Three-year Follow up of GMCSF/bi-shRNA^{furin} DNA-transfected Autologous Tumor Immunotherapy (Vigil) in Metastatic Advanced Ewing's Sarcoma

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Ewing's sarcoma is a devastating rare pediatric cancer of the bone. Intense chemotherapy temporarily controls disease in most patients at presentation but has limited effect in patients with progressive or recurrent disease. We previously described preliminary results of a novel immunotherapy, FANG (Vigil) vaccine, in which 12 advanced stage Ewing's patients were safely treated and went on to achieve a predicted immune response (IFNγ ELISPOT). We describe follow-up through year 3 of a prospective, nonrandomized study comparing an expanded group of Vigil-treated advanced disease Ewing's sarcoma patients (*n* = 16) with a contemporaneous group of Ewing's sarcoma patients (*n* = 14) not treated with Vigil. Long-term follow-up results show a survival benefit without evidence of significant toxicity (no ≥ grade 3) to Vigil when administered once monthly by intradermal injection ($1 \times 10e^6$ cells/injection to $1 \times 10e^7$ cells/injection). Specifically, we report a 1-year actual survival of 73% for Vigil-treated patients compared to 23% in those not treated with Vigil. In addition, there was a 17.2-month difference in overall survival (OS; Kaplan-Meier) between the Vigil (median OS 731 days) and no Vigil patient groups (median OS 207 days). In conclusion, these results supply the rational for further testing of Vigil in advanced stage Ewing's sarcoma.

INTRODUCTION

Ewing's sarcoma is a malignant cancer of the bones with rapid spread to the lungs. It is the second most frequently diagnosed primary malignant bone tumor in the United States with annual incidence of 1 in a million.[1,2] Approximately 20–30% of Ewing's sarcoma cases are diagnosed in the first decade and 10% after age 20. The median age of diagnosis is 14–15 years old.[3,4]

Although less than 25% of patients present with overt metastatic disease, based on relapse patterns subclinical metastases are presumably present in up to 80% of children at diagnosis.[4–8] The median time to relapse in unselected populations is 1.3 years.[4,8] Patients who relapse have a marked reduction in 1- and 5-year survival.

Few recurrent patients respond to second-line therapy and even fewer achieve a second remission,[4,8–13] particularly in those who relapse within 2 years of front-line treatment. In one large retrospective analysis of 714 patients from time of first relapse, the 1-year overall survival (OS) was 43%, 5-year OS was 13%, and 10-year OS was 9%.[4] At time of first relapse, the most significant prognostic factors are time to relapse (<2 versus ≥2 years) and site(s) of recurrence (localized, metastatic, or combined localized and metastatic). For relapses that occur within the first 2 years after initial diagnosis, which make up 72% of relapses,[4] the 2-year OS from relapse is 7%.[10] In two other analyses, 5-year progression-free survival was 5%[13] and 5-year OS 7%.[4]

Regarding the significance effect of metastatic disease as an independent factor related to response,[14] in one assessment, metastatic disease at presentation in 24 patients (13 with lung metastases, 12 with distant bone metastases, and 1 with bone marrow involvement) the 5-year OS from presentation with metastatic disease was 27 versus 85% for patients with localized disease (*n* = 77, *P* < 0.0001) and the 5-year progression-free survival was 28 versus 73% (*P* < 0.001).

Second-line chemotherapy for relapsed Ewing's sarcoma generally shows limited efficacy with only 9 to 13% of patients achieving a second disease-free remission.[11,13,15] Notably, the National Comprehensive Cancer Network guidelines do not provide standard of care recommendations for second-line treatment. Regimens such as topotecan/cyclophosphamide, irinotecan/temozolomide, or docetaxel/gemcitabine have been utilized in second-line or later treatments and may prolong life for those who respond.[16–29] However, none of these regimens have been determined to have a significant advantage in randomized clinical assessments.[30] The irinotecan/temozolomide regimen appears to be the most common second-line regimen utilized today.

Third-line treatment for Ewing's sarcoma is associated with cumulative toxicity (hematologic and neurologic) in addition to an even lower response rate. There is no standard of care treatment for Ewing's sarcoma in the third-line or greater setting.

