Double-Blind Trial of a Polyvalent, Shed-Antigen, Melanoma Vaccine[1]

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ABSTRACT

A polyvalent melanoma vaccine prepared from shed antigens stimulates humoral and cellular immune responses and improves survival compared with historical controls. We conducted a double-blind, prospectively randomized, placebo-controlled trial to assess whether this vaccine could slow the progression of resected melanoma. Thirty-eight patients with resected melanoma metastatic to regional nodes (American Joint Committee on Cancer stage III) who had a particularly poor prognosis on the basis of the nodes being clinically positive or two or more histologically positive nodes were randomly assigned in a 2:1 ratio to treatment with 40 μg of melanoma or placebo (human albumin) vaccine, both of which were bound to alum as an adjuvant. Immunizations were given intradermally into the extremities every 3 weeks × 4, monthly × 3, every 3 months × 2, and then every 6 months for 5 years or until disease progression. Twenty-four patients were treated with the melanoma, and 14 patients were treated with the placebo vaccine. The groups were evenly balanced with respect to prognostic factors. Median length of observation was 2.5 years. There was no local or systemic toxicity. By Kaplan-Meier analysis, median time to disease progression was two and a half times longer in patients treated with melanoma vaccine compared with that in patients treated with placebo vaccine, *i.e.*, 1.6 years (95% confidence interval, 1.0–3.0 years) compared with 0.6 year [95% confidence interval, 0.3–1.9 year(s)]. By Cox proportional hazards analysis, this difference was significant at $P = 0.03$. Overall survival was 40% longer in the melanoma vaccine-treated group (median overall survival of 3.8 years *versus* 2.7 years), but this difference was not statistically significant. In a double-blind and placebo-controlled trial, these results suggest that immunization with a melanoma vaccine may be able to slow the progression of melanoma. Although statistically significant, these results must be interpreted with caution because they are based on a small number of patients.

INTRODUCTION

Malignant melanoma is one of the cancers whose incidence is increasing the most rapidly (1). It is estimated that there were >44,000 new cases and 9,200 deaths from this cancer in the United States in 1999 (2). Surgical resection of early-stage localized disease is the only curative treatment. Once melanoma metastasizes to regional nodes (AJCC[3] stage III disease), only one-half of patients survive for 5 years (3, 4). The only therapy to demonstrate a statistically significant survival benefit in resected AJCC stage III melanoma is high-dose IFN-α2b (5). However, the improvement in outcome is modest, and the morbidity and cost of treatment are significant. Thus, there is a continued need for additional treatments for patients with melanoma at high risk of recurrence.

One approach is the use of vaccines. These are intended to stimulate protective immune responses against tumor-associated antigens. Vaccines have been demonstrated to be effective in preventing melanoma in animals models (6). Moreover, they have few side effects in humans and may stimulate humoral and/or cellular immune responses against melanoma antigens (7–20). Comparisons of vaccine-treated patients with untreated historical series point to the possibility of improved clinical outcome (11, 12, 15, 17–19). However, the clinical effectiveness of melanoma vaccines has not been confirmed in randomized, double-blind trials (20).

A number of strategies are available to construct cancer vaccines (19). These must address several requirements to be effective. The two most important requirements are as follows: (*a*) the vaccine must contain tumor antigens that can stimulate clinically effective antitumor immune responses; and (*b*) some of these antigens must be expressed on the patient's own tumors. Unfortunately, the identity of tumor antigens that can stimulate tumor-protective immune responses is largely unknown, as is the antigenic phenotype of most cancers at the time they are treated. One way to address these requirements is to prepare a polyvalent vaccine that contains a broad range of different tumor antigens (19) because this will increase the likelihood that

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[3] The abbreviations used are: AJCC, American Joint Committee on Cancer; ECOG, Eastern Cooperative Oncology Group; CI, confidence interval.

the vaccine will contain antigens that both stimulate protective immunity and are expressed by the tumor to be treated. Polyvalent vaccines have several additional properties that improve their chances of being clinically effective. They can stimulate immune responses against multiple targets on cancer cells, which is more likely to result in tumor cell kill than responses directed to a single antigen; they can more readily circumvent HLA-dependent and -independent (21) heterogeneity in the ability of patients to develop immune responses to individual tumor antigens; and they may minimize the development of antigen-loss tumor cell variants that can escape immune recognition. These advantages probably outweigh the disadvantages of the approach, which are as follows: (*a*) the methods used to prepare broadly polyvalent vaccines result in irrelevant material being present in the vaccine; and (*b*) to the extent that this occurs, it reduces the concentration of the relevant but still unknown antigens that mediate tumor protective immunity.

