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Intratumoral talimogene laherparepvec injection with concurrent preoperative radiation in patients with locally advanced soft-tissue sarcoma of the trunk and extremities: phase IB/II trial

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The safety data of patients treated in the Phase Ib part of the study was presented as an abstract at the Connective Tissue Oncology Society Meeting at Lisbon, Portugal in 2016.

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ABSTRACT

Background Soft-tissue sarcomas (STS) in the extremities and trunk treated with standard-of-care preoperative external beam radiation therapy (EBRT) followed by surgical resection are associated with local and distant relapses. In preclinical studies, oncolytic virotherapy in sarcoma has demonstrated antitumor effects via direct intratumoral oncolysis and cytotoxic T-cell–mediated immune responses. Talimogene laherparepvec (TVEC) is a replication-competent, immune-enhanced, oncolytic herpes simplex virus type 1 engineered for intratumoral injection; it has been approved by the FDA for the treatment of locally advanced and metastatic melanoma.

Methods We explored a novel combination of TVEC with standard-of-care EBRT administered preoperatively in patients with locally advanced STS of the extremities and trunk in a phase IB/II clinical trial. Thirty patients with primary STS >5 cm for which EBRT was indicated to achieve negative margins were enrolled. FDA-approved TVEC doses were used. Immune correlative studies in peripheral blood, biopsy and resected tumor tissues were performed.

Results No dose-limiting toxicity was observed. Adverse events were similar to those reported in prior studies with TVEC. One patient with myxoid liposarcoma exhibited a partial response. Seven of the 29 (24%) evaluable patients achieved 95% pathological necrosis. None of the patients developed a herpes infection due to the treatment. Eight of the 29 (27%) patients developed postoperative wound complications, which is consistent with previous studies. None of the patients developed local recurrence after surgical resection of the primary sarcoma. 2-year progression-free and overall survival were 57% and 88%, respectively. Caspase-3 demonstrated increased expression of both in TVEC-treated tissue samples as compared with control samples treated with radiation alone.

Conclusion Preoperative intratumoral TVEC with concurrent EBRT for locally advanced STS is safe and well-tolerated. This combination treatment may enhance immune responses in some cases but did not increase the proposed rate of pathological necrosis. The Caspase-3 biomarker may be associated with a positive effect of TVEC in sarcoma tumor tissue and should be explored in future studies.

Trial registration number NCT02453191.

INTRODUCTION

Soft-tissue sarcomas (STS) account for 1% of all solid tumors, with an annual incidence of 5–6 cases per 100,000 persons.[1] Surgical resection of the primary STS with negative margins remains the primary treatment. The addition of neoadjuvant external beam radiation therapy (EBRT) improves the rate of negative surgical margins and results in higher local control rates; hence, it is currently accepted as a standard of care.[2 3] Neoadjuvant and adjuvant cytotoxic chemotherapy, though an emerging treatment option in specific high risk STS subtypes, is associated with significant toxicities.[4] Despite these aggressive standard-of-care treatments, approximately 50% of patients with localized STS of the extremities or trunk develop metastatic disease with associated mortality.[5] Hence, novel treatments are needed that can effectively synergize with and augment the current standard of care treatment to improve survival.

Several preclinical studies evaluated the role of oncolytic viruses (OVs) in sarcomas.[6–9] OVs mediate their antitumor effects through multiple mechanisms. They can directly infect tumor cells following intratumoral injection. Once in the cell, the virus replicates, leading to cell lysis and virus progeny release into the

tumor microenvironment where they can infect other neighboring tumor cells, leading to further lysis. Second, the virus can mediate an indirect effect by enhancing the activation of innate and adaptive immune responses specific to cancer cell antigens, resulting in augmented systemic antitumor immunity. Ionizing radiation induces direct cellular DNA damage and is routinely used in management of STS. Multiple preclinical and clinical studies have shown a synergistic therapeutic effect when OVs are combined with radiation to treat cancer.[10 11] Radiation treated tumors can increase viral uptake and lead to gene expression and replication and resulting in cell death by way of apoptosis and or necrosis; and in turn the viruses may act as radio sensitizing agents. Therefore, the combination of OV and radiation may help with local tumor control and help with enhancing systemic antitumor response in the form of abscopal effect.[12]

Talimogene laherparepvec (TVEC) is replication-competent, immune-enhanced, oncolytic herpes simplex virus type 1 (HSV-1) engineered for intratumoral injection. It contains the coding sequence for human granulocyte-macrophage colony-stimulating factor, which in addition to the above two mechanisms enhances antitumor responses by inducing the production of proinflammatory cytokines. TVEC has been shown to improve melanoma response rates and has been approved by the FDA for the management of locally advanced unresectable and metastatic melanoma.[13 14] It is being studied in several other solid cancers including head and neck cancer.[15 16] Radiation therapy in combination with immunotherapies has shown synergistic activity;[10 11] therefore, we explored a novel combination of neoadjuvant TVEC and standard-of-care EBRT in locally advanced STS of the extremities and trunk.

PATIENTS AND METHODS

This was a phase IB/II, open-label, non-randomized, single-center trial conducted in patients with a histological diagnosis of STS of the extremities and trunk who received intratumoral injections of TVEC in the primary sarcoma tumor together with standard-of-care neoadjuvant EBRT followed by definitive surgery. The phase IB primary objective was to examine the safety and tolerability of TVEC combined with EBRT based on the incidence of dose-limiting toxicities (DLTs). The phase II primary objective was to determine the efficacy of this combination based on near pathological complete response (pCR) defined as ≥95% tumor necrosis of locally advanced STS that were initially unresectable with clear, wide margins and for which neoadjuvant or preoperative radiotherapy was considered appropriate.

