A Phase 2 Trial of Enhancing Immune Checkpoint Blockade by Stereotactic Radiation and *In Situ* Virus Gene Therapy in Metastatic Triple-Negative Breast Cancer

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ABSTRACT

Purpose: A Phase 2 trial of stereotactic radiotherapy and *in situ* cytotoxic virus therapy in patients with metastatic triple-negative breast cancer (mTNBC) followed by pembrolizumab (STOMP) was designed to evaluate dual approach of enhancing single-agent immune checkpoint blockade with adenovirus-mediated expression of herpes-simplex-virus thymidine-kinase (ADV/HSV-tk) plus valacyclovir gene therapy and stereotactic body radiotherapy (SBRT) in patients with mTNBC.

Patients and Methods: In this single-arm, open-label Phase 2 trial, patients with mTNBC were treated with ADV/HSV-tk [5×10^{11} virus particles (vp)] intratumoral injection, followed by SBRT to the injected tumor site, then pembrolizumab (200 mg, every 3 weeks). The primary endpoint was clinical benefit rate [CBR; complete response (CR), partial response (PR), or stable disease (SD) ≥ 24 weeks per RECIST version1.1 at non-irradiated site]. Secondary endpoints included duration on treatment (DoT), overall survival (OS), and safety. Exploratory endpoints included immune response to treatment assessed by correlative tissue and blood-based biomarkers.

Results: Twenty-eight patients were enrolled and treated. CBR was seen in 6 patients (21.4%), including 2 CR (7.1%), 1 PR (3.6%), and 3 SD (10.7%). Patients with clinical benefit had durable responses, with median DoT of 9.6 months and OS of 14.7 months. The median OS was 6.6 months in the total population. The combination was well tolerated. Correlative studies with Cytometry by Time of Flight (CyTOF) and imaging mass cytometry (IMC) revealed a significant increase of CD8 T cells in responders and of myeloid cells in non-responders.

Conclusions: The median OS increased by more than 2-fold in patients with clinical benefit. The therapy is a well-tolerated treatment in heavily pretreated patients with mTNBC. Early detection of increased effector and effector memory CD8 T cells and myeloids correlate with response and non-response, respectively.

Introduction

Triple-negative breast cancer (TNBC) is an aggressive, difficult-to-treat disease. The median overall survival (OS) of locally advanced unresectable or metastatic TNBC (mTNBC) is 8 to 13 months (1, 2). Compared with other breast cancer types, TNBC has higher expression levels of programmed death-ligand 1 (PD-L1; ref. 3) that makes immune checkpoint blockade (ICB) a potentially appealing treatment option. When checkpoint inhibitors as monotherapy were studied in mTNBC, durable responses were seen in those who responded. However, only 5.2% to 25.9% of patients benefited from monotherapy treatment and the responses were seen mostly in patients with high PD-L1 expression in earlier lines of treatment (4–7). Thus, there is an unmet need of treatment strategies to enhance the antitumor activity of ICB.

Radiotherapy has traditionally been applied for local tumor control, based on its cytocidal activity through direct or indirect DNA damage. Recent studies have shown that radiotherapy can modify the tumor microenvironment to induce a systemic antitumor immune response through proinflammatory cytokines and engagement of the innate and adaptive immune systems (8, 9) leading to abscopal effect and response to ICB in distant non-irradiated sites (10, 11). Abscopal effect was more often observed when fractionated instead of a single-dose radiotherapy was given (12, 13). The advent of stereotactic body radiotherapy (SBRT) allows for hypofractionated treatment with precise delivery of high radiotherapy doses, potentially achieving an ideal balance of tumor ablation and immune activation (14).

Even though abscopal effect has been reported, the overall occurrence rate is low (15). Different strategies have been applied to enhance abscopal effect. Adenovirus-mediated expression of herpes-simplex-virus thymidine-kinase (ADV/HSV-tk) can catalyze the phosphorylation of acyclovir to a toxic form capable of inhibiting DNA synthesis and resulting in selective cytotoxicity to cells expressing HSV-tk (16). ADV/HSV-tk gene transduction followed by acyclovir prodrug ganciclovir or valacyclovir therapy has shown both local and systemic antitumor activity in several cancer models (17–19). It was found to induce a pronounced intratumoral

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Translational Relevance

In this Phase 2 trial in metastatic triple-negative breast cancer (TNBC) patients, evaluated the efficacy and safety of combining two additional modalities to enhance efficacy of immune checkpoint blockade. Patients were treated with the combination of *in situ* adenovirus-mediated expression of herpes-simplex-virus thymidine-kinase (ADV/HSV-tk) plus valacyclovir gene therapy, followed by radiation and then pembrolizumab (STOMP).

