Phase II Study of Lucanix™ (Belagenpumatucel) a Transforming Growth Factor β2 (TGF-β2) Antisense Gene Modified Allogeneic Tumor Cell Vaccine in Non Small Cell Lung Cancer (NSCLC)

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Date: 9/21/2006

KEY Words: TGF-β2, vaccine, non small cell lung cancer, gene therapy

ABSTRACT

Background

Belagenpumatucel is a non-viral gene based allogeneic tumor cell vaccine which demonstrates enhancement of tumor antigen recognition as a result of TGF-β2 inhibition.

Methods

We performed a randomized dose variable phase II trial involving stage II, IIIA, IIIB and IV NSCLC. Each patient received one of 3 doses (1.25, 2.5, 5.0 x 10^7 cells/injection) of Belagenpumatucel on a monthly or every other month schedule to a maximum of 16 injections. Immune function, safety and anticancer activity were monitored.

Results

Seventy five patients (2 stage II, 12 IIIA, 15 IIIB, 46 IV), received a total of 550 vaccinations. No significant adverse events were observed. A dose-related survival difference was demonstrated in patients who received ≥ 2.5 x 10^7 cells/injection (p=0.0069). Focusing on the sixty-one late stage (IIIB/IV) evaluable patients, a 15% partial response rate was achieved. The estimated probabilities of surviving 1 and 2 years were 68% and 52% for the higher-dose groups combined and was 39% and 20% for the low dose group. Immune function was explored in the 61 advanced stage (IIIB/IV) patients. Increased cytokine production (at week 12 as compared with patients with progressive disease) was observed among clinical responders (IFN-γ, p=0.006; IL-6, p=0.004; IL4, p=0.007) who also displayed an elevated antibody mediated response to vaccine HLA antigens (p=0.014). Further, positive ELISPOT reactions to Belagenpumatucel showed a correlation trend (p-0.086) with clinical responsiveness by patients achieving stable disease or better.

Conclusions

Belagenpumatucel is well tolerated and survival advantage justifies further phase III evaluation.

INTRODUCTION

NSCLC is the most common and one of the most debilitating cancers affecting society [1]. The majority of patients present at an advanced stage [2] for which chemotherapy, despite its limited efficacy, is usually the only therapeutic option [3-5]. Expected survival of patients who progress after initial chemotherapy is 8 months or less and generally fewer than 30% of patients survive one year [6-11].

Recently, we demonstrated evidence of antitumor activity in advanced stage NSCLC patients with an adenoviral gene based autologous tumor vaccine (GVAX) [12, 13] designed to enhance tumor antigen recognition by immune effector cells. Although encouraging, the requirement for harvest of autologous tumor necessarily imposed limits on this approach. Therefore, to further explore anticancer immune therapy through enhancement of effective immunogenic tumor-antigen recognition we studied the use of a non-viral gene based allogeneic vaccine (Belagenpumatucel) incorporating TGF-β2 antisense gene modification of tumor cells.

TGF-β is one of a family of multi-functional proteins that regulate growth and function of normal and neoplastic cells [14-17]. Elevated levels of TGF-β2, in particular, are linked to immunosuppression in cancer patients, [15-20] and the level of TGF-β2 is inversely correlated with prognosis in patients with NSCLC [21]. TGF-β2 has antagonistic effects on Natural Killer cells, lymphokine activated killer cells and dendritic cells [22-27]. Using an antisense (AS) gene to inhibit TGF-β2, we and others have demonstrated inhibition of cellular TGF-β2 expression with resultant increased immunogenicity of gene modified

cancer cells [28-36]. The results of this work led us to conduct a phase II trial of vaccination with Belagenpumatucel in NSCLC patients.

METHODS

Study Design

This was an open-label, three-arm, Phase II study designed to evaluate the safety and efficacy of intradermal immunization with 3 dose levels once each month of an allogeneic tumor cell vaccine. Patients were randomized to one of the three dose cohorts (1.25×10^7, 2.5×10^7, or 5×10^7 cells/injection) and were treated after a minimum workout of 30 days from prior cytotoxic therapy.

Tumor staging was performed at baseline and at weeks 8, 16 and quarterly thereafter. Patients were serially monitored for immune response every 4 weeks up to week 28 and quarterly thereafter. Toxicity was monitored throughout the study. Patients demonstrating benefit from treatment at 16 weeks could be given up to 12 additional vaccinations. All patients signed local IRB-approved informed consent. No patients received concurrent radiation therapy.

Study Population

Eligible patients for this study were 18 years or older with histological confirmation of stage II, IIIA, IIIB or IV NSCLC and an estimated total tumor burden volume of ≤ 125 cc (excluding nodal or bone disease). Patients were required to have completed or refused conventional therapy, have an ECOG performance status of ≤2, an absolute granulocyte count ≥ 1,500/mm^3, platelet count ≥ 100,000/mm^3, total bilirubin ≤ 2 mg/dL, and AST and ALT ≤ 2x Upper Limit of Normal.

Humoral immune response assessment

Antibodies reactive against the immunizing cell lines were evaluated using an ELISA. Briefly, 2 × 10^4 target cells were plated on flat-bottom 96-well plates (Costar, Inc., Cambridge, MA) and allowed to adhere overnight at 37°C in a humidified 10% CO_2 atmosphere. The next day, the cells were incubated at 4°C for 1 hr with the test sera. The plates were washed, incubated with an enzyme-conjugated anti-human IgM or IgG (Sigma Aldrich, St. Louis, MO) for 30min at 23°C), followed by a chromogenic substrate (30-60 min). Bound antibodies were quantified by spectrophotometric OD using an ELISA plate reader (Bio-Tek Instruments, Winooski, VT). Titration curves were generated with OD measurements of serially (1:3) diluted serum. Stimulation indices were calculated by determining the increase in titer between the pre-treatment and post-treatment samples.

Multiplex flow cytometric analysis was used to determine both the presence and HLA specificity of lymphocytotoxic antibodies in sera. Reactivity was determined with microbeads coated with purified Class I or Class II HLA antigens (LABScreen, LabScreen City, CA). Antibody reactivity in each test serum was determined with the use of the Lambda Array Beads Multianalyte System (LABMAS, Canoga Park, CA) and LABScan 100 flow analyzer for data acquisition and analysis.

Enzyme-linked immunospot assay (ELISPOT) for interferon-gamma (IFN-γ) release

Multiscreen, high protein binding, Immobilon-P Membrane 96 well plates were coated with the primary anti IFN-γ monoclonal antibody (BD Biosciences ELISPOT Set), and incubated overnight at 4ºC. Frozen pre treatment and post treatment peripheral blood mononuclear cells (PBMCs) collected at week 12 post-vaccination were thawed and revitalized in culture medium overnight at 37º C without further stimulation. Microwells were blocked with human AB serum (2 h), then seeded with PBMC at 1-2x10^5 cells/well and the mitogens phorbol myristate acetate and Ionomycin (PMA, 5ηg/m + I, 500ηg/ml)

or target cells (Belagenpumatucel, 1-2 x10^5 cells/well) for 24 hr at 37ºC to attain target:effector cell ratios of 2:1, 1:1 and 1:2. The wells were washed and incubated with a biotinylated detection antibody (BD Biosciences ELISPOT Set), enzyme reagent, and the chromogenic substrate according to manufac turer's protocol. Positive reactions were analyzed using ImmunoSpot® Analyzer. Results in Table 3 represent reactiosn from a target: effector ration of 2:1.

Detection of Intracellular Cytokines in pre and post treatment PBMCs.

Cytokines levels in pre and post treatment PBMC were analyzed by Immunofluorescent staining of intracellular cytokines by flow cytometric analysis. Fresh, unstimulated PBMCs in whole blood were incubated with protein transport blocking reagent (BrefeldinA) for 4hr followed by RBC lysis using PhamLyse solution (BD Biosciences). The PBMCs were fixed and permiabilized using the Cytofix/Cytofirm™ solution (BD Biosciences), followed by incubation with fluorochrome conjugated antibody to the cytokine. The stained cells were analyzed by flow cytometry (Becton Dickinson FACScan) with the CELLQUEST software (Becton Dickinson).