Patients aged ≥18 years with localized, histologically confirmed, STS >5 cm, amenable to direct or ultrasound-guided injection, not suitable for surgical resection alone due to an inability to achieve acceptably wide margins, and for which preoperative EBRT was indicated were included. Patients with metastatic STS as defined by lung nodules>1 cm on staging CT scan were included only if radiation and resection of the primary tumor were indicated. Diagnostic pathology tests were performed using core-needle or incisional biopsy. Surgery was performed with limb-sparing intent and diligence was taken to ensure negative margins. A multidisciplinary team comprising medical oncologists, surgical/orthopedic oncologists, plastic surgeons, and radiation oncologists evaluated the patients before they consented to participation. The eligibility criteria also included an Eastern Cooperative Oncology Group performance status of 0 or 1 and adequate laboratory test values. Patients with retroperitoneal and visceral sarcomas; on anticoagulation therapy; with autoimmune diseases; and prior exposure to TVEC, tumor vaccines, or radiation to the same tumor bed were excluded. Those with histologies including extraosseous Ewing's sarcoma, primitive neuroectodermal tumors, osteosarcoma or chondrosarcoma, Kaposi's sarcoma, or angiosarcoma of the scalp/face were also ineligible. The study was conducted according to the Declaration of Helsinki and Good Clinical Practice guidelines. All patients signed an informed consent form.

The intratumoral TVEC injection sites were marked with ink by the orthopedic surgeon with the intention of including those sites in the resection specimen. TVEC was administered intratumorally directly or under ultrasound guidance at an initial dose of 10^6 PFU/mL up to a volume of 4 mL on day 1 of week 1, followed by the administration of a target dose of 10^8 PFU/mL up to a volume of 4 mL 3 weeks later (on day 1 of week 4). Weekly TVEC injections were continued until surgery. Tumors were injected using a single-entry point at the inked site, but TVEC was delivered along multiple tracts within the lesion to achieve maximum dispersion. Acetaminophen 650 mg and indomethacin 50 mg were administered orally 1 hour before intratumoral injection. Dose delays or reductions were followed per protocol. Safety evaluations were done weekly, with adverse events (AEs) defined and graded according to the National Cancer Institute's Common Terminology Criteria for Adverse Events, V.4.0[17] (CTCAE 4.0).

Neoadjuvant EBRT doses of 50 Gy delivered over 25 fractions were administered by either 3D conformal or intensity-modulated radiation therapy as per sarcoma NCCN guidelines.[18] Weekly TVEC injections were continued during radiation and until surgery for all patients. Surgery was performed 4–6 weeks from EBRT completion to allow for adequate tissue healing and resolution of EBRT-induced acute toxicities (figure 1).

Sarcoma histological subtypes (as defined by the WHO) were determined following examination by a single pathologist with expertise in sarcomas. Tumors were graded based on features such as mitosis rate, necrosis, cellularity, pleomorphism, and differentiation, and tumor size (or the greatest dimension) and the margin description (including centimeters or millimeters to margin) were reported. Tumors were sampled with at least one section per 1 cm of the greatest tumor dimension. Absence of

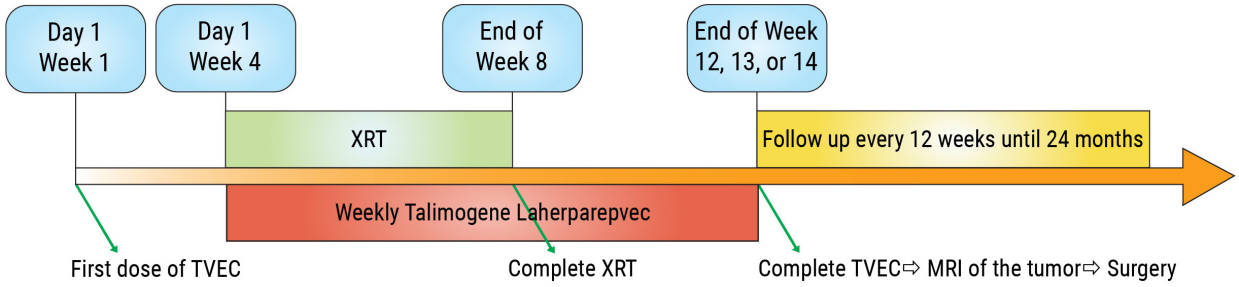

Figure 1 Study schema.TVEC, Talimogene laherparepvec; XRT, radiation therapy; MRI, magnetic resonance imaging.

ink on the tumor was accepted as a negative margin. Near-pCR was assessed according to the percentage of viable tumor remaining and percentage necrosis in the post-treatment resected specimen.[19] Necrosis was calculated for each block of full-face section and then averaged. Clinical response was assessed using tumor measurements performed at screening and preoperative MRI or CT. Response Evaluation Criteria in Solid Tumors (RECIST) V.1.1 was used for target tumor categorization as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD).

Serum samples were collected at day 1 of week 1 (w1d1) as baseline prior to the first injection, day 1 of week 4 (w4d1)—prior to second injection, day 1 of week 8 (w8d1)—after completion of EBRT, day 1 of week 12 (w12d1)—preoperatively, and day 1 of week 18 (w18d1)—after surgery. Luminex Human Magnetic Assay (LXSAHM-36, R&D Systems, online supplemental table S1 summarized all 36 cytokines) was used to determine cytokine concentrations as per the company's instructions for all serum samples and analyzed using the Luminex MAGPIX Instrument (Luminex).

Immunoperoxidase stains were performed on 4-µm-thick sections from the archived paraffin-embedded tissues. Caspase-3 (Cell Signaling #9661 rabbit polyclonal 1:100; retrieval: Citrate Buffer, pH 6.0, in Decloaker (Biocare) 110*C 15 min; Secondary: Dako Rabbit Envision) and Granzyme-B (Leica clone 11F1, dilution 1:40, using High pH HIER, in DAKO Autostainer Link48, using DAKO's detection system) stains and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay were used to highlight apoptotic tumor cells. Cytoplasmic and nuclear expression were considered positive and the apoptotic index was calculated using the following formula: number of immunoreactive cells present in a section as a fraction of the total number of tumor cells.