Previously,[31] we reported preliminary results of 12 patients with advanced disease (multiply recurrent or treatment failure within 2 years) Ewing's sarcoma treated with Vigil (formerly known as FANG) immunotherapy. Safety and immune responses were characterized and a preliminary 1-year survival of 75% was observed. We now report long-term follow-up of the previously reported 12 and an additional four patients with advanced Ewing's sarcoma. We compared the results in these 16 patients to 14 contemporaneous patients with advanced Ewing's sarcoma who fulfilled the same inclusion criteria and underwent similar surgical procedure and vaccine construction but did not receive Vigil.

Our observed preliminary results and now long-term follow-up of our expanded group of patients are a provocative observation in support of further randomized trial testing.

RESULTS

Patient characterization

All 30 Ewing's sarcoma patients who signed MCCRC Institutional Review Board approved consent and qualified for phase 1 testing

Table 1 Ewing's sarcoma phase 1 demographics

	Vigil	No Vigil[a]
Tumor location harvest (lung/soft tissue/other)	13/0/3	11/2/1
Sex (M/F)	12/4	7/7
Age median (range)	19 (59–12)	17 (30–12)
Performance (Eastern Cooperative Oncology Group 0, 1)	16	14
Ethnicity (Caucasian/other)	13/3	12/2
Prior systemic Tx (frontline/2nd/≥3rd)	1/5/10	3/4/7
Surgical candidate (yes/no)	16/0	14/0
Tissue harvested (yes/no)	16/0	14/0
Median time consent to surgery	1 day	1 day
Metastatic disease (yes/no)	16/0	14/0

[a]Three insufficient viable tumor cells, six contaminants, five sought other management.

with Vigil were evaluated in this analysis. All had late stage (*i.e.*, ≥ third-line chemotherapy; $n = 17$) or relapse < 2 years of front-line treatment ($n = 13$). One hundred sixty-three vaccine vials were successfully manufactured. Sixteen patients had successful vaccine construction and received Vigil. Median follow up at the time of reporting was 2 years 11 days. Median time from procurement to first treatment was 53 days. Five treated patients had between 104 and 294 days delay from procurement to treatment. An additional chemotherapy regimen was administered after procurement but prior to Vigil treatment in three of these five patients. Limited field palliative radiation therapy was administered to the other two patients with Vigil delivery. Three of the five remain alive and two passed away 37 days and 430 days after Vigil treatment. Fourteen patients in the comparative group did not receive Vigil after undergoing similar surgery and vaccine construction process. Nine of the latter were unable to have vaccine released (six contaminant, three insufficient viable tumor cells) and five chose other treatment management. One patient in the No Vigil group had progression and mortality before reaching the median time for vaccination in the treatment group. This patient fulfilled the same inclusion criteria as all other patients at time of procurement for surgical justification and study engagement. All vaccines constructed fulfilled release criteria of GMCSF production (one exception with insufficient material for GMCSF testing) and TGFβ1, β2 knockdown. Demographics of patients entered in the long-term follow up analysis are shown in **Table 1**. Though not statistically significant, there was a higher ratio of male patients in the Vigil arm.

Safety

No significant toxicity was observed in the 16 Vigil-treated patients. Specifically no product related Grade 3, four toxic effects were demonstrated during the treatment course or in long-term follow-up. Ninety-three injections of Vigil have been administered to the 16 patients. Adverse events reported are shown in **Table 2**.

There were 11 serious adverse events reported involving 7 participants (See **Table 3**). None of the serious adverse events

Table 2 Ewing's sarcoma patients who received Vigil, definitely or probably related adverse events and long-term follow up

Preferred term	Common toxicity criteria grade	Relationship to study drug	Number of subjects	Number of events
Bruising	1	Definitely related	1	1
Erythema @ injection site	1	Definitely related	1	1
Fatigue	1	Probably related	1	4
Induration/fibrosis injection site reaction—induration	1	Definitely related	2	2
Injection site reaction—induration	1	Definitely related	1	2
Injection site reaction—Erythema	1	Definitely related	11	31
Injection site reaction—induration	1	Definitely related	12	55
Injection site reaction—pain	1	Definitely related	3	3
Injection site reaction—pruritus	1	Definitely related	1	3
Injection site reaction—swelling	1	Definitely related	2	3
Injection site reaction—tenderness	1	Definitely related	1	1
Joint-function	1	Probably related	1	2
Pain—back	1	Probably related	1	1