Vaccine Production

The 4 NSCLC (2 adenocarcinoma, 1 squamous, 1 large cell) lines (NCI-H-460, NCI-H-520, SK-LU-1, Rh-2) were tested for sterility, clonogenicity, and TGF-β2 expression. All demonstrated significant TGF-ß2 knockdown following antisense transfection. Aliquots were thawed and transfected with pCHEK/HBA2, a vector containing the TGF-β2 antisense transgene. The cell lines were frozen in aliquots as (1) Unmodified Master Cell Banks (umMCB), (2) gene-modified Master Cell Banks (gmMCB), and (3) gene-modified Working Cell Banks (gmWCB) and stored in liquid nitrogen. Aliquots were shown to be negative for the presence of viral agents (HIV 1&2, HBV, HCV, EBV, CMV,

HH-6, and HTLV 1&2) by Molecular Diagnostics Associates. Lots achieving >35% blocking of TGF-ß2 secretion were expanded, irradiated with 10,000 cGy and frozen.

Antisense Plasmid

The TGF-β2 antisense vector is a DNA fragment containing base pairs 6-935 of the human TGF-β2 cDNA was ligated in reverse orientation in the HindIII-XhoI sites of the pCEP-4 vector. Expression of the antisense molecule in pCEP-4 is driven by the cytomegalovirus promoter of the vector. The pCEP-4 vector also contains the hygromycin resistance gene driven by the herpes simplex virus thymidine kinase promoter, the Epstein-Barr virus origin of replication, and the gene for the Epstein-Barr virus nuclear associated protein 1 [36].

The plasmid used for transfection was pCHEK/HBA-2 (pCHEK/human ß2 antisense). It contains an EBV origin of replication and EBNA-1 genes, a hygromycin resistance gene under the control of SV-40 promoter, and a 929 base pair fragment of the human TGF-ß2 molecule in antisense orientation under the control of the CMV immediate-early promoter and enhancer. The SV-40 promoter/intron unit was incorporated to increase expression of the hygromycin resistance gene used to facilitate selection of gene modified cells in culture. The pCHEK/HBA-2 vector was electroporated into each cell line *ex vivo*.

Investigational Product

The Belagenpumatucel vaccine (NovaRx Corporation, San Diego, CA) was provided in frozen (liquid nitrogen) vials (1 ml of material). Vials contained 5 x 10^6, 1 x10^7 and 2 x 10^7 cells per cell line were used; for cohort 2, 1 x10^7 cells; and for cohort 3, 2 x 10^7 cells.

Statistical Methods

Statistical analyses were carried out to investigate adverse events, reactivity, ELISPOT response, cytokine response, survival and progression-free survival. Throughout all analyses the type I error rate was controlled at the 0.05 level.

In analyzing adverse events, a “severity” analysis approach was pursued. Homogeneity in the rate of serious adverse events across dose cohorts was tested using a Pearson chi-square statistic.

A Pearson chi-square statistic was also used to test for association between reactivity and clinical response status. Analysis of the association at each of the three clinical sites was undertaken with a Fisher Exact test due to small patient counts per site. Overall survival and survival by dose cohort were illustrated using the Kaplan-Meier estimator. The Cox proportional hazards model was fit to the data with dose cohort as the independent factor. A Bonferroni adjustment was used to control the type I error for these two tests. This adjustment is conservative, and hence both adjusted and unadjusted significance levels are presented. To investigate differences in survival between cohorts among advanced stage patients only, a term denoting the effect of advanced stage (IIIB/IV) as well as an interaction term between cohort and advanced stage was added to the model. Appropriate linear contrasts of the parameters from this model for cohort comparisons were then estimated and tested. Bonferroni multiplicity adjustments were also used for these comparisons. Survival estimates and the accompanying confidence intervals for groupings of cohorts were obtained via the Kalbfleisch-Prentice estimator. Assumption of proportional hazards was investigated graphically. A Kaplan-Meier curve was constructed for progression-free survival (PFS), and differences across dose cohorts for PFS were tested using a log-rank statistic.

Statistical analysis of the effect of tumor vaccination on intracellular cytokine expression was performed to compare pretreatment value with post-treatment samples for advanced stage patients, or by cohort or clinical response. The nonparametric Kruskal-Wallis test was used for cohort analyses. The Fisher Exact test was used in the contingency table analysis of HLA-Ab expression or ELISPOT response with clinical responsiveness. A Pearson chi-square test was used for the combined-site analysis of ELISPOT response with clinical response. All tests performed were two-tailed.

RESULTS

Seventy-five patients were entered into the trial between 5/30/02 and 3/26/04. Demographics of advanced stage (20 in cohort 1, 20 in cohort 2, and 21 in cohort 3) and early stage patients are summarized separately in Table 1. Toxicity and survival were evaluable in all patients. One patient was not evaluable for time to progression. Immune function samples were not evaluable from site 004 due to handling complications. Five hundred and fifty vaccinations were administered. Demographic comparison of sex, age, stage (IIIB, IV), tumor volume (< 12 cm^3, ≥ 12 cm^3), prior chemotherapy and date of diagnosis found no statistical difference among cohorts.

Safety

Analysis of severity of adverse events demonstrated no detectable difference in the rate of serious adverse events across dose cohorts ($p=0.5698$). All toxic events occurring at a $\geq 5\%$ frequency are shown in Table 2. Separation of toxic events between late stage and early stage patients and between cohorts suggest no difference. All grade 3/4 events were attributed to disease progression except 2 events (Table 2).

Response

The analysis of patient response in the advanced stage patients (using RECIST criteria)

revealed a partial response rate of 15% at week 16. Cohort-specific response of patients with measurable disease (n-40) was 1/16, 3/11, and 2/13 in cohorts 1, 2 and 3, respectively (Figure 4). The median tumor shrinkage at 16 weeks was 63% (maximum shrinkage 80%). Fifty-nine percent of all patients showed no evidence of progression at week 16. Progression-free survival across dose cohorts was not different (p=0.3816). Median age of the 6 responding patients was 72 (54-81) years, all 6 were females, 3 had stage IIIB disease, 3 had stage IV disease, and 3 were adenocarcinoma, 2 squamous, and one large cell. Three had received combination chemotherapy and radiation therapy and all of these 3 had achieved an initial responses to the combination. A fourth patient received radiation therapy without chemotherapy and demonstrated progressive disease along with 2 other patients who demonstrated progressive disease following attempt at surgical resection without follow-up adjuvant therapy. The median number of measurable lesions per patient was 3 (range 7-7). Disease was limited to multiple lung sites in 4 of the 6 patients. Prior treatment was discontinued a median of 3 months range (3-8 months) prior to randomization of vaccination of all 6 responding patients. Median volume of measurable disease was 5.67cm^3 (range 0.27 – 51 cm^3).

Survival

Range of follow up for surviving patients was 351 days to 967 days. Median overall survival of all three cohorts was 441 days (Figure 2). Dose related survival differences were found between cohorts, p=0.0155 (Figure 3). The analysis suggested that at doses ≥ 2.5 x 10^7 cells/injection (cohorts 2 and 3) there was a significant difference in survival over cohort 1, p=0.0069. This difference is below the required 0.05/2 = 0.0250 cutoff specified by the Bonferroni adjustment. The estimated 1- and 2-year survival probabilities were 68% (95% CI: 55%, 80%) and 52% (95% CI: 35%, 68%) for the two higher-dose groups combined, and 39% (95% CI: 22%, 56%) and 20% (95% CI: 4%,

36%) for the low dose group. There was no significant difference in survival between cohorts 1 and 2 vs. 3 (p=0.9198).

An estimated 64% percent (95% CI: 50%, 78%) and 47% (95% CI: 30%, 65%) of the advanced stage patients in combined cohorts 2 and 3 survived 1 year and 2 years respectively, compared to 37% (95% CI: 18%, 55%) and 18% (95% CI: 2%, 35%) in cohort 1. The overall difference in survival across dose cohorts for advanced stage patients only was marginally significant (p=0.0605). Estimated median survival of 581 days in dose cohorts 2 and 3 was significantly higher than that for dose cohort 1 (252 days) (p=0. 0186) (Figure 2). Median survival of all IIIB/IV patients was 441 days. One year and 2 year survival was 54% (95 CI: 42%, 67%) and 35% (95 CI: 20%, 51%) respectively. The difference in survival between dose cohorts 1 and 2 vs. 3 for advanced stage patients was not significant (p=0.5148).