Statistical considerations

The phase IB primary objective was to confirm that the current standard dose of TVEC combined with EBRT was well-tolerated. A standard 3+3 design was used with the current standard as the starting dose (4.0 mL of 10^8 PFU/mL weekly beginning at week 3) and one additional de-escalated dose (4.0 mL of 10^8 PFU/mL every 2 weeks) according to the investigator brochure that was included should de-escalation be warranted. The initial dose administered to all patients was up to 4.0 mL of 10^6 PFU/mL. The recommended phase II dose was defined as the highest dose at which at least one out of six patients showed a DLT, which was defined as grade 3 immune-mediated AEs, allergic reactions, or plasmacytoma of any grade. Any unexpected grade 3 or greater hematological or non-hematological toxicity (except for alopecia of any grade, EBRT-related skin toxicity, grade 3 or higher myalgia or arthralgia, fatigue, fever, or diarrhea and vomiting responding to supportive care) was also considered a DLT. The incidence of treatment-emergent AEs attributable (possibly, probable, or definite) to TVEC was descriptively summarized by type and severity based on the maximum grade noted for each patient. Phase IB results were reported for the DLT-evaluable safety population and a full safety profile included all patients (n=30) who received at least one TVEC dose.

The phase II primary objective was to evaluate the preliminary evidence of antitumor activity. The primary endpoint was the proportion of patients with near-pCR. Previous literature suggests that treatment with neoadjuvant EBRT alone results in pathological tumor necrosis ≥95% in approximately 8%–10%[20 21] of patients with extremity sarcomas. Thus, a near-pCR rate of ≤12% was defined as essentially being no different than that associated with EBRT alone whereas a rate of at least 35% may warrant further investigation. Sample size requirements were based on an optimal Simon two-stage design with 80% power and a significance level of 5%. Nine patients were to be enrolled in the first stage in phase 2, and the study would have been terminated if one or fewer had pathological tumor necrosis ≥95%. Otherwise, an additional 14 evaluable patients were to be enrolled in the second stage. If 6 or more of the total 23 patients had pathological tumor necrosis ≥95%, the treatment would be deemed worthy of further investigation.

Survival probabilities were estimated and plotted using the Kaplan-Meier method. Estimates along with 95% pointwise CIs were reported. Progression-free survival (PFS) was defined as the time from treatment initiation to the date of the first documented disease progression or death due to any cause. Otherwise, subjects were censored for progression at their last radiographic assessment. Overall survival (OS) was defined as the time from treatment initiation to death due to any cause. Patients

alive at the time of submission were censored on the date at which they were last known to be alive. Mixed effects regression models were used to assess the change in Caspase-3 Granzyme-B, CD3, CD4, CD56, CD8, and Foxp3 from pretreatment to post-treatment and to compare the change in TUNEL positive cells between treatment groups. Wilcoxon rank sum tests were used to compare Caspase-3 and Granzyme-B between treatment groups.

RESULTS

Six patients were enrolled in phase IB; 24 patients (23 in whom pathological necrosis was evaluable and 1 who opted against surgery) were enrolled in phase II from July 2015 to June 2018 at the Holden Comprehensive Cancer Center. At the time of data analysis (January 2021), 13 patients have had disease progression of which 12 have died. Two out of these 13 patients had known metastasis to the lungs at study enrollment. The median patient age was 65 years (range 28–80) (table 1).

All patients had a measurable tumor >5 cm in size (range 5–22 cm) of the primary site to which neoadjuvant EBRT was delivered following a multidisciplinary group discussion. One patient with undifferentiated pleomorphic sarcoma refused surgery and was excluded from the

Table 1 Patient demographics, histological subtypes, and percentage necrosis in different phases of the trial

		Phase		
Covariate	**Level**	**I N=6**	**II N=24**	**Total N=30**
Sex	F	1 (16.7)	8 (33.3)	9 (30.0)
	M	5 (83.3)	16 (66.7)	21 (70.0)
Age (median)		60 (36–79)	66 (28–80)	65 (28–80)
Pathology	Dedifferentiated liposarcoma	0 (0)	2 (8.3)	2 (6.7)
	Epithelioid sarcoma	0 (0)	1 (4.2)	1 (3.3)
	Myxofibrosarcoma	1 (16.7)	1 (4.2)	2 (6.7)
	Myxoid liposarcoma	0 (0)	5 (20.8)	5 (16.7)
	Pleomorphic liposarcoma	0 (0)	1 (4.2)	1 (3.3)
	Spindle cell rhabdomyosarcoma	1 (16.7)	0 (0)	1 (3.3)
	Synovial sarcoma	0 (0)	2 (8.3)	2 (6.7)
	Undifferentiated pleomorphic sarcoma	4 (66.7)	9 (37.5)	13 (43.3)
	Undifferentiated sarcoma with myxoid stroma	0 (0)	1 (4.2)	1 (3.3)
	Undifferentiated spindle cell sarcoma	0 (0)	2 (8.3)	2 (6.7)
Grade*	1	0 (0)	5 (20.8)	5 (16.7)
	2	4 (66.7)	10 (41.7)	14 (46.7)
	3	2 (33.3)	9 (37.5)	11 (36.7)
HSV serology—pretreatment	Negative	1 (16)	8 (33)	9 (30)
	Positive	5 (83)	16(66)	21(70)
Overall best response	PR	0 (0)	1 (4.2)	1 (3.3)
	SD	4 (66.7)	16 (66.7)	20 (66.7)
	PD	2 (33.3)	7 (29.2)	9 (30.0)
≥95% necrosis	No	4 (66.7)	18 (78.3)	22 (75.9)
	Yes	2 (33.3)	5 (21.7)	7 (24.1)
Progression	No	4 (66.7)	13 (54.2)	17 (56.7)
	Yes	2 (33.3)	11 (50.0)	13 (43.3)
Deceased	No	4 (66.7)	14 (58.3)	18 (60.0)
	Yes	2 (33.3)	10 (41.7)	12 (40.0)
# TVEC injections† (median)		11 (9–11)	10 (5–11)	10 (5–11)
Necrosis (median)†		75% (11%–99%)	78% (8%–100%)	78% (8%–100%)
Median follow-up (months)†		48.9 (2.9–57.7)	22.2 (1.5–50.7)	23.7 (1.5–57.7)