We observed an enhanced clinical benefit rate using this combination strategy in a previously heavily treated population, including patients with low/negative PD-L1 tumors and patients with liver metastases. Patients with clinical benefit had durable responses. Our correlative immune profile analysis using Cytometry by Time of Flight (CyTOF) and imaging mass cytometry (IMC) on paired blood samples and biopsies suggested increase of CD8 T cells and myeloid cells as predictive markers of response and non-response, respectively. We believe these findings will be of interest to the readers in the era of immunotherapy.

infiltrate of macrophages, $CD4^+$ and $CD8^+$ T cells, and a cytokine profile similar to Th1 immune response (20). Combing ADV/HSV-tk plus ganciclovir gene therapy and radiotherapy appeared to result in increased $CD4^+$ T-cell tumor infiltration, additive killing activities, and enhanced systemic antitumor activity in both prostate cancer and mammary tumor models (21, 22).

Studies have shown the antitumor effect of ICB can be enhanced by both radiotherapy and ADV/HSV-tk plus ganciclovir gene therapy. Combining radiotherapy with ICB demonstrated enhanced systemic $CD8^+$ T-cell–dependent antitumor activity in several mouse cancer models (23, 24), and improved responses in solid tumors (25–27). In a study evaluating the combination of ADV/HSV-tk plus ganciclovir gene therapy and PD-L1 checkpoint inhibitor in glioblastoma, the combination therapy upregulated PD-L1 expression and improved long-term survival (28).

Here, we explored the approach of using SBRT and ADV/HSV-tk plus valacyclovir gene therapy before pembrolizumab to enhance pembrolizumab efficacy in patients with mTNBC.

Patients and Methods

Patients

Patients with mTNBC who had relapsed on or were refractory to standard-of-care therapy were eligible for the study. Eligible participants were at least 18 years old, had histologically confirmed TNBC or low estrogen/progesterone receptor breast cancer (estrogen/progesterone receptor <10%, and HER2 negative), an Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2, measurable disease based on RECIST v1.1, a target lesion of at least 1 cm in diameter for SBRT, and a measurable distant metastasis apart from the irradiated site of at least 1 cm to evaluate response. Patients with previously treated brain metastases were eligible for the study provided they were stable (without evidence of progression by imaging for at least 4 weeks before the first dose of trial treatment and any neurologic symptoms have returned to baseline), have no evidence of carcinomatous meningitis, or new/enlarging brain metastases, and have not used steroids for at least 7 days before trial treatment. Other required inclusion criteria included a willingness and ability to provide informed consent for the trial, a life expectancy of at least 6 months, and adequate organ functions.

Key exclusion criteria were bone metastasis–only disease; prior treatment with immunotherapy, including anti–PD-1, anti–PD-L1, or anti–PD-L2 agents; prior treatment with gene vector therapy; immunodeficiency or receipt of any form of systemic immunosuppressive therapy (including glucocorticoids) within 7 days before the first dose of trial treatment; active autoimmune disease that required systemic treatment for the preceding 2 years; supplemental oxygen dependence; carcinomatous meningitis; known active hepatitis B or hepatitis C infection; or major surgery within 4 weeks before study enrollment.

Study oversight

Study design and subject consent forms were reviewed and approved by the hospital institutional review board. All patients provided written informed consent. The study was sponsored by Merck & Co., Inc. and was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice. Adverse events (AE) were recorded regularly according to the protocol and were subject to mandatory reporting by the investigators. Any serious AEs were reported to Merck within 24 hours.

Trial design

STOMP was designed as a single-center Phase 2 study evaluating the approach of enhancing the abscopal effect of SBRT with ADV/HSV-tk plus valacyclovir gene therapy in augmenting the efficacy of pembrolizumab monotherapy in patients with mTNBC. ADV/HSV-tk was created by using a backbone Ad5-dl309 with a Rous sarcoma virus promoter that drives the herpes simplex virus thymidine kinase gene insert (Center for Gene and Cell Therapy, Houston Methodist Hospital) as previously reported (29). ADV/HSV-tk [5×10^{11} virus particles (vp)] in a 2-mL total volume was injected intratumorally to a single target lesion per patient on day 0 of the study using imaging guidance along with fiducial placement for SBRT. Valacyclovir was administered orally at a dose of 2 g three times a day for 14 days from days 1 to 15. The injected target lesion was radiated. Metastatic target lesions were treated with SBRT (30 Gy; 6 Gy $\times$ 5 fractions) and primary breast/chest wall sites were treated with SBRT, IMRT, or 3DCRT (30 Gy; 6 Gy $\times$ 5 fractions, BED10 = 48 Gy or 33.6 Gy; 4.2 Gy $\times$ 8 fractions, BED10 = 47.71 Gy) at the treating physician's discretion. SBRT was administered over 2 weeks from days 2 to 16 of the study. Pembrolizumab at a dose of 200 mg was administered intravenously every 21 days after radiotherapy. Pembrolizumab monotherapy was continued until disease progression, development of unacceptable side effects, withdrawal of consent, or up to 24 months (35 cycles) in patients without disease progression.