Activiation of immune responses

Mononuclear cell cytokine production was measured prior to vaccination, and at weeks 4 and 8 after vaccination without further stimulation. The frequency of Intracellular cytokine producing cells from week 8 samples is shown in Figure 3. Vaccinated patients collectively demonstrated a higher frequency of TNF-α producing cells at week 8 (p=0.01; Wilcoxon). Further, patients achieving stable disease or better displayed a higher frequency of IFN-γ (p=0.006), IL-6 (p=0.004) and IL-4 (p=0.04) producing cells at week 12 than patients with progressive disease, although other cytokines in the panel did not exhibit marked differences between the treatment groups.

Prior to vaccination, nine of 36 patients lacked a significant ELISPOT response when stimulated with PMA + I (Table 3). However, 7 of these 9 generated a positive ELISPOT polyclonal response (PMA+I) at week 12 following Belagenpumatucel vaccination.

Further, a majority of advanced stage patients with stable disease or better produced a markedly elevated IFN-γ response (ELISPOT activity) when challenged with Belagenpumatucel (Table 3). Twelve of 20 patients with clinical response (PR or SD) displayed a ≥ 2-fold response. By comparison, 5 of 16 of patients with progressive disease (p=0.086; Pearson's chi-square, n=36) produced an elevated ELISPOT response (Table 3).

Antibody-mediated immune response

Positive clinical outcomes were correlated with development of HLA antibody response to the vaccine (Table 3). Eleven of 20 patients with stable disease or better developed novel HLA-antibody reactivity to one or more allotypes of the vaccinating cell lines, as compared with 2 of 16 from progressive disease patients (p=0.014, Fisher's exact test). Antibody activity was directed against the vaccinating haplotypes and cross-reacting epitopes.

Antibodies reactive with Belagenpumatucel were determined. Seven of 57 patients showed an increase in an IgM titer ≥ 2.0, 42/57 patients showed an increase in IgG titer ≥ 2.0. For IgG, 13/22 progressive disease patients had a stimulation index ≥ 2.0 compared to 29/35 of the stable disease or better patients (p=0.066).

DISCUSSION

Historically, NSCLC has generally been regarded as a non-immunogenic cancer [37]. Immunotherapy for lung cancer has yielded little benefit in humans [38-40] although preliminary results of more recent vaccine studies designed to enhance tumor antigen recognition have demonstrated beneficial outcome in subsets of patients [12, 13, 41-43]. In the current study we defined a clear dose related effect of Belagenpumatucel involving both early and late stage patients. We also found an impressive survival advantage at

dose levels ≥ 2.5 x 10^7 cells/injection, with an estimated 2-year survival of 47% in response to Belagenpumatucel in advanced stage patients. This compares favorably to the historical 2-year survival rate of less than 20% of stage IIIB/IV NSCLC patients. Furthermore, a correlation of positive clinical outcome with induction of immune enhancement of tumor antigen was observed.

Hypotheses as to why previous immune approaches to NSCLC have yielded disappointing results include ineffective priming of tumor specific T-cells, lack of high avidity tumor specific T-cells, and physical or functional disabling of primed tumor specific T-cells by primary host and/or tumor related mechanisms. For example, a high proportion of the tumor infiltrating lymphocytes (TIL) in NSCLC tissue are immunosuppressive T regulatory cells ($CD4^+CD25^+$) with spontaneous TGF-β secretion and constitutive high level expression of CTL A-4 [44, 45]. These have been shown to specifically prevent effective immune activation [44 46-52] and so could lead to tolerance (or cross-tolerance) of T cells rather than cross-priming [44, 45, 53-57]. In support of this hypothesis, elevated levels of IL-10 and TGF-β are found in patients with NSCLC.

Indeed, evidence suggests that TGF-β is a key immunosuppressive factor in NSCLC [15-21]. TGF-β is able to convert $CD4^+CD25^-$ naïve T cells to $CD4^+CD25^+$ regulatory T cells by induction of transcription factor Foxp3 [58, 59]. The process of cancer recruitment and subsequent recognition by immune processing cells, e.g., NK T cells, γδ T cells, macrophages, and $CD8^+$ αβ T cells, is dependent on ligand-receptor interactions such as the NKG2D C-type lectin-like homodimeric receptor on immune modulating cells and the stress-inducible MICA ligand on tumor cells including lung [60, 61]. TGF-β2 downregulates NKG2D on $CD8^+$ αβ T cells and NK cells. Further, it inhibits the transcription of MICA [62]. Silencing of TGF-β2 using siRNA targeting prevents NKG2D

downregulation and enhances transcription of MICA [62]. Moreover, both $CD4^+CD25^+$ T regulatory cells and lipopolysaccharide activated B cells demonstrating surface bound TGF-β2 produce anergy in $CD8^+$ T cells [63-65]. As a consequence, immunotherapy which attempts to improve immune effector function (TIL, IL-2), as opposed to antigen recognition, might be expected to be less effective [37, 44, 53, 66]. In animal models, immune suppression that was mediated by TGF-β has been attributed to impairment of high affinity IL-2 receptor function [27, 67-70] which leads to inhibition of cytotoxic T cell activation [22, 24, 27, 67, 69-73]. TGF-ß suppression of activation of effector function in memory $CD8^+$ T cells is effected even if the presence of exogenous IL-2 [74]. One could hypothesize that approaches which lead to enhancement of activation of effector cells (GVAX, dendritic therapy, GP96-Ig) may be more likely to provide greater activity, not excluding the fact that all such immune modulations are, in effect, multifactorial. Specifically, as noted above, TGF-ß has recently been shown to suppress the acquisition of effector function in memory $CD8^+$ T cells [74] and this may be mediated, in part, by inhibition of T-bet [75]. The introduction of antisense pCEP-4/TGF-ß2 into Morris hepatoma rat cells (MRH) reduced TGF-ß2 production from 1571 pg/ml to 92 pg/ml/10^6 cells/24 hours. Five days following implantation of wild-type MRH cells into Buffalo rats, the rodents were inoculated with irradiated MRH cells transfected with pCEP-4 antisense TGF-ß2. All 10 animals remained disease free compared with 50% injected with irradiated MRH cells modified with pCEP-4 vector alone [76]. Recent demonstrations of several dramatic responses with "priming" vaccine approaches are also consistent with this explanation [13, 77-79].

There is evidence for shared antigens in lung cancers [46-52, 79] as is seen in other tumor types [80, 81]. Dendritic cells, as opposed to tumor cells, may be responsible for the induction of anti-tumor immunity in tumor-bearing hosts by a process of antigenic cross presentation [82, 83]. One of the critical factors in dendritic cell based immunization is the activation status of dendritic cells. Immature dendritic cells are able to process antigen

but are not able to stimulate potent immune responses. The secretion of TGF-β2 by cancer cells may contribute to immunosuppression by blocking maturation of dendritic cells [84] and reversibly regulating dendritic cell chemotaxis by modifying chemokine receptor expression [85]. TGF-β2 has been shown to immobilize dendritic cells within the tumor and reduce the number of dendritic cells migrating to drainage lymph nodes [86]. Those dendritic cells reaching the drainage nodes are less likely to be completely activated thereby favoring the induction of cross-tolerance rather than cross-priming [87].

Our findings suggest that overall immune activation may contribute to a favorable clinical outcome. The current vaccination protocol employed whole tumor cells and the immunodominant antigens have not been characterized. Hence immune response evaluations have been limited to the use of unfractionated Belagenpumatucel preparations to encompass allogeneic as well as potential tumor-specific activational events. The selected immune assays represented attempts to document collateral immune activation events in the cellular (ELISPOT analyses) and humoral components (overall antibody production and HLA-directed Ab responses) that accompany Belagenpumatucel vaccination. Vaccination induced alloreactivity indicates that Belagenpumatucel is effective across allogeneic barriers, and is likely to translate into clinical effectiveness among immunocompetent patients who have the capacity to mount antigen-presentation/ activation events against novel allo- and/or tumor-associated antigens. Our findings of upregulated immune responses that correlated with clinical responsiveness supports the possibility of enhanced tumor antigen sensitization may be achieved through multiple treatments with the TGF-β2 antisense modified cancer vaccine.

In conclusion, in view of the acceptable safety profile and suggested survival advantage, further investigation is recommended. Data presented support the hypothesis that

inhibition of TGF-β2 in an allogeneic cell vaccine results in sufficient repression of inhibiting factors to allow for immune recognition and effector cell activation in patients with advanced NSCLC.

ACKNOWLEDGMENTS

The authors acknowledge Brenda Marr and Jennifer Sala for manuscript preparation, Linda Jennings, PhD for statistical analysis, Dr. Douglas Smith for HLA-related analyses, and Dr. Steven Dubinett for the cell line RH-2 analysis. This study was partially supported by SBIR grant 1 R44 CA96025 to HF.