*Grading of sarcomas according to the French Federation of Cancer Centers Sarcoma Group.
†Numbers within parentheses indicate the range.
HSV, herpes simplex virus; PD, progressive disease; PR, partial response; SD, stable disease; TVEC, talimogene laherparepvec.

efficacy analysis but included in the safety analysis. Per the preoperative radiographic evaluation, the majority of subjects (66.7%) had SD according to RECIST V.1.1[22] and none showed CR. One patient with myxoid liposarcoma had PR. Two patient deaths occurred during the study due to PD. The treatment-related toxicities noted were similar to previous studies using TVEC.[23 24] Headaches (3%), chills and rigors (19%), and fatigue (22%) were manageable with single repeat doses of acetaminophen, indomethacin, and meperidine 4–6 hours before each intratumoral injection and with supportive care. Ten per cent of patients developed significant lymphopenia which eventually returned to baseline. None of the patients developed a herpes viral infection. All toxicities attributed to intratumoral TVEC injections are outlined in table 2.

No DLTs were noted in the six subjects enrolled in Phase1b. Two of the first six patient sarcomas showed ≥95% tumor necrosis. Hence, the preliminary safety of the standard TVEC dose of 4 mL of 10^6 PFU as the initial dose followed by 4 mL of 10^8 PFU weekly was established and the study proceeded to phase II. Twenty-four subjects were enrolled in phase II. One subject refused surgery. Three patients who showed no evidence of metastatic disease at enrollment developed metastatic disease at the preoperative disease assessment time point. They underwent primary tumor resection as planned and then received systemic therapies. One patient developed regional lymph node progression before surgery. One patient developed local progression during treatment and underwent amputation. All other patients underwent limb sparing surgery of their primary tumor. None of the patients who underwent limb sparing surgery developed local recurrence. A total 8 out of 29 patients (27%) who underwent surgery developed wound healing complications. Six of them had acute complications needing incision and debridement and two (6%) patients had long-term open wounds.

Per the study design, nine patients were enrolled in the first stage and three showed ≥95% tumor necrosis. The study proceeded to the second stage, in which an additional 14 subjects were enrolled. Only 5 of the 23 evaluable patients in phase II achieved ≥95% tumor necrosis; thus, the study did not show a statistical improvement in the near-pCR rate. Sarcoma tumors in 7 out of 29 (24%) evaluable patients from phases I and II who completed treatment and underwent surgery achieved near-pCR as indicated by the swimmer plot (figure 2).

Additional efficacy endpoints including the overall response rate, tumor necrosis, PFS, and OS have been summarized for all patients enrolled in phases IB and II (table 1). As of January 2021, the 2-year PFS is 57% (95% CI 37% to 72%) and 2-year OS is 77% (95% CI 57% to 88%) (figure 3A,B). Median PFS and OS has not been reached. Pretreatment HSV serology did not affect pathological necrosis or radiographic responses. No significant association was noted between AEs and response.

Table 2 Maximum grade adverse event per patient (Grade 2–4 only as per CTCAE V.4.0) attributed as possibly, probably, or definitely related to talimogene laherparepvec

Toxicity	Grade		
	2	3	4
Anemia	3	2	0
Diarrhea	2	0	0
Nausea	6	0	0
Vomiting	4	2	0
Chills	18	1	0
Leg edema	1	0	0
Fatigue	19	3	0
Fever	4	0	0
Influenza like symptoms	6	1	0
Injection site reaction	5	0	0
Malaise	2	0	0
Pain	5	1	0
Skin infection	1	0	0
Lymphopenia	6	2	2
Weight loss	3	1	0
Anorexia	1	1	0
Back pain	1	0	0
Generalized muscle weakness	2	1	0
Pain in extremity	3	3	0
Dizziness	1	0	0
Dysgeusia	1	0	0
Headache	2	1	0
Neuralgia	1	0	0
Depression	2	0	0
Proteinuria	0	1	0
Renal and urinary disorders	1	0	0
Hiccups	1	0	0
Hypertension	1	1	0
Hypotension	1	0	0
Total	65	17	2

CTCAE, Common Terminology Criteria for Adverse Events.

Correlative analysis

Caspase-3 staining was performed on the diagnostic biopsy tissue specimens and a H score value was calculated (signal intensity (weak, moderate, or strong) * percentage of positive cells). The H scores were compared between diagnostic biopsy specimens with the corresponding TVEC plus EBRT treated resected specimens. A significant increase in the of H score was noted between pre and post treatment tissue specimens (p=0.05) figure 4A. Similarly, Granzyme-B was also noted to be significantly increased between corresponding pretreatment and post-treatment tissue specimens (p=0.05) (figure 4B).