Table 3 Phase 1 Ewing's sarcoma: serious adverse events reported and long-term follow up

ID#	Last dose date prior to AE	Onset date	Resolved date	Reported term	Grade	Date of death	Drug-related assessment
058	10/01/12	10/16/12	10/19/12	Left hip pain	3	10/29/12	Unrelated
058	10/01/12	10/25/12	10/29/12	Disease progression of Ewing's sarcoma	5	10/29/12	Unrelated
063	03/12/13	03/19/13	03/26/13	Pain (bone)	3	04/17/13	Unrelated
063	03/12/13	03/19/13	03/26/13	Constipation	3	04/17/13	Unrelated
092	02/03/15	02/16/15	03/06/15	Possible infection	2	N/A	Unrelated
102	01/12/15	02/09/15	02/10/15	Malignant neoplasm, right upper lobe of lung	2	N/A	Unrelated
104	08/08/14	08/27/14	08/29/14	Intractable cancer pain	3	N/A	Unrelated
107	09/12/14	09/16/14	09/25/14	Intractable pain right posterolateral chest	3	09/25/14	Unrelated
107	09/12/14	09/16/14	09/25/14	Disease progression of Ewing's sarcoma	5	09/25/14	Unrelated
121	05/15/15	06/02/15	06/03/15	Pneumonia	2	06/28/15	Unrelated
121	06/12/15	06/28/15	06/28/15	Disease progression of Ewing's sarcoma	5	06/28/15	Unrelated

AE, adverse events; N/A, not applicable.

were related to Vigil. Seventeen deaths (7 Vigil, 10 No-Vigil) have occurred, none of which were determined to be related to Vigil.

Response

Figure 1 shows the results of the Kaplan-Meier analysis of the survival data. Overall survival from time of procurement revealed a 17.2-month improvement in survival in the Vigil-treated patients compared to the no Vigil group. The median survival for the Vigil-treated group from time of treatment was 22.7 months (689 days).

The actual 1-year survival of patients who received Vigil (11/15, 73%) was higher than those who did not receive Vigil (3/13, 23%) (one in each of the Vigil and no Vigil have not yet achieved the 1-year survival time point).

One patient was previously reported as achieving partial response as per RECIST 1.1 criteria (at 2.7 months after immunotherapy initiation the sum of target lung tumor diameters had decreased from 9.3 to 5.3 cm; a 46% decrease) (#089). He progressed 20.6 months after procurement (17.9 months after treatment initiation). Six of the other 15 patients maintained stable disease ≥ 3 months. Another patient (#062), who demonstrated recurrent disease 20.4 months (622 days) after prolonged stable disease, underwent a second harvest to procure tumor for treatment for recurrent disease as previously described.[31] She received no further systemic therapy since second treatment with Vigil harvested from recurrent disease and now no longer demonstrates evidence of Ewing's sarcoma by imaging 42.4 months (1,291 days) after initial procurement.

DISCUSSION

The majority of relapsed Ewing's sarcoma patients who do not achieve a second remission, as well as those who go into a second remission but subsequently relapse, do not derive significant benefit from additional chemotherapy. Previously published results of 161 patients treated with second-line chemotherapy, comparing non-responding patients (SD/PD) to responding patients (PR/CR) revealed a 5-year survival of 4 versus 25% (2-year survival 10 versus 45%).[15] Likewise, response versus no response to second-line chemotherapy was a significant ($P = 0.0001$) prognostic factor

(5-year survival 48 versus 0% respectively) in another series of 55 patients.[11] In a third analysis of 195 patients, 86% of the patients did not achieve second remission; 97% of these patients died with a median survival of 11.7 months and no patient achieved disease control. Of the 26 patients who did go into a second remission, 12 relapsed again and did not reach a third remission (10/12 died and in the first year and 2 were living with uncontrolled disease at 6 and 13 months).[13] Several targeted and/or targeted plus chemotherapy combination studies are under early clinical or preclinical development (i.e., inhibition of insulin-like growth factor receptor I, midostaurin, YK-4–279, bevacizumab/topotecan/cyclophosphamide, olaparib, enzastaurin, zoledronic acid/ifosfamide) but none have thus far shown significant benefit.[30] As such, there is a need for treatment development in patients with metastatic Ewing's sarcoma that is refractory to systemic therapy or has progressed after second-line treatment.