FIGURE LEGEND

Figure 1: Dose related survival between cohorts for all patients (n=75, p=0.0155).

Figure 2: Overall survival for cohorts 1 vs. 2 and 3 for advanced stage patients (n=61, p=0.0186).

Figure 3: Cytokine response of peripheral blood lymphocytes from advanced stage patients receiving Belagenpumatucel. The frequency of cytokine-producing cells was determined by flow analysis, using cytokine-specific antibodies and an intracellular cytokine detection assay. Mean frequencies for individual patient cohorts pre-vaccination and at 8 weeks post-vaccination are shown.

Figure 4: Radiographic evidence of response comparing week 16 assessment (post therapy) to baseline (pre therapy) of patients #11, #20, and #38.

Figure 1

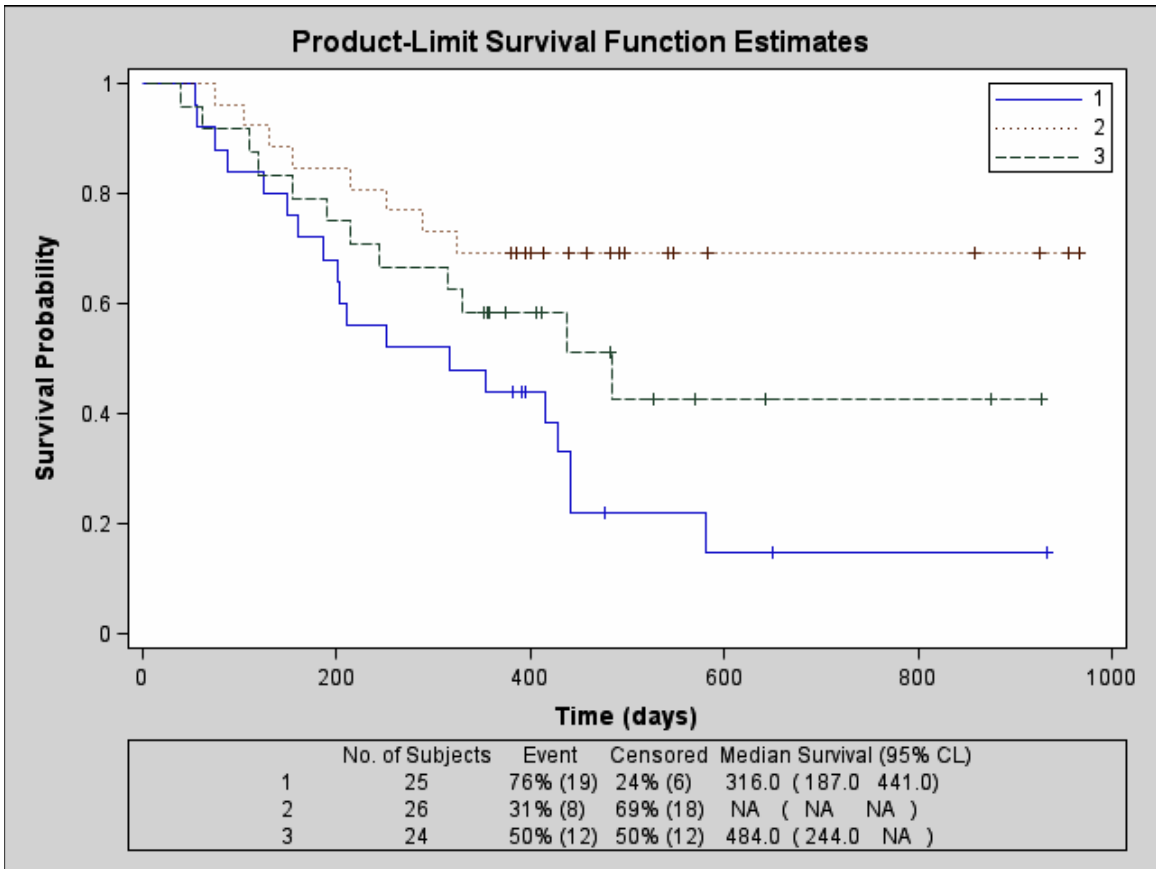
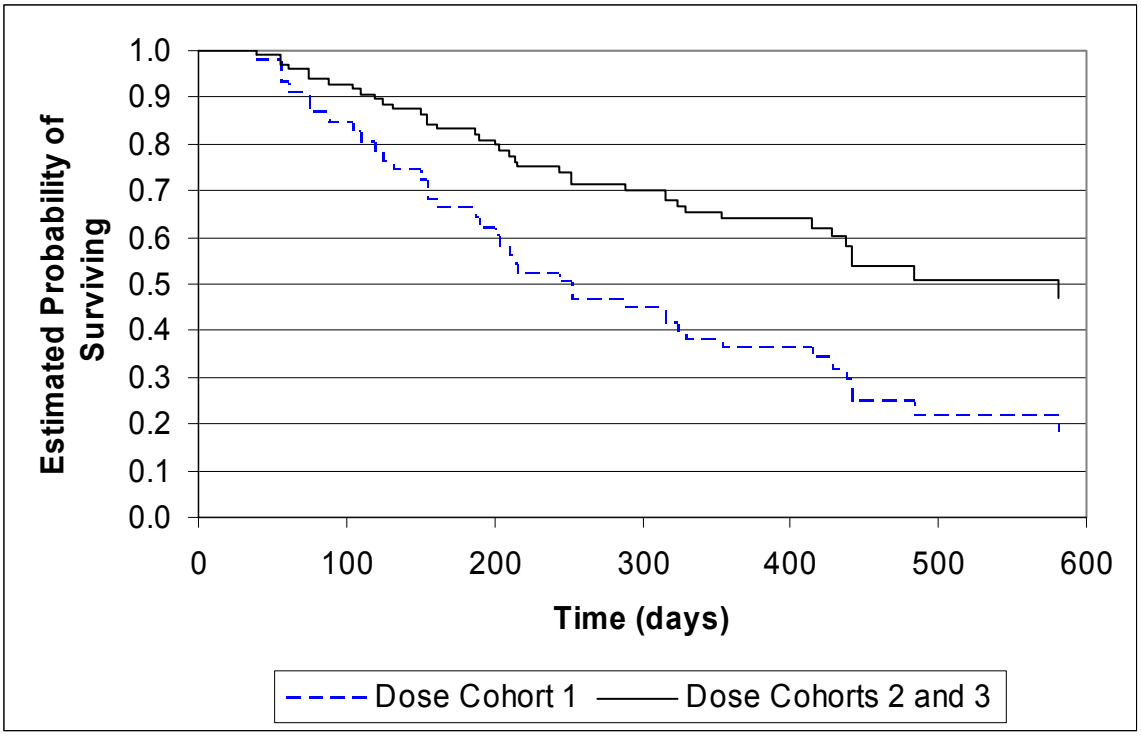

Product-Limit Survival Function Estimates
Survival Probability
1
0.8
0.6
0.4
0.2
0
0
200
400
600
800
1000
Time (days)
1
2
3
No. of Subjects Event Censored Median Survival (95% CL)
1 25 76% (19) 24% (6) 316.0 (187.0 441.0)
2 26 31% (8) 69% (18) NA (NA NA)
3 24 50% (12) 50% (12) 484.0 (244.0 NA)

Figure 2


Estimated Probability of Surviving
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0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
100
200
300
400
500
600
Time (days)
Dose Cohort 1
Dose Cohorts 2 and 3