Peripheral blood for immune correlative analysis was collected on days 1 (baseline), 17, and 38 and two panels of Cytometry by Time of Flight (CyTOF) on paired peripheral blood mononuclear cells were performed. One panel of 35 myeloid markers (M-panel, Supplementary Table S1) and a second panel of 35 T-cell markers (T-panel, Supplementary Table S2) were used on each sample. Barnes-Hut tSNE implementation in Rtsne package was used to plot tSEN. Data were 99th percentile normalized before the analysis, and we used the default tSNE parameters (initial dimensions, 110; perplexity, 30; and theta, 0.5).

In addition, paired core biopsies were performed on days 1 (baseline) and 17. Imaging mass cytometry (IMC) was performed after

staining of these patient samples with a 35-marker panel of metal-tagged antibodies and ablation by Hyperion (Fluidigm Inc.; Supplementary Table S3).

Assessment

PD-L1 expression was measured as the percentage of tumor area involved by PD-L1 positive tumor infiltrating immune cells as assessed by IHC using the Ventana SP142 antibody testing that was validated in the IHC section of our institutional pathology laboratory. PD-L1 expression was also measured as a combined positive score (CPS) using the PD-L1 IHC 22C3 PharmDx kit (Neogenomics performed the staining and results were interpreted by our institutional pathologists) or E1L3N antibody (Cell Signaling Technology, our laboratory performed the staining and results were interpreted by outside pathologists). CPS was defined as the ratio of PD-L1–positive cells out of the total number of tumor cells × 100.

Tumor assessments in non-irradiated sites by CT scans or MRI were performed at screening, every 8 weeks thereafter until completion of the protocol-specified study treatment and/or at the discretion of the treating physician, when clinically indicated, and 30 days after the last dose of pembrolizumab. Tumor response was assessed in these *non-irradiated metastatic lesions* by two independent radiologists according to RECIST v1.1. AEs were evaluated throughout the trial until 30 days after the end of treatment and were graded according to the National Cancer Institute Common Terminology Criteria for AEs (NCI CTCAE), version 4.03.

Statistical analysis

The primary end point was clinical benefit rate [CBR; the proportion of patients with complete response (CR), partial response (PR), or stable disease (SD) for ≥24 weeks]. Secondary end points included OS (the time from intratumoral viral injection to death or last date of contact), duration on treatment (DoT), and safety. Exploratory objectives included immune response to treatment, correlative tissue, and blood-based biomarkers that included PD-1 and PD-L1 expression, immune infiltrates, and cytokine expression (IL1, IL2, IL6, IL12, IFNc, TNFα, and GM-CSF). Efficacy and safety were assessed in all patients who received intratumoral ADV/HSV-tk injection.

With the null and alternate hypotheses of 19% CBR and 39% CBR, respectively, an enrollment of 28 patients with TNBC was planned for the target response rate of 39%. Power study revealed that with a sample size of 28 patients if the observed number of responders is 11 (39.3%), the 95% confidence interval (CI) estimate will have a margin of error of 18.1% points (i.e., 21.2% to 57.4%) based on the normal approximation. The Kaplan–Meier method was used to obtain estimates of 95% CIs for mean and median survival times. Pearson correlation analysis was used to assess the association of response with lactate dehydrogenase (LDH), disease-free interval (DFI), PD-L1 expression assessed by Ventana SP142 antibody testing, and CPS as assessed by IHC using the Dako 22C3 PharmDx kit.

Data availability

The data generated in this study are stored in a secured drive and available upon request from the corresponding author.

Results

Patients

From June 7, 2017 to May 7, 2020, a total of 39 patients were screened and 28 patients with histologically confirmed mTNBC were enrolled (**Table 1**). Median age was 54 years (range, 34 to 78). The majority of patients were heavily pretreated, with a median of two (range, 0–7) previous lines of cytotoxic treatment in the metastatic setting, and 8 patients (25.0%) had received ≥3 prior metastatic lines of chemotherapy. The median duration of response from the last line of treatment was 2 months (range, 1 to 7). Of note, 3 patients (10.7%) had treated brain metastasis at the time of enrollment. The majority of patients were PD-L1 negative; 18 (64.3%) patients had <1% PD-L1 expression by Ventana SP142 antibody testing. Four patients (14.3%) had CPS combined score ≥10 by PD-L1 IHC 22C3 PharmDx kit testing.

At the time of data cutoff on October 22, 2021, patients had received a median of three pembrolizumab doses (range, 0 to 23). Median duration of follow-up was 8.3 months (95% CI, 3.0–10.1 months). One patient (3.6%) remained on treatment at the data cutoff date. Disease progression was the most common reason for treatment discontinuation (89.3%).

Efficacy

Of the 28 patients enrolled, 6 patients (21.4%) had clinical benefit, including 2 with CR (7.1%), 1 with PR (3.6%) and 3 with SD (10.7%; **Table 2**). The median OS was 6.6 months (95% CI, 3.0 to 10.1 months) in the overall population, and 14.7 months (95% CI, 6.5 to 47.5 months) in the patients who had the clinical benefit (**Fig. 1A**). The median DoT was 2.2 months (95% CI, 1.4 to 2.9 months) in the overall population, and 9.6 months (95% CI, 4.8 to 23 months) in the patients who had clinical benefit (**Fig. 1B**). In the overall population, 57.1% were alive at 6 months and 19.5% at 12 months; whereas in the responders, 100% were alive at 6 months and 66.7% at 12 months (**Fig. 1C**).