That the "No Vigil™" subset of patients followed was not a prospectively randomized group precludes ascribing significance to interpretation of results. However, insofar as all of these patients traveled to the treatment site, met all inclusion criteria, exhibited none of the exclusion criteria and underwent similar surgical harvest of tumor tissue for Vigil construction, we submit that comparison is justifiable albeit with interpretation of results as hypothesis-engendering at best. As shown in results, the demographics of the no Vigil versus Vigil groups appear well-balanced viv-a-vis prognostic factors that might be expected to affect survival based on historical data.

Regarding evaluation of the comparative findings; the results in the published literature that consistently reflect the severely limited survival of this population of Ewing's sarcoma patients do not necessarily apply to the selected subset of patients in the current report who, in addition to performance status, are also surgical candidates (particularly so for thoracic surgery that was performed in most of the Vigil/no Vigil patients). Thus, despite a perceived survival advantage of Vigil-treated patients when compared to historical study patients who received alternative systemic therapies, there could be an unintended bias toward a healthier patient population that could have produced skewed

results. However, the comparison to the concurrently accrued "No Vigil™" population would strongly suggest that the reported survival prolongation is indeed meaningful. In addition, these results are also consistent with the data previously presented from the phase 1 study in which a prolongation in median survival was also shown in adult cancer patients treated with Vigil.[32,33]

Given these results of Vigil in advanced Ewing's sarcoma, our study group consensus under FDA IND support is that a randomized assessment of Vigil in this population is justified. To that end, a randomized, controlled, open-label phase 2 study of Vigil versus systemic chemotherapy in ≥ third-line treatment

of patients with metastatic Ewing's sarcoma is now underway (NCT02511132).