Figure 3

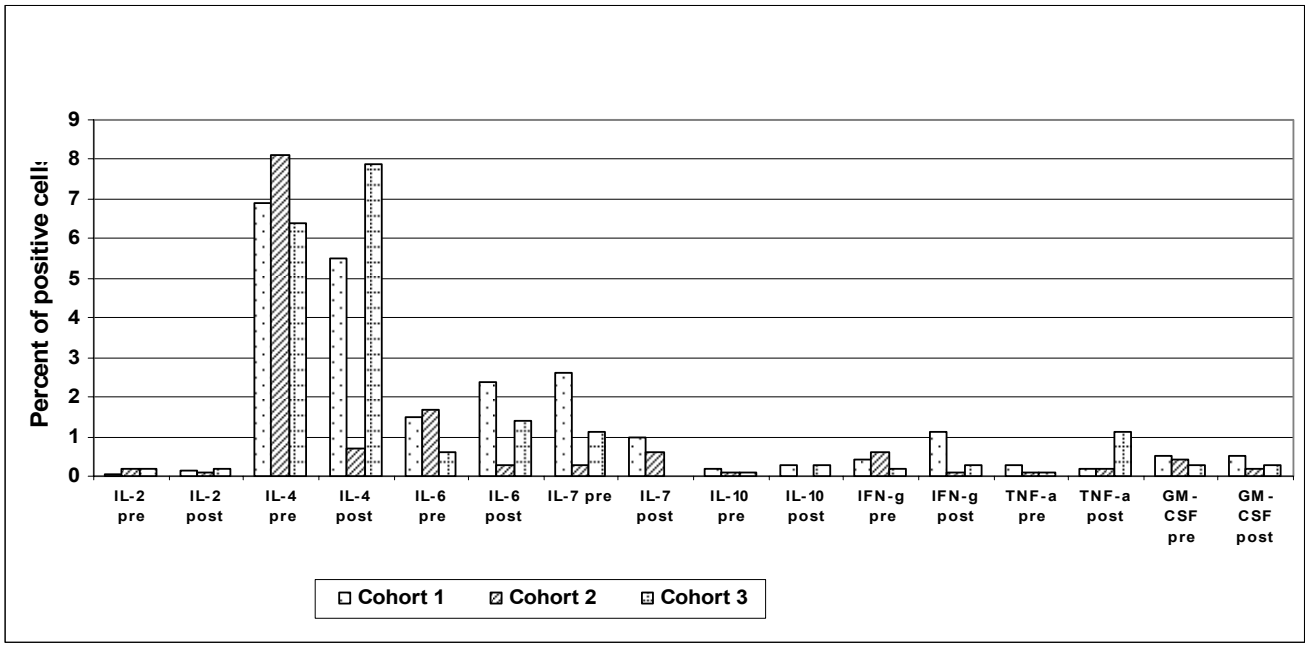
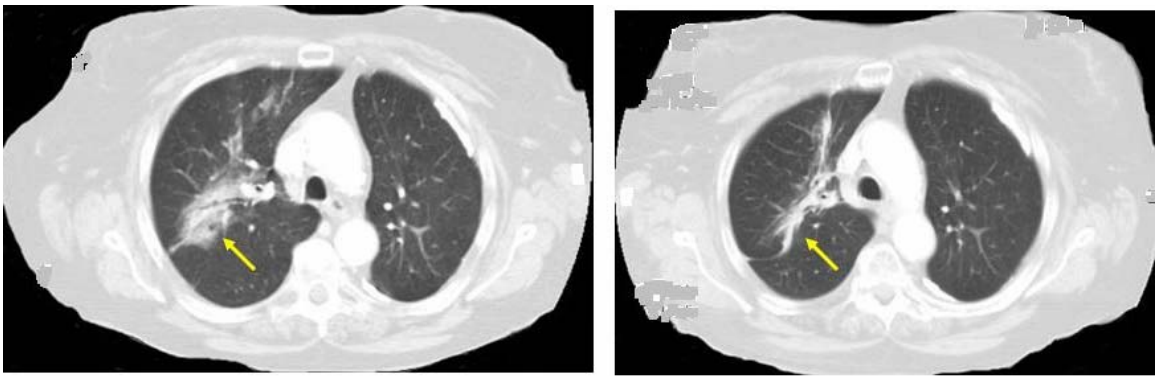

Percent of positive cells
9
8
7
6
5
4
3
2
1
0
IL-2 pre
IL-2 post
IL-4 pre
IL-4 post
IL-6 pre
IL-6 post
IL-7 pre
IL-7 post
IL-10 pre
IL-10 post
IFN-g pre
IFN-g post
TNF-a pre
TNF-a post
GM-CSF pre
GM-CSF post
Cohort 1
Cohort 2
Cohort 3

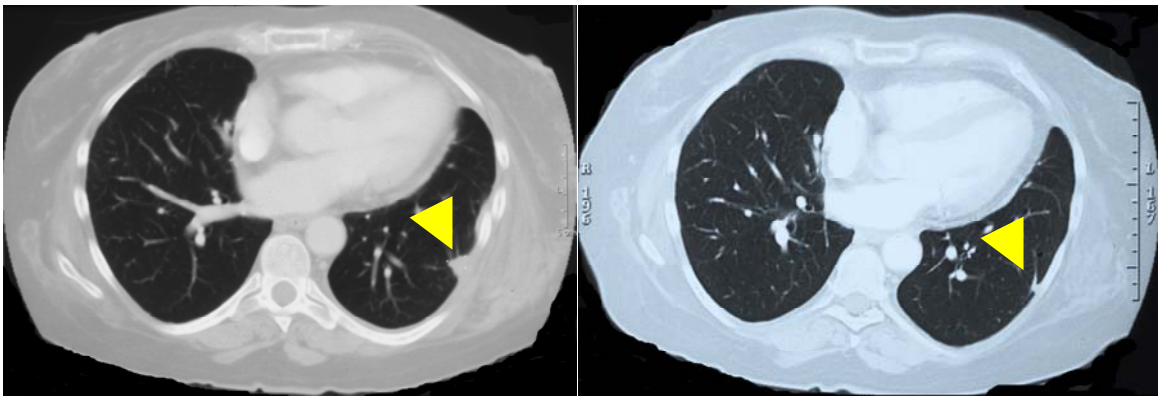
Figure 4

Patient #11



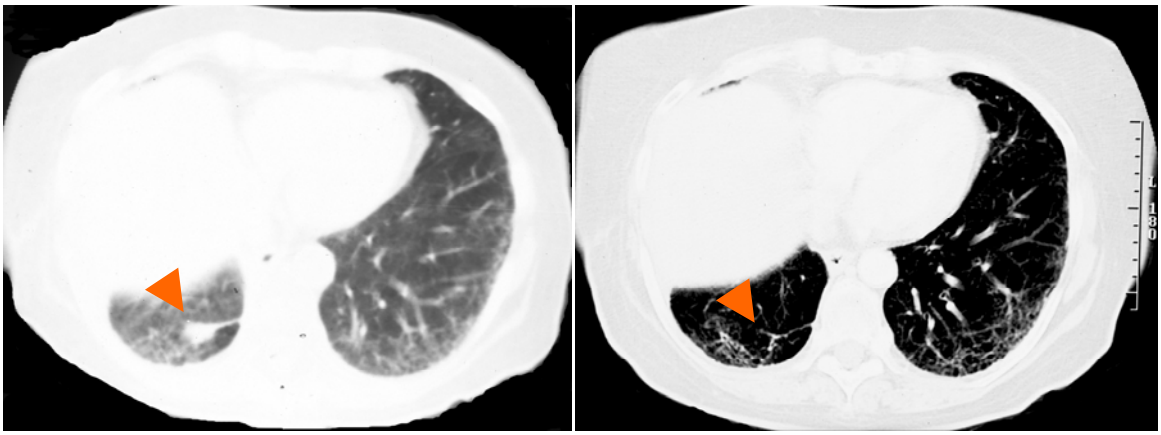
Pre-Therapy Post-Therapy

Patient #20



Pre-Therapy Post-Therapy

Patient #38



Pre-Therapy Post-Therapy

Table 1. Demographic Data

Description	# of Advanced Stage Patients (Stage IIIB/IV)	Early Stage Patients (Stage II/IIIA)
Number of Patients	61	14
Median Age (yrs.)	63	65
Range	41-82	51-77
Sex		
Male	31	11
Female	30	3
Stage		
II		2
IIIA		12
IIIB	15	
IV	46	
Cohort		
1	20 (6 IIIB, 14 IV)	5 (1 II, 4 IIIA)
2	20 (5 IIIB, 15 IV)	6 (0 II, 6 IIIA)
3	21 (4 IIIB, 17 IV)	3 (1 II, 2 IIIA)
Histology		
Adeno	40	6
Squamous	14	4
Other	7	4
# of Prior Cytotoxic Therapy		
0	10	4
1	16	4
2	16	6
>3	19	0
Prior Treatments		
Iressa	22	0
Tarceva	1	0
ECOG		
0	21	6
1	40	8
Stable Brain Metastasis	5	N/A
Tumor Presence		
NED[o]	3	N/A
NMD[o]	18	N/A
MD[o]	40	N/A
Median Tumor Volume (cm^3)*	12.02	N/A