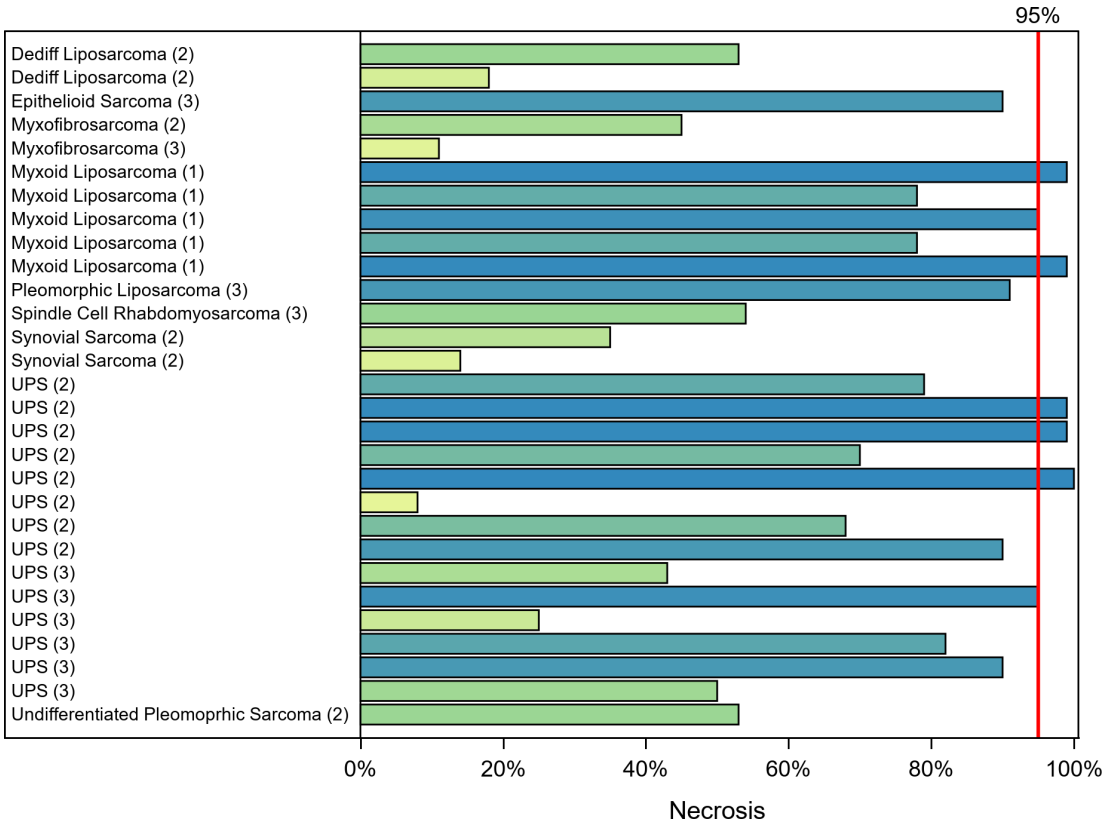

Figure 2 STS histologic subtypes and corresponding percentage tumor necrosis. Tumor grade is shown within parentheses. UPS, Undifferentiated pleomorphic sarcoma; STS, soft-tissue sarcomas.

A similar tissue staining for both Caspase-3 and Granzyme-B was performed on the histology matched radiation only treated historical control primary resected tumor specimens (online supplemental tables S2 and S3). The H-score for Caspase-3 staining was noted to be significantly higher for the TVEC plus EBRT treated tumor specimens as compared with radiation alone specimens (p=0.01) (online supplemental figure S1A) but the difference did not reach significance for the Granzyme-B testing results (p=0.10) (online supplemental figure S1B).

Staining was also performed for T cells using markers against CD3, CD4, CD8, and Foxp3. CD56 staining was performed to identify Natural Killer (NK) cells. Expression of CD3, which is present on all T cells, was significantly increased between pretreatment and post-treatment tissue specimens (p<0.01). Tissue staining for CD4 (p<0.01), CD8 (p<0.01) and CD56 (p<0.03) were also significantly increased in the post-treatment specimens. Foxp3, a transcription factor expressed in regulatory T cells, did not show a significant difference between time points (figure 5).

TUNEL staining performed on resected sarcoma specimens treated with the TVEC plus EBRT combination and compared with historical tumor specimens treated with radiation therapy alone (online supplemental figure s2) showed no statistical significance between the two groups. Multiple cytokine markers were evaluated but

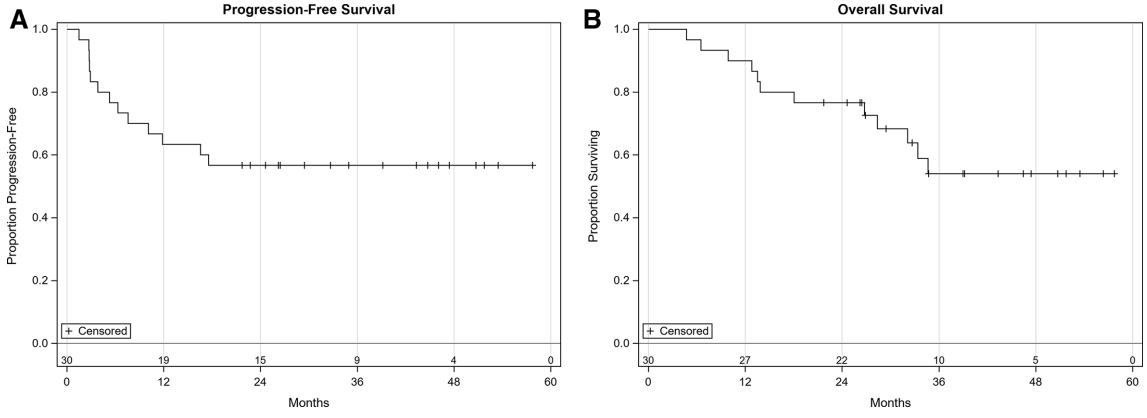

Figure 3 Overall survival and progression-free survival of 30 patients enrolled in the trial.