Two patients who had clinical response were alive at the data cutoff date and 1 patient remained on treatment (**Table 3**). Patient 2 had biopsy-proven recurrent metastatic disease to the lungs and liver, with a 1.2-cm liver lesion diagnosed on PET scan that decreased in size on treatment. She then achieved CR but discontinued pembrolizumab after 18 doses due to Grade 3 pneumonitis. She remains in CR and has not received any systemic treatment for over 47 months. Patient 5 had recurrent biopsy-proven metastatic disease in the liver and had SD on treatment for over 22 months.

We explored the potential predictive values of LDH, DFI, and CPS. Clinical benefit was moderately associated with PD-L1 expression ($r = 0.46$), but not with LDH, DFI, or CPS.

Safety

Treatment-related AEs of any cause were reported in 22 patients (78.6%; **Table 4**). The most common AEs were any-grade constitutional (35.7%), hypothyroidism (21.4%), diarrhea (14.3%), nausea (10.7%), dyspnea on exertion (10.7%), alkaline phosphatase elevation (10.7%), and hyperthyroidism (10.7%). Grade 3 or higher treatment-related AEs were reported in 10 patients (35.7%). Immune-related AEs at any grade occurred in 13 patients (46.4%). The most common immune-related AEs were hypothyroidism (21.4%) and hyperthyroidism (10.7%). Grade 3 or higher immune-related AEs were reported in 2 patients (7.1%), including 1 pneumonitis (3.6%), and 1 transaminitis (3.6%). Two patients (7.1%) discontinued treatment due to Grade 3 pneumonitis and Grade 2 transaminitis. There were no treatment-related deaths.

Immune profile analysis

To investigate the association between response and the immune populations in peripheral blood, CyTOF was performed on 25 patients (5 responders and 20 non-responders) on days 1 and 17 and on 20

Table 1. Patient characteristics at baseline.

	Total population ($n = 28$)	Responders ($n = 6$)	Non-responders ($N = 22$)
Median age (range; years)	54 (34–78)	62 (44–78)	50 (35–79)
Female sex, *n* (%)	28 (100)	6 (100)	22 (100)
Race, *n* (%)			
White	18 (64.2)	6 (100)	12 (54.5)
Black	5 (17.9)	0 (0)	5 (22.7)
Asian	5 (17.9)	0 (0)	5 (22.7)
Others	0 (0)	0 (0)	0 (0)
ECOG PS, *n* (%)			
0	17 (60.7)	6 (100)	11 (50.0)
1	8 (28.6)	0 (0)	8 (36.4)
2	3 (10.7)	0 (0)	3 (13.6)
Germline BRCA1/2, *n* (%)			
Mutation	0 (0)	0 (0)	0
Wild-type	24 (85.7)	5 (83.3)	19 (86.4)
Unknown	4 (14.3)	1 (16.7)	3 (13.6)
Smoking status, *n* (%)			
Never smoker	24 (85.7)	4 (66.7)	20 (90.9)
Former smoker	4 (14.3)	2 (33.3)	2 (9.1)
Current smoker	0 (0)	0 (0)	0 (0)
Location of metastasis, *n* (%)			
Lymph node only	6 (21.4)	2 (33.3)	4 (18.2)
Visceral metastasis	19 (79.2)	4 (66.7)	18 (81.8)
Brain metastasis	3 (10.7)	1 (16.7)	2 (9.1)
Previous lines of treatment in metastatic setting, *n* (%)			
0	8 (28.6)	1 (16.7)	7 (31.8)
1	5 (17.9)	2 (33.3)	3 (13.6)
2	8 (28.6)	2 (33.3)	6 (27.3)
3	3 (10.7)	1 (16.7)	2 (9.1)
≥4	4 (14.3)	0 (0)	4 (18.2)
Median lines of treatment	2 (0–7)	1.5 (0–3)	2 (0–7)
Median duration of response from last line of treatment (range; months)	2 (1–7)	2 (1–6)	2 (1–7)
Prior chemotherapy received, *n* (%)			
Anthracycline	24 (85.7)	5 (83.3)	19 (86.4)
Cyclophosphamide	24 (85.7)	5 (83.3)	19 (86.4)
Taxanes	27 (96.4)	5 (83.3)	22 (100)
Carboplatin	15 (53.6)	3 (50.0)	12 (54.5)
Microtubule inhibitors	7 (25.0)	1 (16.7)	6 (27.3)
Gemcitabine	9 (32.1)	0 (0)	9 (40.9)
Capecitabine	12 (42.9)	4 (66.7)	8 (36.4)
Anti-HER2 therapy	2 (7.1)	0 (0)	2 (9.1)
Anti-hormone therapy[a]	4 (14.3)	1 (16.7)	3 (13.6)
Others[b]	8 (28.6)	4 (66.7)	4 (18.2)
PD-L1%			
<1%	18 (64.3)	2 (33.3)	16 (72.7)
≥1%	9 (32.1)	3 (50.0)	6 (27.3)
Unknown	1 (3.6)	1 (16.7)	0 (0)
CPS			
≥10	4 (14.3)	2 (33.3)	2 (9.1)
<10	16 (57.1)	2 (33.3)	14 (63.6)
Unknown	8 (28.6)	2 (33.3)	6 (27.3)
Irradiated metastatic site			
Lymph nodes/skin/chest wall	13 (46.4)	3 (50.0)	10 (45.5)
Visceral	15 (53.6)	3 (50.0)	12 (54.5)
Baseline LDH			
Normal	9 (32.1)	3 (50.0)	6 (27.3)
>1 ULN	15 (53.6)	3 (50.0)	12 (54.5)
>2 ULN	4 (14.3)	0 (0)	4 (18.2)