The strategy we have adopted to construct polyvalent cancer vaccines is to prepare them from antigens shed by tumor cells. This approach is based on the observation that tumor cells rapidly shed into culture medium a broad range of different tumor antigens that are expressed on their external surface (22). These antigens are partially purified because they are separated from the bulk of cellular material, which is in the cytoplasm and nucleus and is poorly shed (22). The advantages of this approach are that the resulting vaccine is both polyvalent and partially purified and that the antigens it contains are more likely to be biologically relevant because they are expressed on the external surface of tumor cells, where they can be seen and interact with host defense mechanisms. We have applied this approach to construct a vaccine for melanoma that contains multiple melanoma-associated antigens that are immunogenic *in vivo* in humans, as evidenced by their ability to stimulate immune responses in vaccine-treated patients. These responses include: (*a*) antibody responses to antigens of 45, 59, 68, 79, 89, 95, and 110 kDa that are expressed *in vivo* by human melanoma (14); (*b*) CD8+ T-cell responses to the melanoma-associated antigens MAGE-1, MAGE-2, MAGE-3, MART-1, tyrosinase, gp100, and TRP-2. The CD8 responses are directed to multiple peptides derived from each of these antigens and presented by the major class I HLA allotypes present in patients with melanoma (13, 21); and (*c*) cellular immune responses that infiltrate into melanoma nodules *in vivo* (23). Prior trials have demonstrated that the vaccine has minimal toxicity (19). The vaccine appears to be clinically effective because the recurrence-free survival and overall survival of vaccine-treated patients with resected AJCC stage III melanoma are both prolonged by 50% compared with historical controls (7, 19). There is a relation between vaccine-induced immune responses (7, 13) and an improved clinical outcome.

To more objectively investigate the clinical effectiveness of the vaccine, we conducted a double-blind, placebo-controlled, randomized trial in patients with resected AJCC stage III melanoma who had a particularly high risk of disease recurrence.

PATIENTS AND METHODS

Patients. The study was conducted at the New York University Kaplan Comprehensive Cancer Center in patients between the ages of 18 and 76 years with histologically confirmed, surgically resected, AJCC stage III (metastatic to regional nodes) malignant melanoma who had a poor prognosis on the basis of having nodes that were clinically palpable at presentation or ≥2 histologically positive nodes. Patients were required to have intact cellular immunity at baseline as defined by 5 mm or more of induration at 48 h to skin test to one or more of the following recall antigens (PPD intermediate, mumps, *Candida*, SK-SD) or the ability to be sensitized to DNCB. Patients were required to have normal liver, renal, and hematological studies. Patients were excluded if they had metastasis beyond the regional nodes, metastasis to more than one anatomically distinct nodal group, any prior therapy for melanoma other than surgery or local radiotherapy, or a previous cancer other than non-melanoma skin cancer. Patients who were potentially immunosuppressed because of medication (corticosteroids or cytotoxic drugs) within 28 days of starting therapy, HIV infection, or autoimmune disease were ineligible. All patients gave written informed consent to participate in the study, which was approved by the New York University Institutional Review Board.

Construction of Melanoma and Placebo Vaccines. A polyvalent, shed antigen, melanoma vaccine was prepared as described previously from material shed into culture medium by four lines of melanoma cells adapted to long-term growth in serum-free medium (7). Three of the lines were allogeneic, and one was xenogeneic. The presence of xenogeneic antigens in the vaccine may improve its immunological activity because recent studies suggest that the xenogeneic version of an antigen may induce immune responses more effectively than the autologous version of that antigen (24). The shed material was collected, concentrated by ultrafiltration, treated with a final concentration of 0.05% NP40, and ultracentrifuged at 100,000 × *g* for 90 min. Supernatants from the four cell lines were adjusted to contain the same amount of protein, pooled, and filter-sterilized through a 0.22 μm Millipore filter. A placebo vaccine was prepared in a similar fashion from human albumin (human albumin VSP 25%; Armour Pharmaceutical Co., Kankakee, IL). Both vaccines were adjusted to a final protein concentration of 200 μg/ml in normal saline, bound to alum as an adjuvant, and stored in sterile vials.