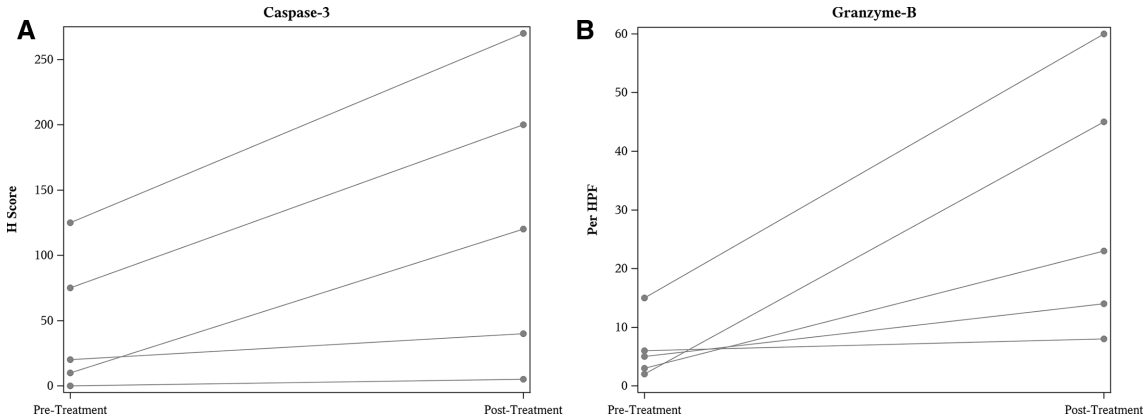

Figure 4 Caspase -3 (p=0.05) stain on tumor cells as determined by H score and Granzyme B staining (p=0.05) on immune cells determined by number of cells positive per high power field (HPF) in pre-treatment biopsy specimens and TVEC plus EBRT post-treatment resection tissue specimens.

no significant differences were found between serum specimens and did not correlate with survival or necrosis (online supplemental table S1).

DISCUSSION

Preoperative radiation is generally favored as it has better toxicity profile in the long run and is determined to be more cost effective, although it does come at a risk of higher wound complicated rates as compared with postoperative radiation.[25 26] Neoadjuvant chemotherapy with ifosfamide and doxorubicin is a commonly used regimen which is associated with radiographic responses, significant toxicities and only marginal survival benefit.[27 28] Recently, a study with neoadjuvant and adjuvant chemotherapy with combination of epirubicin and ifosfamide did not show any benefit as compared with histology tailored chemotherapy regimen in common histologies of localized STS.[4] A study in children and young adults with chemotherapy sensitive localized STS showed that targeted therapy with pazopanib in addition to neoadjuvant chemoradiotherapy had improved 90% pathological responses as compared with chemoradiotherapy alone. With the availability of newer immunotherapy drugs, their safety and efficacy with intratumoral injections warrants exploration in STS which have limited treatment options.

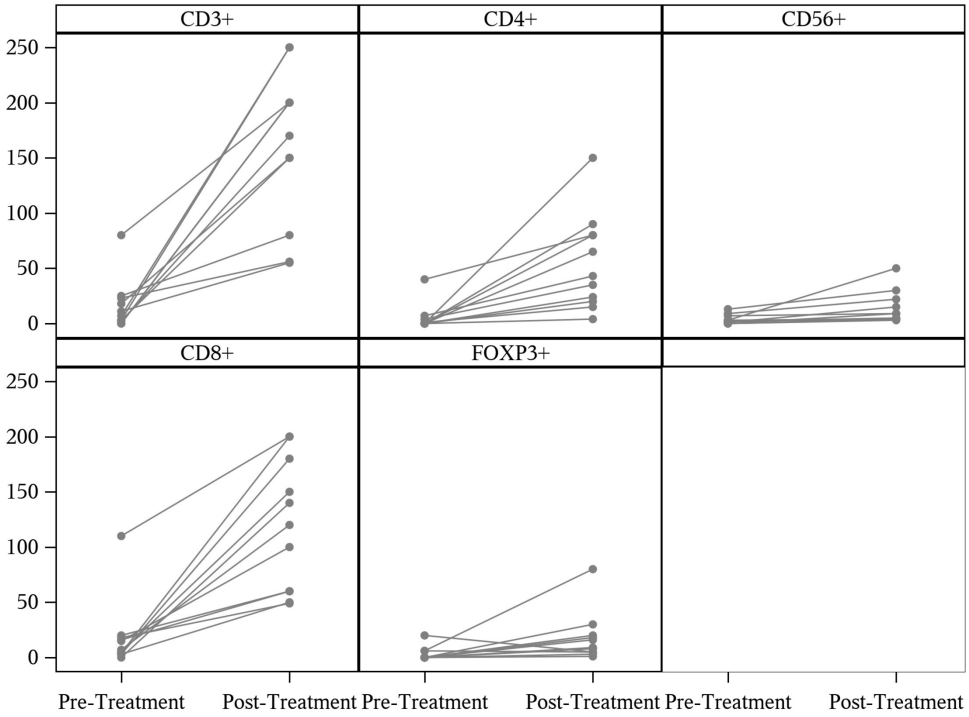

Figure 5 Immune cells with respective tissue staining comparison between pre treatment biopsy tissue specimen and post treatment resection tissue specimen. Significant increases were noted for CD3+ (p<0.01), CD4+ (p<0.01), CD8+ (p<0.01) and CD56+ (p<0.03) cells.

The first application of immunotherapy in sarcoma was reported by Sir William Coley in the 18th century. He injected streptococcal toxins in malignant sarcomas intratumorally.[29] Since then, researchers have explored the efficacy of several immunotherapeutic agents in the treatment of STS. EBRT either preoperatively or postoperatively remains a standard of care for high-risk deep STS with the intent to improve local disease control and metastasis-free survival. A recent literature review indicates that 10% of patients with STS will achieve ≥95% tumor necrosis and approximately 25% will achieve ≥80% tumor necrosis following preoperative EBRT alone. Patients who achieve ≥95% tumor necrosis with preoperative radiation may have improved local and distant control.[20 21] Tumor necrosis has not yet been shown to be a surrogate of OS; however, it is an acceptable end point in various sarcoma studies evaluating novel therapeutics in the neoadjuvant therapeutic space. Furthermore, in the era of immunotherapy and OVs with the potential for an abscopal effect,[12 13 23 24 30 31] the use of near-pCR as a study endpoint remains to be fully explored.