* Nodal disease and bone disease not included in this measurement

[o] NED = no evidence of disease; NMD = no measurable disease but disease detectable (ie bone, ascites, pleural effusion); MD = measurable disease

Table 2. All Toxic Events Occurring at ≥ 5% Frequency.*

Final Event Term	Frequency of AE	# of Patients with at least 1 occurrence of AE	% of Patients (n = 75) with AE
Anemia	22	9	12.0
Anorexia	10	7	9.3
Breathing problem	35	16	21.3
Bruising	5	4	5.3
Constipation	4	4	5.3
Cough	33	19	25.3
Diarrhea	5	4	5.3
Dysphagia	6	5	6.7
Edema	9	4	5.3
Esophogeal problem	7	5	6.7
Fatigue	24	20	26.7
Flu-like symptoms	14	12	16.0
Fracture	5	4	5.3
Headache	13	9	12.0
Nausea	13	10	13.3
Pain	52	30	40.0
Rash	12	8	10.7
Respiratory problems	21	15	20.0
Sore throat	5	5	6.7
Vomiting	9	5	6.7
Weakness	12	10	13.3
Weight loss	12	11	14.7

*2 Grade 3 events possibly attributed to Belagenpumatucel were identified. One advanced stage patient developed grade 3 arm swelling at the injection site. Another stage IIIA patient developed chronic myelocytic leukemia (CML) 5 months after completion of 16 cycles of treatment. Bone marrow aspirates showed the presence of Philadelphia chromosome, a reciprocal translocation between chromosome 9 and 22 t(9:22)(q34;911) containing a chimeric gene composed of the 5' portion of the BCR gene on chromosome 22 and the 3' portion of the ABL gene on chromosome 9. We investigated a potential link between the PCHEK-HBA2 vector, which was used to gene-modify the four NSCLC cell line components of Belagenpumatucel, and the patient's development of CML. A sensitive polymerase chain reaction (PCR) assay to test peripheral blood mononuclear cells (PBMC) for the presence of the PCHEK-HBA2 vector was established. Nine PCR primer pairs to identify different regions of the pCHEK-HBA2 vector were designed. To prevent fortuitous PCR bands, each PCR amplicon was designed to span two different, adjacent DNA fragments in pCHEK-HBA2. The detection limit in this methodology is approximately eight (8) strands of vector. All control primer sets produced strong bands at the expected size for the pCHEK-HBA2 vector DNA, while no PCR products were detected in genomic DNA from the patient's PBMC. We concluded that there is no detectable pCHEK-HBA2 vector DNA or any of its nine fragments in the genomic DNA from the patient's PBMC post CML development. We also developed a series of PCR primers to walk through the ABL fragment of the Philadelphia chromosome isolated from the patient, and used the BCR-B2 segment of the BCR gene as the initial primer. We subsequently cloned the fragment of BCR-ABL fusion gene. It completely matched the expected DNA sequence in the NCBI data base, thereby, confirming lack of insertion of additional elements. The results of these studies suggest that the development of CML in this patient was not related to the vaccine.

Table 3. Immune Analysis Late Stage NSCLC

Site[om]/ Clinical response/ Patient ID	ELISPOT competence prior to vaccination*	ELISPOT (IFN-γ) response at week 12 by		Anti-HLA seroconversion to ****	
		PMA + I	Cancer Vaccines**	Vaccine haplotypes	Non cross-reacting haplotypes
Stable Disease or Better					
M004	**>200**	**>200**	**136 (+++)**	**Yes (A11,24,B40)**	**Yes**
M012	**>200**	**>200**	**76 (++)**	**Yes (A11,24,25,30,68)**	**Yes**
M032	**>200**	**>200**	**31 (+++)**	**No**	**No**
M005	**>200**	**>200**	**27 (++)**	**Yes (A11,24,25,68, B15,18,35)**	**Yes**
M017	**>200**	**>200**	**<20**	**No**	**No**
M033	**>200**	**>200**	**44 (++)**	**Yes (A11,30,B40)**	**Yes**
M031	**>200**	**>200**	**<20**	**No**	**No**
M030	**>200**	**>200**	**<20**	**No**	**No**
M028	**>200**	**>200**	**<20**	**Yes(A11,24,25,30, B15,18,35,40,44)**	**Yes**
M014	**>200**	**>200**	**37 (+)**	**Yes (A24)**	**Yes**
M022	**>200**	**>200**	**<20**	**Yes (B15,18,40)**	**Yes**
M001	**>200**	**>200**	**30 (++)**	**Yes (A11,24,B15,18,35))**	**Yes**
H004	**>200**	**>200**	**<20**	**Yes**	**N/A**
H005***	**>200**	**>200**	**99 (+)**	**No**	**No**
H009	**65**	**164**	**102[@] (+++)**	**No**	**No**
H010	**<20**	**60**	**194[@] (+++)**	**No**	**No**
H011***	**41**	**38**	**290[@] (+++)**	**No**	**No**
H012	**<20**	**<20**	**<20**	**Yes**	**N/A**
H015	**<20**	**>200**	**297 (+++)**	**Yes (B40)**	**Yes**
H017	**<20**	**<20**	**<20**	**No**	**No**
Progressive Disease					
M006	**>200**	**>200**	**55[@] (+)**	**No**	**No**
M024***	**117**	**>200**	**<20**	**No**	**No**
M025	**<20**	**22**	**<20**	**No**	**No**
M003[@]	**>200**	**>200**	**<20**	**No**	**No**
M007	**>200**	**>200**	**36 (++)**	**No**	**No**
M009	**>200**	**>200**	**66[@] (+++)**	**No**	**No**
M013[@]	**>200**	**>200**	**<20**	**No**	**No**
M015[@]	**>200**	**>200**	**<20**	**No**	**No**
M016	**>200**	**>200**	**<20**	**No**	**No**
M019	**>200**	**>200**	**<20**	**No**	**No**
M027	**<20**	**>200**	**<20**	**Yes (A11, 25, 30, 68, B15, 18, 35, 40, 44)**	**Yes**
M023[@]	**153**	**>200**	**<20**	**No**	**No**
M026[@]	**>200**	**>200**	**<20**	**No**	**No**
H003	**<20**	**>200**	**72 (++)**	**No**	**No**
H013	**<20**	**164**	**<20**	**Yes (A11,24)**	**Yes**
H018	**<20**	**65**	**73 (+++)**	**No**	**No**

[om]:M = Mary Crowley Medical Research Center; H = Hoag Cancer Center

*positive ELISPOT response (≥20 spots) to PMA+I.

**2x (+), >/=5x (++), or >/=10x (+++) increase in ELISPOT response as compared with week 0; all replicas differed by ≤25% of mean.

***displays a positive ELISPOT response to vaccine prior to immunization

****by ELISA and multiplex flow analysis using HLA-Ab-conjugated beads, seroconversion = novel reactivity not found prevaccination.

[@]determined with PBMC collected at week 8 since week 12 sample was unavailable. All other samples from week 12.

N/A Antibody Specificities can not be determined.