Study Design and Vaccine Treatment. After verification of eligibility, patients were randomly assigned to treatment with either 40 μg of melanoma vaccine or placebo vaccine. To encourage enrollment into the trial, patients were randomized in a 2:1 ratio between melanoma vaccine and placebo vaccine. To achieve this while maintaining the double-blind status of the trial, patients were randomly allocated to one of three treatment groups using a computer-generated code. Two of the groups were treated with the melanoma vaccine, and one group was treated with the placebo vaccine. Immunizations were administered intradermally, divided into four sites (the volar surface of the forearms and the anterior surface of the upper anterior thighs). No injection was given into an extremity subjected to a lymph node dissection, but an additional injection was administered into the contralateral extremity. Immunizations were given every 3 weeks for four cycles, monthly for three cycles, every 3 months for two cycles, and then every 6 months for a total of 5 years or until disease progression.

Patient Monitoring. Screening tests to determine eligibility were performed before initiation of therapy and included but were not limited to a medical history and physical examination, ECOG performance status evaluation, complete blood count, liver function tests, BUN, creatinine, chest X-ray, and computed tomography of the brain, chest, abdomen, and pelvis. Patients were monitored every 3 weeks during the first 2 months and then monitored at intervals of 1–3 months during the first year and at intervals of 3 months thereafter. Laboratory studies (blood count and liver function tests) were obtained every 3 months, chest X-rays were obtained every 6 months, and computed tomography scans of the brain, chest, and abdomen were obtained once a year or as clinically indicated. Toxicity included all adverse experiences, regardless of whether or not they were treatment related.

Statistical Considerations. The planned sample size was 210 patients, *i.e.*, 140 patients in the vaccine arm and 70 in the placebo arm. This sample size calculation assumed a 2-year recurrence-free survival of 50% in the control group, a 10% withdrawal rate, and a one-sided log-rank test with a significance level of 0.05. Under these assumptions, this sample size ensured 80% power to detect an improvement in recurrence-free survival at 2 years to 70%. However, the study was closed prematurely after the publication by the ECOG that IFN-α2b significantly prolonged relapse-free and overall survival in AJCC stage III melanoma (5). Nonetheless, the actual improvement in recurrence-free survival between the two groups was sufficiently large to reach statistical significance, although the planned sample size was not reached.

The primary end points were time to recurrence and overall survival. Both were measured from time of randomization. Time to recurrence was measured as the time interval to first evidence of disease recurrence, and overall survival as the time interval to death from any cause. Toxicity and disease progression were graded according to the ECOG standard criteria.

The statistical analysis was performed according to the intention-to-treat principle and included all patients who received any treatment, regardless of the duration of therapy. The Kaplan-Meier method was used to estimate the time to disease recurrence and overall survival. (25). As recommended by Peto (26) and to avoid overinterpretation of the tails of the survival curves, which are unstable, survival curves are presented up to the time when at least five patients were still being followed. Comparisons between treatment groups were made by the log-rank test. The Cox proportional hazards model was used to adjust for prognostic factors. The factors evaluated included age, sex, thickness of primary tumor, site of primary tumor, presence of clinically positive nodes, number of histologically positive nodes, and time interval between surgery and protocol entry, which have previously been identified as being of prognostic significance in large studies (3, 4). Continuous variables were analyzed as such, as well as after categorization (above *versus* below median). Indicator variables were used for site of primary tumor (head and neck, trunk, or extremities). The hazard ratios were used to estimate relative risks. All *P*s are two-tailed.