We found the combination of intratumoral TVEC and preoperative EBRT to be safe and well-tolerated in patients with STS of the extremities and trunk. The toxicity profile of TVEC was similar to prior experiences reported in advanced melanoma and head and neck cancers.[13 16 32] There was no treatment-related death. Postoperative wound complications were noted in 27% of the patients, which was consistent with other studies.[26 33 34] The TVEC 10^6 and 10^8 PFU in 4 mL dose used in this study, with its well-studied safety profile, was similar to the dose used in previous melanoma clinical trials. The larger size of the sarcoma tumors perhaps warranted a higher injection dose for better virus distribution, which may translate into better efficacy.

Our results revealed several novel observations. Increased lymphoplasmacytic infiltrate was reported in TVEC-treated specimens compared with that in specimens historically treated with EBRT alone. On further examination, certain tumor specimens showed increased CD8 T cell subset infiltration in comparison to pretreatment biopsies (figure 6). One patient with metastatic undifferentiated pleomorphic sarcoma of the trunk who received protocol therapy but later opted against primary tumor resection showed PD on the preoperative disease assessment scan. A follow-up scan 3 months later without interim treatment showed PR both in the primary tumor and metastatic disease sites in the lung, suggesting a delayed immune response. Given the curative intent, standard surgical timelines were followed in this study, which could have limited treatment efficacy especially in patients with metastatic disease. One patient with undifferentiated pleomorphic sarcoma of the extremities who was treated using protocol therapy but developed lung metastases later underwent metastasectomy. The resected specimen showed lymphoplasmacytic infiltration, suggesting an off-target immune response. This immune-driven phenomenon in the presence of radiation therapy is

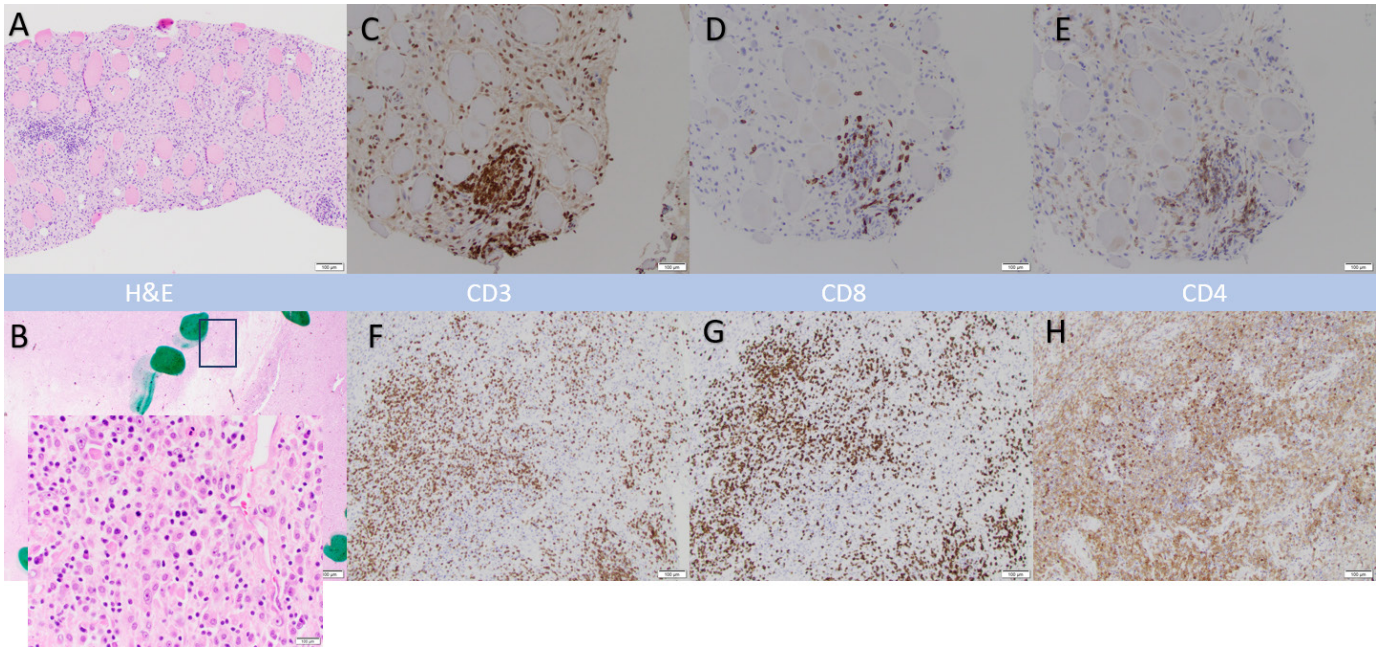

Figure 6 Example of myxofibrosarcoma histopathology sections after TVEC and preoperative EBRT. Hematoxylin and eosin staining showing the histology in the biopsy specimen (Panel A) and a residual tumor (11% necrosis) with admixed lymphocytic infiltrate in panel B (TVEC plus EBRT). The inset in Panel B shows a high power view of dense immune infiltrate. Panel C, D and E show CD3, CD8 and CD4 positive infiltrating T cells in pretreatment biopsy specimens. Panels F, G and H show corresponding changes in those T cell subtypes in post treatment tumor specimens. UPS, Undifferentiated pleomorphic sarcoma; TVEC, Talimogene laherparepvec; EBRT, external beam radiation therapy.

difficult to characterize and attribute to TVEC or EBRT alone.