Abbreviations: CPS, combined positive score; ECOG PS, Eastern Cooperative Oncology Group performance status; LDH, lactate dehydrogenase; ULN, upper limit of normal.

[a]Included aromatase inhibitors, CDK4/6 inhibitors, fulvestrant, and everolimus.

[b]Included pan-NOS inhibitor, PARPi with mTOR or AKT inhibitor, bevacizumab.

Table 2. Efficacy (RECIST 1.1) of non-irradiated metastatic lesion.

	Breast ($N = 28$)
Best overall response, *n* (%)	
Confirmed CR	2 (7.1)
Confirmed PR	1 (3.6)
Stable disease	3 (10.7)
Progressive disease	22 (78.6)
Clinical benefit (CR, PR, and SD)	6 (21.4)

Abbreviations: CR, complete response; PR, partial response; SD, stable disease.

patients (4 responders and 16 non-responders) on day 38. Cell populations were clustered, identified (Supplementary Table S4), and plotted on tSNE plots (**Fig. 2A**). The $\log_2$ fold change between Days 17 or 38 and day 1 of responders and non-responders was calculated and illustrated as heatmaps (**Fig. 2B**).

When the cell populations were compared between responders and non-responders at the same time points (**Fig. 2C**), significantly more T cells [effector memory (Tem) CD4 T cells and Tem CD8 cells, unconventional T cells, and natural killer T (NKT) cells] were seen in the non-responders on day 1. At day 17, these T-cell populations increased in the responders (**Fig. 2B** and **C**), with Tem CD8 and the activated CD8 T-cell (PD-1^+ CD8) showing the most profound increase. In the non-responders, a statistically significant increase of myeloid cells was observed in 14/20 (70%) patients. In those patients who had increased myeloid cells, 12/14 patients (85.7%) had a decrease of T cells. On day 38, there was no continuous increase of CD8 T cells in responders except for 1 patient (**Fig. 2B**). In comparison, continuous increase of myeloid cells was seen in the non-responders (10/16, 62.5%).

The tumor microenvironment was further assessed on paired tumor biopsies by IMC. A detailed description of IMC was previously described (30). In responders, tumors contained myofibroblasts (SMA^+) on day 1 and became infiltrated with immune cells on day 17 after treatment. In contrast, non-responder tumor cells were cohesive with few myofibroblasts present on day 1 and limited immune cell infiltration was observed on day 17 after treatment (Supplementary Fig. S1A). In addition, more CD4 and CD8 T cells were found in responders with few $Ki67^+$ tumor cells, whereas more $CD68^+$ macrophages were found in the non-responders with more $Ki67^+$ tumor cells. The cell populations