Table 1 Baseline characteristics of the patients

Characteristic	Melanoma vaccine	Placebo vaccine
No. of patients		
Enrolled	24	14
Evaluated	24	14
Median age (range) (yr)	58.8 (24–76)	64.2 (34–76)
Sex (%)		
Male	83%	86%
Female	17%	14%
Performance status		
ECOG 0-1	100%	100%
Stage		
CS1/PS2 (high risk)[a]	17%	15%
CS2/PS2[b]	21%	28%
Recurrent[c]	62%	57%
Primary tumor thickness		
Median (range) (mm)	2.3 (0.3–9.2)	2.8 (1.1–7.0)
4 mm or greater	20%	25%
Primary tumor site		
Head/neck	13%	14%
Trunk	48%	43%
Extremities	35%	43%
Unknown primary	4%	0%
Regional nodes (% pts)		
Clinically positive	83%	85%
2 or more histologically positive nodes	67%	64%
Median (range) time interval between node dissection and randomization (weeks)	9.8 (3.6–20.6)	8.5 (5.7–21.3)

[a] Nodes were clinically negative but histologically positive, and at least two nodes were histologically positive.
[b] Nodes were clinically and histologically positive.
[c] Nodes were clinically positive after appropriate surgery for primary melanoma.

RESULTS

Clinical Characteristics of the Patients. Thirty-eight patients with resected AJCC stage III melanoma and a particularly high chance of disease progression, as evidenced by regional nodes that were clinically positive or by two or more nodes that were histologically positive, were randomly allocated to treatment with the melanoma or placebo vaccine. None of the patients referred for treatment were excluded because of absence of cellular immunity as determined by skin tests to recall antigens. Randomization was in a 2:1 ratio between melanoma and placebo vaccines to encourage enrollment into the trial. Twenty-four patients were assigned to the melanoma vaccine group, and 14 were assigned to the placebo vaccine group. Immunizations were given intradermally into the extremities every 3 weeks × 4, monthly × 3, every 3 months × 2, and, finally, every 6 months for a total of 5 years or until disease progression.

All patients were evaluable and included in all analyses. At the time of analysis, patients still alive had been observed for a median of 39 months (range, 14–58.3 months). The clinical characteristics of the patients are summarized in Table 1. The median age of the group treated with melanoma vaccine was 58.8 years, the median thickness of their primary lesions was 2.3 mm (range, 0.3–9.2 mm). A total of 83% of melanoma vaccine-treated patients had clinically positive nodes, 67% had 2 or more

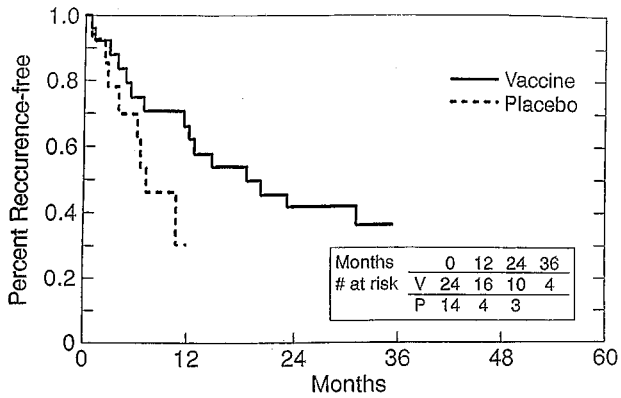

Fig. 1 Kaplan-Meier estimates of time to recurrence among patients treated with a polyvalent, shed-antigen, melanoma vaccine or a placebo vaccine. The estimated median time to recurrence in the melanoma vaccine-treated group was 1.6 years compared with 0.6 year for the placebo-treated group. Data are based on an intention-to-treat analysis.

Table 2 Comparison of recurrence-free and overall survival in melanoma patients treated with melanoma or placebo vaccine

Variable	Patients treated with melanoma vaccine (n = 24)	Patients treated with placebo vaccine (n = 14)	P^a
Median time to recurrence[a,b]	1.6 yrs	0.6 yr	0.03
% recurrence-free at[b]			
1 yr	67%	31%	
2 yrs	42%	23%	
3 yrs	29%	NA[c]	
Median overall survival[b]	3.8 yrs	2.7 yrs	
% alive at			
1 yr	83%	77%	
2 yrs	67%	61%	
3 yrs	53%	33%	

[a] Evaluated by Cox proportional hazards analysis.
[b] Estimated from Kaplan-Meier curves.
[c] NA, not available.

histologically positive nodes, and the median time interval between surgery and randomization into the trial was 8.5 weeks. The average age of the placebo vaccine-treated group was 64.2 years, the median thickness of the primary lesions was 2.8 mm (range, 1.1–7.0 mm), 85% of these patients had clinically positive nodes, 64% had 2 or more histologically positive nodes, and the time interval from surgery to randomization was 9.8 weeks. The time interval between surgery and randomization was longer than that in many large trials, probably because a minimum of 4 weeks was required to elapse between surgery and entry into the trial. This was done to avoid the temporary immunosuppression that can follow surgery. The interval is unlikely to have had an impact on the results of the trial because the interval was similar between the two groups and did not introduce a selection bias because the trial was double-blind.