While trying to draw conclusions about the STS subtype-specific response is difficult given the small number of subjects in the study, some trends were noted. Five patients with low-grade myxoid liposarcoma remain free from local recurrence or distant metastasis, which may reflect the increased radiation sensitivity of this subtype or a good local immune control secondary to TVEC. Two patients with intermediate-grade myxofibrosarcoma (involving the trunk or extremities), one with locally recurrent disease at enrollment and both with microscopic positive margins postoperatively, have not had local or distant relapse >24 months since treatment, suggesting good local disease control similar to TVEC responses noted for in-transit metastasis in melanoma (stage IVM1a).[13] A major concern with intratumoral injection was skin infiltration by tumor cells at the injection sites. During surgical resection, the ink marks used to direct injections were removed en bloc with the tumor specimen, and the sites were not infiltrated with tumor cells.

Our study adds to the growing literature on immune response in patients with an inherent resistance to immunotherapy, which needs to be further explored. There are several limitations of this study. A fixed TVEC dose was injected irrespective of tumor size. Whether this was an appropriate approach was not explored. This limitation could perhaps be overcome by evaluating phased dose escalations of TVEC plus preoperative EBRT based on sarcoma size (eg, 4 mL of TVEC in tumors 5–10 cm in size, 8 mL of TVEC in tumors >10 cm). An ongoing trial is evaluating 8 mL dose of intratumoral TVEC (NCT04599062) in combination with preoperative radiation is currently enrolling patients. Standard EBRT doses were used. Perhaps hypofractionated dosing of EBRT regimens that result in greater tumor cell killing and immune responses may elicit a better immune response.[35 36] Despite careful ultrasound-guided injection to ensure good TVEC distribution to every tumor region, the drug effect could not be confirmed histologically in all parts of the tumor specimens. Whether good drug distribution within the tumor is important to elicit more potent antitumor immune responses remains to be determined. Visceral-site TVEC injections could be considered for metastatic disease. Given the possibility of delayed responses with immunotherapy, as seen in one of our subjects, allowing continued treatment despite first progression may be considered, provided there are limited symptoms. Addition of other immune stimulants, such as systemic anti-PD-1[37–39] or intratumoral TLR 9 receptor agonists, could be considered for more robust localized immune responses. A recent study of TVEC in combination with pembrolizumab in locally advanced or metastatic soft tissue sarcoma showed promising objective responses and the combination is being explored in select sarcoma subtypes.[40] Exploring TVEC in combination with anti-PD1 together with radiation, which can further enhance immunotherapy effects, may be a very valuable approach to be studied in future clinical trials. Radiographic responses determined by comparing pretreatment and preoperative imaging (MRI or CT) did not correspond to pathological necrosis in all cases, which again highlights the lack of good radiographic disease assessment in the neoadjuvant therapeutic space.

Immunohistochemistry revealed significant differences in T cell infiltration into tumor specimens. Total T cells, as measured by CD3 staining, were increased in post-treatment tumor samples compared with pretreatment levels. Additionally, staining of CD4 and CD8 was significantly increased, indicating that there was increased infiltration of both CD4 and CD8 T cells into the tumor after TVEC treatment. Enhanced T cell infiltration to tumor sites, in particular higher numbers of CD8 T cells, correlates with better clinical outcomes in numerous cancer types.[41 42] There is a significant increase in CD56 stained cells in TVEC plus EBRT treated resection specimens as compared with pretreatment biopsy specimens which suggests enhanced NK cell function. The change in CD56 is subtle, while the changes in CD3, CD4 and CD8 are much more substantial. This could also be due to the increased T cell infiltration since some T cell subsets are known to express CD56.

Immunostaining of tissue sections revealed significant differences in multiple immune markers. Expression of granzyme-B was significantly increased in post-treatment tissue specimens compared with pretreatment, although no difference was observed between patients that received TVEC compared with radiation alone controls. Granzyme-B is an effector serine-protease released by cytotoxic CD8 T cells and NK cells that function as a primary mechanism of tumor cell death.[43] Caspase-3 is a protease involved in apoptosis and is downstream of many molecules including granzyme-B. Caspase-3 expression can correlate with increased tumor cell death in a number of cancer types.[44–47] Staining for caspase-3 was increased in the post-treatment specimens. Additionally, caspase-3 staining was significantly increased in those patients that received TVEC treatment compared with those that received radiation alone. These combined data indicate that the TVEC treatment may enhance apoptotic tumor cell death.

No difference in TUNEL staining was demonstrated between TVEC treated patients and those treated with radiation alone. TUNEL staining detects double-stranded DNA breaks, a characteristic of both necrotic and apoptotic cell death; however, recent literature favors it to be a marker of apoptosis.[48] Given the lack of significant difference as compared with radiation alone controls suggest that the response to therapy was maybe due to TVEC's action as an immune stimulant and not as a radiosensitizer. While it is possible that apoptosis may be secondary to radiation therapy

alone, the caspase data suggest that there is an added induction of apoptosis with the combination therapy with increased Granzyme being the major mediator of apoptosis. The reasons for the discrepancy between the TUNEL and caspase data are not readily apparent, but it is possible that caspase-3 measures apoptosis at a 'reversible' stage.[49] Thus, caspase-3 staining may be a more reliable marker for apoptotic cancer cell death related to TVEC use.

CONCLUSION

Preoperative OV treatment with TVEC and concurrent EBRT for locally advanced STS is safe and well-tolerated. The combination however did not increase the proposed pathological necrosis rate. No DLTs were observed. While the combination of TVEC plus EBRT may enhance immune responses, as observed in some cases, long-term survival benefits will be awaited and reported in future. A trial evaluating greater dose level of TVEC corresponding to the tumor size and various combination strategies should be considered.

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Contributors VM, MM, BJM, BA, CAn, SB, DK, MR, CM, and CAb were involved in actively treating the patients on the study. MT, SB, OJ, and JT reviewed and interpreted the histopathology data. LS, SH, SV, JH, LW, and WZ analyzed blood specimens and tissue specimens for immunological markers and interpreting the data. GZ and SM were the biostatisticians involved with this study. All authors reviewed and edited the final manuscript.

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