REFERENCES

1. Parkin DM, Bray FI, Devesa SS. Cancer burden in the year 2000. The global picture. Eur J Cancer 2001;37 Suppl 8:S4-66.
2. Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ. Cancer statistics, 2003. CA Cancer J Clin 2003;53(1):5-26.
3. Chemotherapy in non-small cell lung cancer: a meta-analysis using updated data on individual patients from 52 randomised clinical trials. Non-small Cell Lung Cancer Collaborative Group. Bmj 1995;311(7010):899-909.
4. Grilli R, Oxman AD, Julian JA. Chemotherapy for advanced non-small-cell lung cancer: how much benefit is enough? J Clin Oncol 1993;11(10):1866-72.
5. Schiller JH, Harrington D, Belani CP, et al. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. N Engl J Med 2002;346(2):92-8.
6. Shepherd FA, Dancey J, Ramlau R, et al. Prospective randomized trial of docetaxel versus best supportive care in patients with non-small-cell lung cancer previously treated with platinum-based chemotherapy. J Clin Oncol 2000;18(10):2095-103.
7. Fossella FV, DeVore R, Kerr RN, et al. Randomized phase III trial of docetaxel versus vinorelbine or ifosfamide in patients with advanced non-small-cell lung cancer previously treated with platinum-containing chemotherapy regimens. The TAX 320 Non-Small Cell Lung Cancer Study Group. J Clin Oncol 2000;18(12):2354-62.
8. Hanna N, Shepherd FA, Fossella FV, et al. Randomized phase III trial of pemetrexed versus docetaxel in patients with non-small-cell lung cancer previously treated with chemotherapy. J Clin Oncol 2004;22(9):1589-97.
9. Tsao MS, Sakurada A, Cutz JC, et al. Erlotinib in lung cancer - molecular and clinical predictors of outcome. N Engl J Med 2005;353(2):133-44.
10. Shepherd FA, Rodrigues Pereira J, Ciuleanu T, et al. Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med 2005;353(2):123-32.
11. Kris MG, Natale RB, Herbst RS, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. Jama 2003;290(16):2149-58.
12. Nemunaitis J. Vaccines in cancer: GVAX, a GM-CSF gene vaccine. Expert Rev Vaccines 2005;4(3):259-74.
13. Nemunaitis J, Sterman D, Jablons D, et al. Granulocyte-macrophage colony-stimulating factor gene-modified autologous tumor vaccines in non-small-cell lung cancer. J Natl Cancer Inst 2004;96(4):326-31.
14. Fakhrai H, Gramatikova S, Safaei R. Down-regulation of TGF-ß2 as a therapeutic approach In" Brain Tumor Immunotherapy. Totowa, New Jersey: Humana Press; 2001.
15. Sporn MB, Roberts AB, Wakefield LM, Assoian RK. Transforming growth factor-beta: biological function and chemical structure. Science 1986;233(4763):532-4.
16. Massague J. The TGF-beta family of growth and differentiation factors. Cell 1987;49(4):437-8.
17. Border WA, Ruoslahti E. Transforming growth factor-beta in disease: the dark side of tissue repair. J Clin Invest 1992;90(1):1-7.
18. Bodmer S, Strommer K, Frei K, et al. Immunosuppression and transforming growth factor-beta in glioblastoma. Preferential production of transforming growth factor-beta 2. J Immunol 1989;143(10):3222-9.
19. Jakowlew SB, Mathias A, Chung P, Moody TW. Expression of transforming growth factor beta ligand and receptor messenger RNAs in lung cancer cell lines. Cell Growth Differ 1995;6(4):465-76.

20. Constam DB, Philipp J, Malipiero UV, ten Dijke P, Schachner M, Fontana A. Differential expression of transforming growth factor-beta 1, -beta 2, and -beta 3 by glioblastoma cells, astrocytes, and microglia. J Immunol 1992;148(5):1404-10.
21. Kong F, Jirtle RL, Huang DH, Clough RW, Anscher MS. Plasma transforming growth factor-beta1 level before radiotherapy correlates with long term outcome of patients with lung carcinoma. Cancer 1999;86(9):1712-9.
22. Rook AH, Kehrl JH, Wakefield LM, et al. Effects of transforming growth factor beta on the functions of natural killer cells: depressed cytolytic activity and blunting of interferon responsiveness. J Immunol 1986;136(10):3916-20.
23. Kasid A, Bell GI, Director EP. Effects of transforming growth factor-beta on human lymphokine-activated killer cell precursors. Autocrine inhibition of cellular proliferation and differentiation to immune killer cells. J Immunol 1988;141(2):690-8.
24. Tsunawaki S, Sporn M, Ding A, Nathan C. Deactivation of macrophages by transforming growth factor-beta. Nature 1988;334(6179):260-2.
25. Hirte H, Clark DA. Generation of lymphokine-activated killer cells in human ovarian carcinoma ascitic fluid: identification of transforming growth factor-beta as a suppressive factor. Cancer Immunol Immunother 1991;32(5):296-302.
26. Naganuma H, Sasaki A, Satoh E, et al. Transforming growth factor-beta inhibits interferon-gamma secretion by lymphokine-activated killer cells stimulated with tumor cells. Neurol Med Chir (Tokyo) 1996;36(11):789-95.
27. Ruffini PA, Rivoltini L, Silvani A, Boiardi A, Parmiani G. Factors, including transforming growth factor beta, released in the glioblastoma residual cavity, impair activity of adherent lymphokine-activated killer cells. Cancer Immunol Immunother 1993;36(6):409-16.
28. Fakhrai H, Mantil JC, Liu L, et al. Phase I Clinical Trial of TGF-β Antisense-modified Tumor Cell Vaccine in patients with Advanced Glioma. Cancer Gene Therapy (in press).
29. Liau LM, Fakhrai H, Black KL. Prolonged survival of rats with intracranial C6 gliomas by treatment with TGF-beta antisense gene. Neurol Res 1998;20(8):742-7.
30. Dorigo O, Shawler DL, Royston I, Sobol RE, Berek JS, Fakhrai H. Combination of transforming growth factor beta antisense and interleukin-2 gene therapy in the murine ovarian teratoma model. Gynecol Oncol 1998;71(2):204-10.
31. Tzai TS, Shiau AL, Liu LL, Wu CL. Immunization with TGF-beta antisense oligonucleotide-modified autologous tumor vaccine enhances the antitumor immunity of MBT-2 tumor-bearing mice through upregulation of MHC class I and Fas expressions. Anticancer Res 2000;20(3A):1557-62.
32. Tzai TS, Lin CI, Shiau AL, Wu CL. Antisense oligonucleotide specific for transforming growth factor-beta 1 inhibit both in vitro and in vivo growth of MBT-2 murine bladder cancer. Anticancer Res 1998;18(3A):1585-9.
33. Marzo AL, Fitzpatrick DR, Robinson BW, Scott B. Antisense oligonucleotides specific for transforming growth factor beta2 inhibit the growth of malignant mesothelioma both in vitro and in vivo. Cancer Res 1997;57(15):3200-7.
34. Park JA, Wang E, Kurt RA, Schluter SF, Hersh EM, Akporiaye ET. Expression of an antisense transforming growth factor-beta1 transgene reduces tumorigenicity of EMT6 mammary tumor cells. Cancer Gene Ther 1997;4(1):42-50.
35. Kettering JD, Mohamedali AM, Green LM, Gridley DS. IL-2 gene and antisense TGF-beta1 strategies counteract HSV-2 transformed tumor progression. Technol Cancer Res Treat 2003;2(3):211-21.
36. Fakhrai H, Dorigo O, Shawler DL, et al. Eradication of established intracranial rat gliomas by transforming growth factor beta antisense gene therapy. Proc Natl Acad Sci U S A 1996;93(7):2909-14.
37. Salgaller ML. The development of immunotherapies for non-small cell lung cancer. Expert Opin Biol Ther 2002;2(3):265-78.

38. Ratto GB, Zino P, Mirabelli S, et al. A randomized trial of adoptive immunotherapy with tumor-infiltrating lymphocytes and interleukin-2 versus standard therapy in the postoperative treatment of resected nonsmall cell lung carcinoma. Cancer 1996;78(2):244-51.
39. Lissoni P, Meregalli S, Fossati V, et al. A randomized study of immunotherapy with low-dose subcutaneous interleukin-2 plus melatonin vs chemotherapy with cisplatin and etoposide as first-line therapy for advanced non-small cell lung cancer. Tumori 1994;80(6):464-7.
40. Ratto GB, Cafferata MA, Scolaro T, et al. Phase II study of combined immunotherapy, chemotherapy, and radiotherapy in the postoperative treatment of advanced non-small-cell lung cancer. J Immunother 2000;23(1):161-7.
41. Salgia R, Lynch T, Skarin A, et al. Vaccination with irradiated autologous tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor augments antitumor immunity in some patients with metastatic non-small-cell lung carcinoma. J Clin Oncol 2003;21(4):624-30.
42. Soiffer R, Hodi FS, Haluska F, et al. Vaccination with irradiated, autologous melanoma cells engineered to secrete granulocyte-macrophage colony-stimulating factor by adenoviral-mediated gene transfer augments antitumor immunity in patients with metastatic melanoma. J Clin Oncol 2003;21(17):3343-50.
43. Morse MA. Technology evaluation: BLP-25, Biomira Inc. Curr Opin Mol Ther 2001;3(1):102-5.
44. Woo EY, Chu CS, Goletz TJ, et al. Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. Cancer Res 2001;61(12):4766-72.
45. Woo EY, Yeh H, Chu CS, et al. Cutting edge: Regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. J Immunol 2002;168(9):4272-6.
46. Sienel W, Varwerk C, Linder A, et al. Melanoma associated antigen (MAGE)-A3 expression in Stages I and II non-small cell lung cancer: results of a multi-center study. Eur J Cardiothorac Surg 2004;25(1):131-4.
47. Yamazaki K, Spruill G, Rhoderick J, Spielman J, Savaraj N, Podack ER. Small cell lung carcinomas express shared and private tumor antigens presented by HLA-A1 or HLA-A2. Cancer Res 1999;59(18):4642-50.
48. Weynants P, Thonnard J, Marchand M, Delos M, Boon T, Coulie PG. Derivation of tumor-specific cytolytic T-cell clones from two lung cancer patients with long survival. Am J Respir Crit Care Med 1999;159(1):55-62.
49. Bixby DL, Yannelli JR. CD80 expression in an HLA-A2-positive human non-small cell lung cancer cell line enhances tumor-specific cytotoxicity of HLA-A2-positive T cells derived from a normal donor and a patient with non-small cell lung cancer. Int J Cancer 1998;78(6):685-94.
50. Yamada A, Kawano K, Koga M, Takamori S, Nakagawa M, Itoh K. Gene and peptide analyses of newly defined lung cancer antigens recognized by HLA-A2402-restricted tumor-specific cytotoxic T lymphocytes. Cancer Res 2003;63(11):2829-35.
51. Jiang F, Yin Z, Caraway NP, Li R, Katz RL. Genomic profiles in stage I primary non small cell lung cancer using comparative genomic hybridization analysis of cDNA microarrays. Neoplasia 2004;6(5):623-35.
52. Melloni G, Ferreri AJ, Russo V, et al. Prognostic significance of cancer-testis gene expression in resected non-small cell lung cancer patients. Oncol Rep 2004;12(1):145-51.
53. Neuner A, Schindel M, Wildenberg U, Muley T, Lahm H, Fischer JR. Prognostic significance of cytokine modulation in non-small cell lung cancer. Int J Cancer 2002;101(3):287-92.

