, respectively (34). Furthermore, 9 samples were analyzed on days 2-3 and only 22% were positive for CGTG-401, while 80% and 77% of samples collected at similar times were positive for patients treated with Ad5/3-D24-Cox2L and Ad5/3-D24-GMCSF (34), respectively. These data suggest that the hTERT promoter employed in CGTG-401, and/or CD40L expression, may lower virus replication or shedding of the virus from tumors into blood.

Th1 and Th2 type cytokines were assessed before treatment and one and two months later. The most evident switch from Th2 to Th1 type immune response was seen for patient T181 in both after-treatment time points. This patient seemed to benefit from the treatment since he showed SD in RECIST evaluation, 56%
reduction in serum thyreoglobulin (a tumor marker) and survived for 340 days, which is unusual for thyroid cancer patients with widely metastatic high load cancer refractory to all other therapies. However, since the serum Th1/Th2 data was not as logical in all patients, we conclude that it may be more useful to look at cytokine profiles at the tumor level. Therefore, malignant ascites and pleural effusion for patients R8 and T181 were analyzed before and after treatment. T181 showed clear increase in Th1 type cytokines IFN-γ and IL-2 whereas Th2 type cytokine IL-10 disappeared completely. Patient R8 showed 10-fold local increase in IFN-γ, while IL-2 levels were constantly low. These data are in accordance with tumor specific expression of CD40L.

Interestingly, R8 showed also clear increase in local IL-10 concentration. Increased IL-10 might represent local anti-inflammatory compensation in response to pro-inflammatory cytokines IFN-γ and IL-2. It will be interesting to study these aspects in larger patient cohorts. In patients with malignant ascites (such as R8, R73), intraperitoneal administration could be a particularly useful approach for tumor immunotherapy, taking into account the large accessible tumor surface area and the possibility of the peritoneum acting as an immunological organ (40). In patients lacking tumor associated effusions, tumor biopsy before and after treatment could yield interesting data.

Immunotherapy is becoming an increasingly appealing approach as knowledge on tumor tolerance versus recognition is accumulating. Even though a wide range of tumor associated antigens (TAAs) have been identified (41), they are often self antigens of low immunogenicity (42, 43), and in addition tumors commonly lack danger signals needed to provoke recognition and rejection (44, 45). In contrast,
adenoviral antigens are highly immunogenic (46), providing danger signals useful also for generation of anti-tumor immunity (47). The strong initial adenovirus-triggered immune response is likely to synergize with anti-tumor effects through many mechanisms. Efficient anti-viral epitope recognition can lead to “epitope spreading”, where an initial narrow immune response to a few (adenoviral) antigens is followed by a broader immune response directed to a multiplicity of TAAs (48, 49) released in oncolysis from tumor cells for adaptive T cell priming. Also, accumulating evidence suggests that non-apoptotic cell killing, such as resulting from oncolysis, can be a useful additional danger signal supportive of immunity versus tolerance (50). These effects are likely to be potentiated by the effects of CD40L on antigen presenting cells.

Multiple rounds of treatment are likely to boost anti-tumor immunity, including both the innate and adaptive arms. Interestingly, initially strong anti-adenoviral T-cell responses declined over time (Figure 3C), while the anti-tumoral immune responses often increased and remained elevated for weeks (Figures 3A-B). Rapid antiviral response could reflect the higher immunogenicity of adenoviral epitopes, perhaps facilitated by a memory response since most humans have sustained prior adenovirus infections. In contrast, tumor epitopes may be weaker, partially self, and thus require boosting for the full magnitude of the response to be realized.

In summary, the data presented here suggests that CGTG-401 is safe for the treatment of advanced solid tumors and some evidence supporting anti-tumor immunity and efficacy was also seen. It is clear that further work is needed to understand these aspects in different patient populations. However, as CGTG-401 was designed to be active in cancers featuring hTERT activity, thus including most
solid tumors, it can be viewed as promising that possible signs of activity were seen in many types of malignancies.