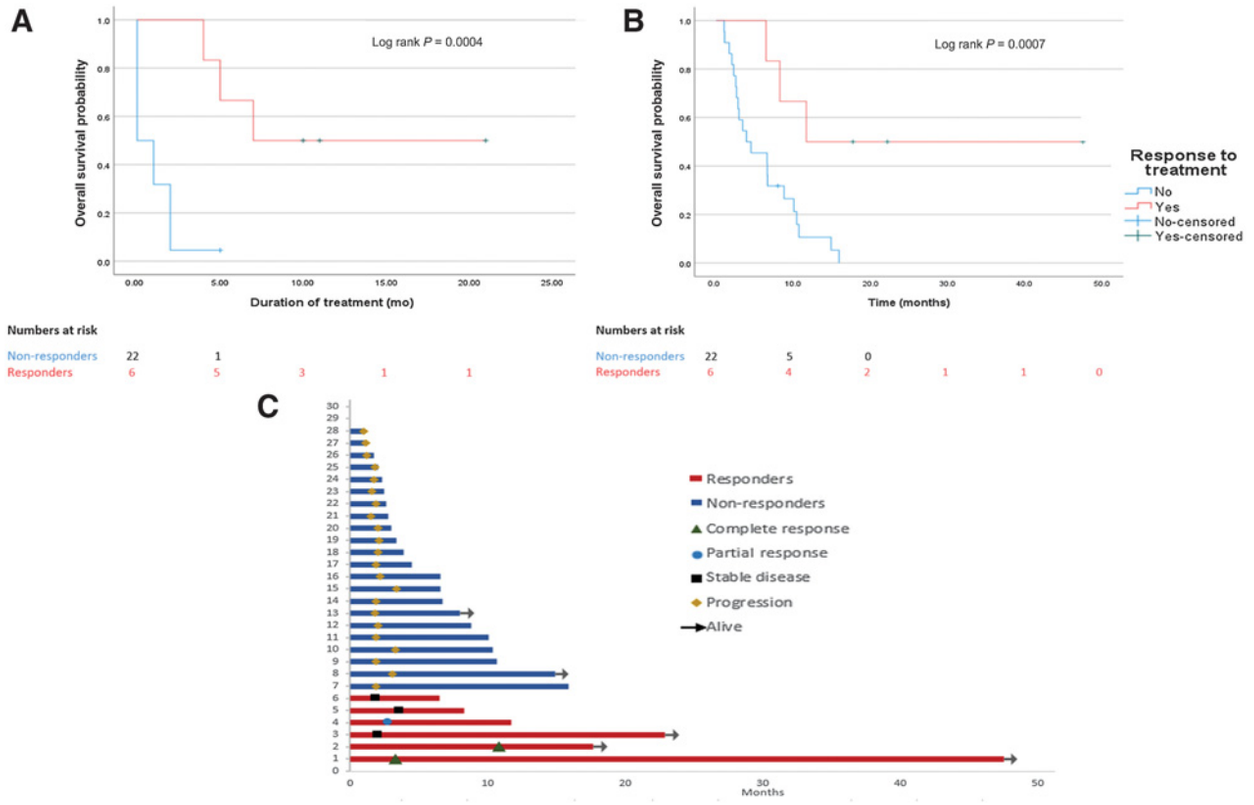

Figure 1.

Kaplan–Meier curve of overall survival and duration of treatment in responders and non-responders. **A,** Overall survival in responders (red line) and non-responders (blue line). The comparison of survival curves shows that patients who responded to the treatment had a significantly higher probability of survival ($P = 0.007$). **B,** Kaplan–Meier curve of duration on treatment in responders (red line) and non-responders (blue line) showing significantly longer duration of treatment in responders versus non-responders ($P = 0.0004$). **C,** Swimmer plot of patient survival by response. The *x*-axis represents survival in months and *y*-axis represents patients. Bars are colored by RECIST v1.1 response with red representing responders and blue representing non-responders. For patients who were responders (red bar), a green triangle represents complete response, a dark blue dot represents partial response, and a black square represents stable disease. For non-responders, a yellow diamond represents progression. A horizontal arrow at the end of a bar represents patient alive status. Responders, patients who had clinical benefit, including those who had CR, PR, or SD ≥ 24 weeks per RECIST v1.1 at non-irradiated metastatic sites.

Table 3. Characteristics of patients with clinical benefit.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age	44	62	58	67	77	60
ECOG PS	0	0	0	0	0	0
Location of metastasis	Chest wall, axillary and mammary LNs	Lung and liver	Lung, liver, brain, adrenal gland	Supraclavicular LN, pleura, bone	Liver	Mediastinal and axillary LN
Number of previous therapies for metastatic disease	2	1	2	1	0	3
Previous chemotherapy	Paclitaxel and bevacizumab, carboplatin	Docetaxel/ pan-NOS inhibitor	Nab-paclitaxel/ carboplatin, docetaxel/ pan-NOS inhibitor	Ixabepilone/ capecitabine	NA	Capecitabine, carboplatin/XRT, docetaxel/pan-NOS inhibitor
Duration of response on prior chemotherapy (mo)	3	1	2	2	NA	6
DFI (mo)	10.0	4.0	8.1	44.0	44.2	0.5
Irradiated site	Chest wall	Lung mass	Lung mass	Lymph node	Liver mass	Lymph node
LDH level at baseline (U/L)	189	210	322	285	291	170
PD-L1 expression by Ventana SP142	2	1	Unknown	0	0	30
CPS by PD-L1 IHC 22C3 PharmDx kit	10	80	Unknown	1	1	30
Best clinical response	CR	CR	PR	SD	SD	SD
DoT (mo)	12.4	11.5	7.7	5.7	22.9	4.8
Overall survival (mo)	17.7	47.5	11.7	8.3	22.9	6.5

Abbreviations: CR, complete response; DFI, disease-free interval; DoT, duration on treatment; ECOG PS, Eastern Cooperative Oncology Group performance status; LDH, lactate dehydrogenase; LN, lymph node; mo, months; PR, partial response; SD, stable disease; XRT, radiation.

from 3 responders and 14 non-responders were then clustered and identified by unsupervised algorithms (Supplementary Fig. S1B) and the $\log_2$ fold change between days 1 and 17 was plotted in a heatmap (Supplementary Fig. S1C). Consistent with CyTOF findings, there was a significant increase of M2-like monocytes ($CD163^+CD14^+$) in non-responders ($P = 0.0012$).