MATERIALS AND METHODS

The construction and current good manufacturing practice adherence of Vigil immunotherapy are summarized in **Figure 2**. Briefly, Vigil vector utilizes the pUMVC3 vector backbone in which the granulocyte-macrophage colony-stimulating factor (GMCSF)-encoding complementary DNA and the DNA encoding the furin bifunctional shRNA are under transcriptional control of the cytomegalovirus immediate-early promoter. The final construct was confirmed by bi-directional sequencing. Following protocol-specific informed consent, the
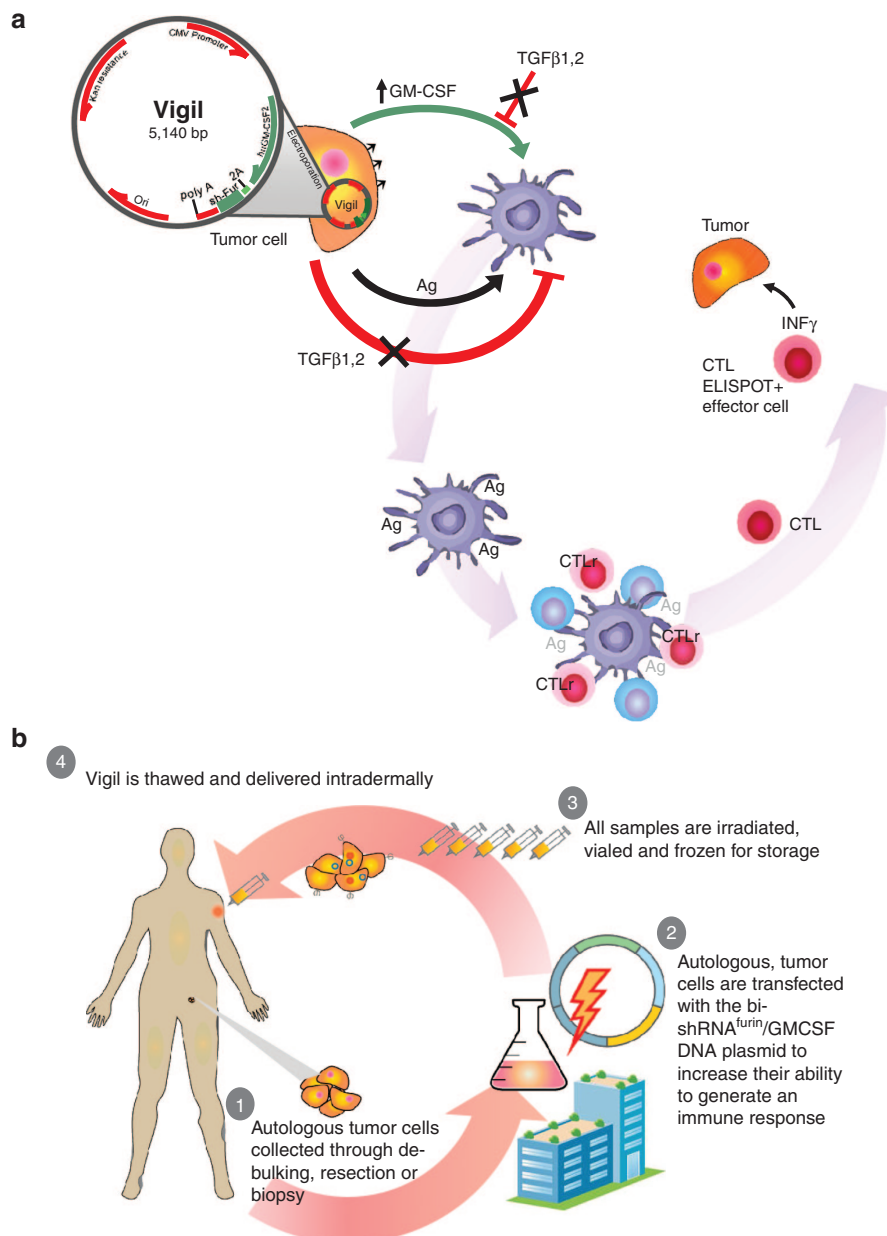
Figure 1 (**a**) Vigil is a 5,140 bp plasmid of a bifunctional shRNA-furin DNA sequence which prevents cleavage of TGFβ precursor into functional TGFβ$_1$ & TGFβ$_2$ and a GMCSF DNA sequence which stimulates antigen presentation and adaptive immune response when expressed after placement by electroporation into individual autologous tumor tissue which provides the full tumor antigen (Ag) profile and has demonstrated in phase 1, 2 testing induction of circulating cytotoxic T lymphocytes capable of specific lytic activity (ELISPOT and response) to autologous tumor. (**b**) Vigil constructing is portrayed.
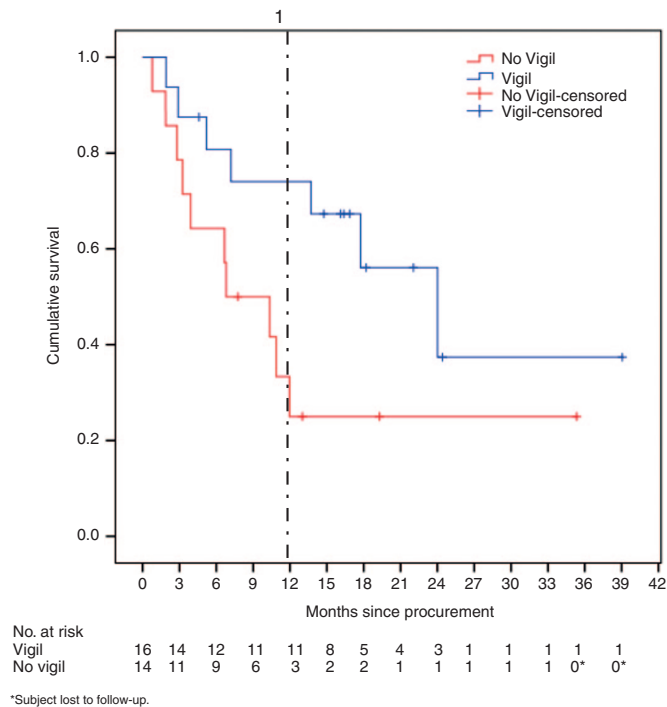
Group	N	No. of deaths	Mean Survival (months)	Median Survival (months)	P-value (Log rank)
No Vigil™	14	10	13.4 (409days)	6.8 (207 days)	.056
Vigil™	16*	7	23.1 (704days)	24 (731 days)	