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REFERENCES


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\(^a\), Performance status at the time of treatment; cyclo po, cyclophosphamide orally 50 mg/day; VP, viral particles; CGTG-103, Ad5-RGD-D24-GMCSF; CGTG-101, Ad5-D24-GMCSF; CGTG-201, Ad3-hTERT-E1; empty indicates not available. <500 indicates that sample was positive for virus DNA but the titer was below the quantitative range of the assay.
Table 2. Induction of neutralizing antibodies (NAb) and treatment efficacy.

<table>
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<tr>
<th>Code</th>
<th>Concomitant low-dose (50mg/d) cyclophosphamide</th>
<th>NAb titer Weeks post-treatment</th>
<th>TREATMENT RESPONSES</th>
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<tr>
<td>P251</td>
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Blanks indicates not available. *, Data are presented as a serum dilution factor causing 80% inhibition of gene transfer with Ad5/3Luc1 (capsid identical to Ad5/3-hTERT-E1A-hCD40L) to 293 cells. RECIST, Response Evaluation Criteria in Solid Tumors version 1.1. PR, partial response (>30% reduction); CR, complete response; SD, stable disease; PD, progressive disease (>20% increase); PET Response Criteria in Solid Tumors; MMR, minor metabolic response; SMD stable metabolic disease based on SUVmax. **, alive at 3rd November 2011.
FIGURE LEGENDS

Figure 1. Malignant ascites (resulting from peritoneal tumor masses) was removed from the peritoneal cavity of patient R8 before and 28 days after virus administration. Soluble CD40L (sCD40L; A) and RANTES (B) concentrations in the fluid were assessed and compared to systemic levels of these cytokines. Both sCD40L and RANTES levels increased locally at the tumor whereas no increases in systemic levels were seen. Similarly, high amounts of virus particles (VP) suggestive of replication in the tumor virus were found in ascites fluid (C) and in cells isolated from ascites (D) on day 28 after virus treatment, whereas no virus was detected in the serum on the same day.

Figure 2. Unstimulated total PBMCs were isolated and pulsed with an adenovirus 5 penton-derived and survivin-derived peptide pools to assess the activation of (A) adenovirus-specific and (B) tumor-specific cytotoxic T-lymphocytes with interferon gamma ELISPOT without pre-stimulation. Stars indicate the days of virus administration, PBMC were collected immediately prior to virus injections. The use of concomitant low dose cyclophosphamide (50 mg/d) is indicated for each patient separately (CP+ or CP-).

Figure 3. Pre-stimulated and clonally expanded PBMCs were pulsed either with commercial tumor-derived peptide pool (specified for each patient according to tumor type) or commercial adenovirus-derived peptide pool. The relative numbers of TNF-alpha / IFN-gamma double positive (A) tumor-specific CD8+ T-cells, (B) tumor-
specific CD4+ T-cells, and (C) adenovirus-specific CD4+ T-cells were assessed with intracellular cytokine staining. Stars indicate the days of virus administration, PBMC were collected immediately prior to virus injections.

**Figure 4.** Overall survival (A, p=0.01) and progression free survival (B, p=0.146) of CGTG-401 treated patients. Historical controls had similar inclusion and exclusion criteria and were treated with an oncolytic adenovirus with an identical capsid but lacking a transgene (Ad5/3-cox2L-D24, (32)). (C) PET-CT analysis of injected liver lesions of patient C239 before and after three rounds of virus treatment.
Figure 1. Pesonen et al.
A: Adenovirus penton; IFN-gamma ELISPOT

Weeks after 1st treatment

Figure 2A. Pesonen et al.
B: Survivin; IFN-gamma ELISPOT

Weeks after 1st treatment

Figure 2B. Pesonen et al.
Figure 3. Pesonen et al
C: PET-CT analysis of injected liver lesions (C239)

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</table>

Figure 4. Pesonen et al