We then performed neighborhood analysis to assess the spatial relationship between the identified cell populations (Supplementary Fig. S2). Comparing before and after treatment in responders, the epithelial cells or cancer cells (cluster 3) were separated from most immune populations (cluster 17–24) at baseline, but became surrounded by these immune cells after treatment; CD8 T cells (cluster 23) were surrounded by immunosuppressive $CD15^+$ neutrophils (cluster 27) at baseline, but were freed from these neutrophils after treatment. Comparing responders and non-responders, the non-responders already had some immune populations surrounding epithelial cells or cancer cells (cluster 3) at baseline, which is consistent with our previous observation that non-responders had more CD4 T cells at baseline (**Fig. 1C**). These results demonstrate distinct spatial patterns of tumor microenvironment and dynamic change with treatment in responders and non-responders.

Discussion

In this Phase 2 study to evaluate the efficacy of enhancing pembrolizumab monotherapy by engaging innate and adaptive immunity through SBRT and intratumoral ADV/HSV-tk gene therapy in patients with mTNBC who had relapsed on or were refractory to standard-of-care therapy, the treatment demonstrated an acceptable safety profile. The incidence of grade 3 or higher treatment-related AEs was 35.7% and immune-related AEs was 7.1%. The two grade 3 or higher immune-related AEs were managed with glucocorticoids and discontinuation of treatment. There were no treatment-related deaths. The safety profile of this trial was consistent with that seen in previous studies (5–7, 31).

ICB monotherapy in mTNBC yields low ORR of 7.6% to 10% in second line and beyond metastatic setting (5, 6), 2.6% to 5% in patients with low or negative PD-L1 (6, 7), and nearly no response in patients with liver metastases. Primary and acquired immune resistance involving antigen-presenting machinery and T-cell dysfunction play important roles in the low response rate to ICB in mTNBC (32). Both radiotherapy and ADV/HSV-tk plus ganciclovir gene therapy can induce tumor antigen release, increase T-cell infiltration and activation and PD-L1 expression (23, 24, 28); adding ICB to either modality can potentially overcome PD-L1 inhibitory signals and enhance antitumor response. Indeed, many animal studies of combining radiotherapy or ADV/HSV-tk plus ganciclovir gene therapy with ICB have shown enhanced antitumor effect (12, 23, 24). However, combining either SBRT or virus therapy with ICB only yielded moderate response in early-phase clinical trials. In TONIC trial that explored different immune induction strategies with chemotherapy agents or radiotherapy in mTNBC, the radiotherapy arm had an ORR of only 8% (33). In contrast with an animal study showing radiotherapy can induce T-cell infiltration and activation (25), biopsy after treatment did not show change of tumor-infiltrating lymphocytes or CD8 cells in TONIC trial. Even though two Phase 2 studies combining SBRT with ICB in treated mTNBC (34, 35) showed an ORR of 17.6% to 22%, the median duration of response was only 2.5 to 5 months and no response was observed in patients with liver metastases. Similarly, oncolytic virus type I herpes-simplex-virus Talimogene laherparepvec (TVEC, IMLYGIC) in combination with ICB in mTNBC yielded only one PR in a Phase 1 study (36).

Combining both SBRT and ADV/HSV-tk plus valacyclovir gene therapy with ICB may result in complementary mechanisms of DNA damage, alter and potentiate tumor-specific antigenicity, and

Table 4. Adverse events in all patients.

Events	Total population ($N = 28$)	
	Any grade	Grade 3 or 4
Treatment-related AEs	22 (78.6)	10 (35.7)
AEs leading to discontinuation	2 (7.1)	2 (7.1)
Treatment-related death	0 (0)	0 (0)
GI		
Mucositis	1 (3.6)	0 (0)
Nausea	3 (10.7)	0 (0)
Vomiting	0 (0)	0 (0)
Colitis	1 (3.6)	1 (3.6)
Diarrhea	4 (14.3)	0 (0)
Constipation	1 (3.6)	0 (0)
ALT elevation	2 (7.1)	0 (0)
AST elevation	2 (7.1)	1 (3.6)
ALP elevation	3 (10.7)	1 (3.6)
Abdominal pain	1 (3.6)	1 (3.6)
Decreased appetite	1 (3.6)	0 (0)
Hematologic toxicity		
Anemia	2 (7.1)	2 (7.1)
Leukocytosis	1 (3.6)	0 (0)
Pulmonary toxicity		
Pneumonitis	2 (7.1)	1 (3.6)
SOB	2 (7.1)	0 (0)
DOE	3 (10.7)	0 (0)
Cough	2 (7.1)	0 (0)
Endocrine		
Hypothyroidism	6 (21.4)	0 (0)
Hyperthyroidism	3 (10.7)	0 (0)
Infection		
Pneumonia	1 (3.6)	0 (0)
Shingles	1 (3.6)	1 (3.6)
Sepsis	1 (3.6)	1 (3.6)
Oral thrush	1 (3.6)	0 (0)
Skin	0 (0)	0 (0)
Constitutional	10 (35.7)	2 (7.1)
Immune-related AEs		
Hypothyroidism	6 (21.4)	0 (0)
Hyperthyroidism	3 (10.7)	0 (0)
ALT elevation	2 (7.1)	0 (0)
AST elevation	2 (7.1)	1 (3.6)
Pneumonitis	2 (7.1)	1 (3.6)