Figure 2 Survival from surgical procurement of advanced Ewing's patients successfully harvested for Vigil construction (n = 30). Comparison is made of those who received Vigil (n = 16) versus those who did not receive Vigil ((n = 14) as a result of construction failure or choice of other management). All patients are censored alive as of dates provided on 19 October 2015.

tumor was excised, placed in sterile transport media, and brought to the Gradalis manufacturing facility (Carrollton, TX) for vaccine manufacture as previously described.[32]

Study design. Briefly, each patient entered into study received monthly doses of Vigil the concentrations of which were based on cell yield from procured tumor ($1 \times 10e6$ cells/injection, $4.0 \times 10e6$ cells/injection, $8.3 \times 10e6$ cells/injection, $1 \times 10e7$ cells/injection or $2.5 \times 10e7$ cells/injection in 1 ml volume). A minimum of 4 doses to a maximum of 12 doses were administered via intradermal injection, alternating between the right and left upper arms. Patients were discontinued from study for progressive disease or intolerable toxicity. The trial was performed after approval by a local Ethics Committee and in accordance with an assurance filed with and approved by the Department of Health and Human Services.

Patient population. The study population involved patients with Ewing's sarcoma (n = 30) that was multiply recurrent or progressed within 2 years of treatment who, following study consent, underwent surgical tumor extraction and subsequent tumor processing for vaccine construction. These Ewing's sarcoma patients were a subset of the phase 1 solid tumor study previously published.[32] Specific inclusion criteria included a histologically confirmed advanced or metastatic non-curable Ewing's sarcoma following completion of ≥1 disease appropriate standard of care therapy; recovery from all treatment-related toxicities to ≤ Grade 1 (except alopecia); availability of tumor in sufficient quantity (a minimum of 2–8 g of solid tumor tissue or at least 500 ml of pleural/ascites fluid) for vaccine processing; history of brain metastases allowed if treatment completed ≥2

months prior to enrollment with magnetic resonance imaging confirmation of no active disease; presence of ≥1 measurable or evaluable lesion; patient age ≥12 years; Eastern Cooperative Oncology Group performance status of 0–1; a signed, Institutional Review Board-approved, protocol-specific written informed consent document; a negative pregnancy test for women of child-bearing potential; and normal organ and marrow function defined as follows: absolute granulocyte count ($>1,500/mm^3$), platelets ($>100,000/mm^3$), total bilirubin ($<2 mg/dl$), AST(SGOT)/ALT(SGPT) ($<2\times$ institutional upper limit of normal), and creatinine ($<1.5 mg/dl$).

Exclusion criteria included: surgery involving general anesthesia, chemotherapy, radiotherapy, or immunotherapy within 4 weeks of study entry; use of other investigational agents within 30 days prior to study entry; known immune compromised state or autoimmune disease; prior malignancy (excluding non-melanoma skin cancer) unless in remission for ≥2 years; uncontrolled intercurrent illness or psychiatric illness/social situations that would limit compliance with study requirements; confirmation that patient was pregnant or nursing; HIV; or chronic hepatitis B or C infection (except in patients with hepatocellular carcinoma).

All patients receiving vaccine (n = 16) were treated in the outpatient facilities of Mary Crowley Cancer Research Centers (MCCRC), Dallas, TX. Twelve of the 16 patients treated with Vigil in this analysis were previously described[31] for early safety and response. Fourteen patients did not receive Vigil following procurement and vaccine manufacture (n = 14) and were followed as a comparative, nonrandomized but contemporarily alternatively treated group to the Vigil-treated patients. Thirteen of these patients were previously described[31] as "consecutively harvested but not treated". This analysis now describes, for the first time, comparison of survival through up to 3 years of both study groups (Vigil treated and non-Vigil treated).

Statistical analysis. Statistical analyses were performed using IBM SPSS Version 22 (IBM Corporation). Overall survival from time of surgical procurement or from time of treatment was analyzed using Kaplan-Meier curves. Actual 1-year survival difference between treatment groups was determined using the Fisher's Exact test. All patients were censored alive using the last known date alive.

ACKNOWLEDGMENTS

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