54. Neuner A, Schindel M, Wildenberg U, Muley T, Lahm H, Fischer JR. Cytokine secretion: clinical relevance of immunosuppression in non-small cell lung cancer. Lung Cancer 2001;34 Suppl 2:S79-82.
55. Dohadwala M, Luo J, Zhu L, et al. Non-small cell lung cancer cyclooxygenase-2-dependent invasion is mediated by CD44. J Biol Chem 2001;276(24):20809-12.
56. Schwartz RH. Models of T cell anergy: is there a common molecular mechanism? J Exp Med 1996;184(1):1-8.
57. Lombardi G, Sidhu S, Batchelor R, Lechler R. Anergic T cells as suppressor cells in vitro. Science 1994;264(5165):1587-9.
58. Chen W, Jin W, Hardegen N, et al. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med 2003;198(12):1875-86.
59. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. J Immunol 2004;172(9):5149-53.
60. Groh V, Rhinehart R, Secrist H, Bauer S, Grabstein KH, Spies T. Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. Proc Natl Acad Sci U S A 1999;96(12):6879-84.
61. Bauer S, Groh V, Wu J, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 1999;285(5428):727-9.
62. Friese MA, Wischhusen J, Wick W, et al. RNA interference targeting transforming growth factor-beta enhances NKG2D-mediated antiglioma immune response, inhibits glioma cell migration and invasiveness, and abrogates tumorigenicity in vivo. Cancer Res 2004;64(20):7596-603.
63. Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. J Exp Med 2001;194(5):629-44.
64. Annunziato F, Cosmi L, Liotta F, et al. Phenotype, localization, and mechanism of suppression of CD4(+)CD25(+) human thymocytes. J Exp Med 2002;196(3):379-87.
65. Parekh VV, Prasad DV, Banerjee PP, Joshi BN, Kumar A, Mishra GC. B cells activated by lipopolysaccharide, but not by anti-Ig and anti-CD40 antibody, induce anergy in CD8+ T cells: role of TGF-beta 1. J Immunol 2003;170(12):5897-911.
66. Castelli C, Rivoltini L, Andreola G, Carrabba M, Renkvist N, Parmiani G. T-cell recognition of melanoma-associated antigens. J Cell Physiol 2000;182(3):323-31.
67. Roszman T, Elliott L, Brooks W. Modulation of T-cell function by gliomas. Immunol Today 1991;12(10):370-4.
68. Smith KA. Interleukin-2: inception, impact, and implications. Science 1988;240(4856):1169-76.
69. Smith KA. Lowest dose interleukin-2 immunotherapy. Blood 1993;81(6):1414-23.
70. Tigges MA, Casey LS, Koshland ME. Mechanism of interleukin-2 signaling: mediation of different outcomes by a single receptor and transduction pathway. Science 1989;243(4892):781-6.
71. Fontana A, Frei K, Bodmer S, et al. Transforming growth factor-beta inhibits the generation of cytotoxic T cells in virus-infected mice. J Immunol 1989;143(10):3230-4.
72. Hirte HW, Clark DA, O'Connell G, Rusthoven J, Mazurka J. Reversal of suppression of lymphokine-activated killer cells by transforming growth factor-beta in ovarian carcinoma ascitic fluid requires interleukin-2 combined with anti-CD3 antibody. Cell Immunol 1992;142(1):207-16.
73. Ranges GE, Figari IS, Espevik T, Palladino MA, Jr. Inhibition of cytotoxic T cell development by transforming growth factor beta and reversal by recombinant tumor necrosis factor alpha. J Exp Med 1987;166(4):991-8.
74. Ahmadzadeh M, Rosenberg SA. TGF-beta 1 attenuates the acquisition and expression of effector function by tumor antigen-specific human memory CD8 T cells. J Immunol 2005;174(9):5215-23.

75. Gorelik L, Constant S, Flavell RA. Mechanism of transforming growth factor beta-induced inhibition of T helper type 1 differentiation. J Exp Med 2002;195(11):1499-505.
76. Maggard M, Meng L, Ke B, Allen R, Devgan L, Imagawa DK. Antisense TGF-beta2 immunotherapy for hepatocellular carcinoma: treatment in a rat tumor model. Ann Surg Oncol 2001;8(1):32-7.
77. Hirschowitz EA, Foody T, Kryscio R, Dickson L, Sturgill J, Yannelli J. Autologous dendritic cell vaccines for non-small-cell lung cancer. J Clin Oncol 2004;22(14):2808-15.
78. Raez LE, Cassileth PA, Schlesselman JJ, et al. Allogeneic vaccination with a B7.1 HLA-A gene-modified adenocarcinoma cell line in patients with advanced non-small-cell lung cancer. J Clin Oncol 2004;22(14):2800-7.
79. Nemunaitis J MT, Senzer N, Cunningham C, Anthony, S, Vukelja S, Berman B, Sarmiento S, Arzaga R, Cheever M. Phase I Trial of Sequential Administration of Recombinant DNA and Adenoviral Expressing L523S Protein in Early Stage Non Small Cell Lung Cancer (NSCLC). Submitted.
80. Fong L, Engleman EG. Dendritic cells in cancer immunotherapy. Annu Rev Immunol 2000;18:245-73.
81. Boon T, Cerottini JC, Van den Eynde B, van der Bruggen P, Van Pel A. Tumor antigens recognized by T lymphocytes. Annu Rev Immunol 1994;12:337-65.
82. Nestle FO, Burg G, Fah J, Wrone-Smith T, Nickoloff BJ. Human sunlight-induced basal-cell-carcinoma-associated dendritic cells are deficient in T cell co-stimulatory molecules and are impaired as antigen-presenting cells. Am J Pathol 1997;150(2):641-51.
83. Enk AH, Jonuleit H, Saloga J, Knop J. Dendritic cells as mediators of tumor-induced tolerance in metastatic melanoma. Int J Cancer 1997;73(3):309-16.
84. Yamaguchi Y, Tsumura H, Miwa M, Inaba K. Contrasting effects of TGF-beta 1 and TNF-alpha on the development of dendritic cells from progenitors in mouse bone marrow. Stem Cells 1997;15(2):144-53.
85. Sato K, Kawasaki H, Nagayama H, et al. TGF-beta 1 reciprocally controls chemotaxis of human peripheral blood monocyte-derived dendritic cells via chemokine receptors. J Immunol 2000;164(5):2285-95.
86. Weber F, Byrne SN, Le S, et al. Transforming growth factor-beta1 immobilises dendritic cells within skin tumours and facilitates tumour escape from the immune system. Cancer Immunol Immunother 2005;54(9):898-906.
87. Overwijk WW. Breaking tolerance in cancer immunotherapy: time to ACT. Curr Opin Immunol 2005;17(2):187-94.