Effect of Vaccine Treatment on the Recurrence of Melanoma. By Kaplan-Meier analysis (see Fig. 1), the estimated median time to recurrence for patients treated with the melanoma vaccine was 1.6 years (95% CI, 1.0–3.0 years) compared with 0.6 year [95% CI, 0.3–1.9 year(s)] for the group treated with the placebo vaccine (P = 0.26; Table 2). The estimated proportion of recurrence-free patients at 1 and 2 years after the onset of therapy was twice as high in the melanoma vaccine-treated group compared with the placebo-treated group (*i.e.*, 67% and 42% compared with 31% and 23%, respectively).

Because the progression of stage III melanoma is influenced by a number of risk factors, we then evaluated the significance of the difference in time to recurrence after adjusting for those factors using the Cox proportional hazards model. We first identified which of the known risk factors for progression of stage III melanoma (3, 4) were associated with recurrence in our data. The factors examined were age, sex, thickness and location of the primary lesion, presence of clinically positive nodes, number of histologically positive nodes, and time interval between surgery and randomization into the trial. The results are presented in Table 3. Tumor site and the presence of >2 histologically positive nodes were the only variables statistically associated with time to progression in this study. The association of melanoma vaccine treatment with increased time to progression became statistically significant (P = 0.04) after adjusting simultaneously for these two factors. Adjusting in addition for the other factors that can influence melanoma progression [*i.e.*, age (<60 years *versus* >60 years), sex, primary tumor thickness (<2.5 mm *versus* 2.5 mm), presence of clinically positive nodes (yes *versus* no), and time interval between surgery and protocol entry (<2 months *versus* >2 months)] gave a P of 0.03.

Overall Survival. The estimated median overall survival for patients treated with the melanoma vaccine was 3.8 years (95% CI, 1.6–5.4 years) compared with 2.7 years (95% CI, 1.6–4.9 years) for the placebo-treated group (see Fig. 2). Although the median overall survival of melanoma vaccine-treated patients was 40% longer than that of the control group, this difference was not statistically significant even after adjusting for risk factors. The estimated proportion of patients alive 3 years after randomization was 53% in the melanoma vaccine-treated group and 33% in the placebo-treated group. This was not statistically significant.

Adverse Events. There was a small urticarial reaction at the site of vaccine injection in both groups. The reaction appeared within 12 h, ranged in diameter from 20–40 mm, and faded completely in several days. A small asymptomatic granuloma, less than 4 mm in size, persisted at the injection site in some patients in both groups and was probably a reaction to the alum adjuvant. There was no pain or ulceration. There were no systemic adverse events in either group.

DISCUSSION

These results suggest, in a double-blind and placebo-controlled trial, that immunization with a melanoma vaccine may be able to slow the progression of melanoma. Although statistically significant, these results must be interpreted with caution because they are based on a small number of patients.

There have been a number of prior trials of melanoma vaccines as treatment for resected stage III or stage IV melanoma (20). These include trials of vaccines prepared from whole irradiated melanoma cells (11), melanoma cell lysates (18), viral

Table 3 Relative risks for recurrence and overall survival associated with patient characteristics

Variable	Recurrence		Death	
	Relative risk (95% CI)	*P*	Relative risk (95% CI)	*P*
Age (>60 yrs)	1.18 (0.55–2.57)	N.S.[a]	1.43 (0.58–3.50)	N.S.
Sex (female)	1.10 (0.41–2.93)	N.S.	1.32 (0.44–3.94)	N.S.
Thickness of primary tumor (>2.6 mm)	1.82 (0.77–4.26)	N.S.	1.31 (0.53–3.26)	N.S.
Primary tumor site[b]				
Extremities	0.24 (0.07–0.84)	0.03	0.41 (0.10–1.67)	N.S.
Trunk	0.34 (0.11–1.10)	0.07	0.69 (0.19–2.51)	N.S.
Clinically positive nodes	0.82 (0.31–2.19)	N.S.	0.45 (0.13–1.55)	N.S.
≥2 histologically positive nodes	2.99 (1.31–6.81)	0.007	1.68 (0.68–4.12)	N.S.
Time interval between surgery and randomization (>66 days)	1.51 (0.69–3.30)	N.S.	1.43 (0.58–3.54)	N.S.

[a] N.S., nonsignificant.
[b] Reference group = head and neck.

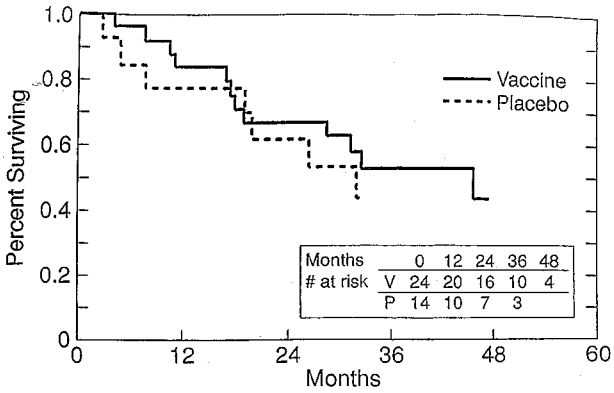

Fig. 2 Kaplan-Meier estimates of overall survival among patients treated with a polyvalent, shed-antigen, melanoma vaccine or a placebo vaccine.

oncolysates (27), hapten-conjugated autologous cells (12), single or multiple melanoma-associated peptides (10, 15–17), and anti-idiotype monoclonal antibodies (8). Whereas these trials have shown survival benefits for vaccine-treated patients, the control groups have been historical, so that the significance of the results is uncertain. A randomized but nonblinded trial of a GM2 ganglioside vaccine admixed with *Bacillus Calmette-Guérin* compared with *Bacillus Calmette-Guérin* alone has been conducted in patients with resected stage III melanoma pretreated with cyclophosphamide by Livingston *et al.* (9). There was a trend for improved recurrence-free and overall survival in the vaccine-treated group, which became statistically significant for recurrence-free survival once patients with elevated prestudy levels of GM2 antibodies were excluded from analysis. However, there was no improvement in survival compared with IFN-α2b-treated patients in a subsequent randomized trial. Two other concurrently randomized trials of melanoma vaccines constructed from viral oncolysates (28) or cell lysates (29) did not show a statistically significant advantage for the melanoma vaccine-treated groups.

In the present trial, patients with resected stage III melanoma and a particularly high chance of disease recurrence were randomly allocated to treatment with a polyvalent, shed antigen, melanoma vaccine or with a placebo vaccine, both admixed with alum as an adjuvant. The study was double-blind. The trial was small ($n = 38$) because it was closed prematurely after publication by the ECOG that IFN-α2b significantly improves survival in stage III melanoma. Patients treated with the melanoma vaccine had a prolongation in recurrence-free survival that was statistically significant ($P = 0.03$) following Cox multivariate analysis. The improvement was clinically relevant because recurrence-free survival was increased 2.5-fold, from a median of 0.6 year to 1.6 years. It is difficult to compare these results with those of other clinical trials in stage III melanoma because our study was restricted to patients with a subset of stage III melanoma with a particularly high chance of disease recurrence by virtue of having regional nodes that were clinically positive or having two or more nodes that were histologically positive. Overall survival was also prolonged by 40% in melanoma vaccine-treated patients. However, this difference was not statistically significant, perhaps because the number of patients evaluated for survival was small. Although based on a small number of patients, these results are provocative because the trial was both double-blind and placebo-controlled.

The trial was conducted with an alum adjuvant formulation of the vaccine. This adjuvant is safe but not very potent. Because the clinical effectiveness of vaccines is related to their ability to stimulate antitumor antigen immune responses (9–12), we believe our results could be improved by using one of the more potent vaccine adjuvants now available, such as dendritic cells, interleukin 2 liposomes, granulocyte macrophage colony-stimulating factor liposomes, QS21, or others.

In conclusion, a shed polyvalent melanoma vaccine appears to prolong the recurrence-free survival of patients with resected melanoma metastatic to regional nodes and may affect overall survival. Thus, shed antigens may provide a novel approach to construct clinically effective cancer vaccines. Because shedding is a general phenomenon, this approach may be useful to construct vaccines against other cancers.

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