Abbreviations: AE, adverse event; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; DOE, dyspnea on exertion; GI, gastrointestinal; SOB, shortness of breath.

elicit a stronger immune response (22, 37). By combining three modalities, a CBR of 21.4% (2 CR, 1 PR, and 3 SD), a median DoT of 9.6 months and two long-term responders were observed. Although cross-trial comparisons could be confounded by different trial designs, patient populations, and other factors, trimodality combination strategy improved ORR and DoT numerically compared with SBRT and ICB combination strategy (34, 35). Response observed was not associated with the line of treatment delivered in metastatic setting or PD-L1% or CPS. Among patients who had received chemotherapy in metastatic setting, 5/20 (25%) responded to the combination treatment, and 2/18 (11.1%) patients with negative PD-L1 had clinical benefit. Out of the 6 responders, 3 had CPS ≥10 and 2 patients had CPS <10, consistent with the observation that CPS is not predictive of response beyond first-line metastatic setting (38). Taken together, these data suggest the enhanced antitumor effect of SBRT, ADV/HSV-tk plus valacyclovir gene therapy and ICB combination therapy in pretreated patients and in patients with low or negative PD-L1.

We observed some atypical patterns of response in our patients, in particular in patients with liver metastases. Of the 6 responders in our study, 2 patients had liver metastases (**Table 3**). Patient 2 had biopsy-proven *lung metastases* and *one liver lesion* on PET scan that decreased in size with treatment. She then achieved CR but discontinued pembrolizumab after 18 doses due to Grade 3 pneumonitis. She remains in *complete remission without any systemic treatment* for over 47 months. Patient 5 had biopsy-proven relapsed metastatic disease in the liver and had remained on treatment for over 22 months with stable disease. Visceral metastases, especially liver metastases, have been a poor prognostic factor in mTNBC (39, 40). Liver metastases have the lowest PD-L1 level among metastatic sites (41). Historically, ICB monotherapy, and ICB and SBRT combination therapy had very low response rates in those patients (5, 34, 36). In contrast, previous studies of ADV/HSV-tk plus valacyclovir gene therapy demonstrated significant regression of liver metastases in breast cancer and lung cancer mouse models (42, 43). In the Phase 1 study of combining oncolytic type I herpes simplex virus TVEC (IMLYGIC) with ICB in patients with mTNBC and liver metastases, 1 out of 7 patients had a PR (36). The durable responses in these 2 patients in our study further suggest the role of ADV/HSV-tk plus valacyclovir gene therapy in re-priming T cells and potential added synergistic effect of ADV/HSV-tk plus valacyclovir gene therapy and SBRT in enhancing ICB in these with liver metastases, an observation that warrants further investigation.

In non-responders, immune profile analysis revealed high baseline T cells; however, there was no increase of these T cells after treatment suggesting that these T cells may be tolerant to the tumor and are not proliferating. In contrast, in responders, the increase of T cells suggests that these T cells are newly proliferating in response to the treatment and can antigen-specifically target tumor cells. In addition, neighborhood analysis suggested that these immune cells were actively engaged with cancer cells for tumor killing. Myeloid cells in the tumor can exhibit suppressive activity on T cells. In responders, CD8 T cells were distant from $CD15^+$ neutrophils after treatment, indicating that the immune populations were freed from immunosuppressive neutrophils. In contrast, myeloid cells increased in non-responders after treatment. These findings further support that myeloid cells play an important role in immunosuppression and treatment response. Six non-responders exhibited immune profiles similar to that of responders with increased Tem CD8 cells and $PD\text{-}L1^+$ CD8 T cells on D17. Among these 6 patients, 3 had increased $PD\text{-}L1^+$ monocytes at D17 and 2 exhibited delayed increase of myeloid cells on D38. Moderate upregulation of Tregs and exhausted CD4 T cells were also seen in one of these 6 non-responders. These observations are consistent with previously reported mechanisms of ICB resistance (44–46).

There are several limitations to this study: (i) As a single-arm study, patients were not randomized and the exact contribution of single modality to the enhanced antitumor effect is unknown. Given that oncolytic virus alone (47) or in combination with ICB (36) had a low response rate of 0%–12.5%, and ICB and SBRT combination only improved response with short duration of response in pre-treated patients without liver metastases (34), the results from our study suggest that all three treatment modalities contributed to the observed enhanced antitumor effect. However, larger randomized studies are needed to elucidate the exact role of each modality and their additive effects on tumor microenvironment response. (ii) Initial trial design defined PD-L1 expression by IHC using the Ventana SP142 antibody.

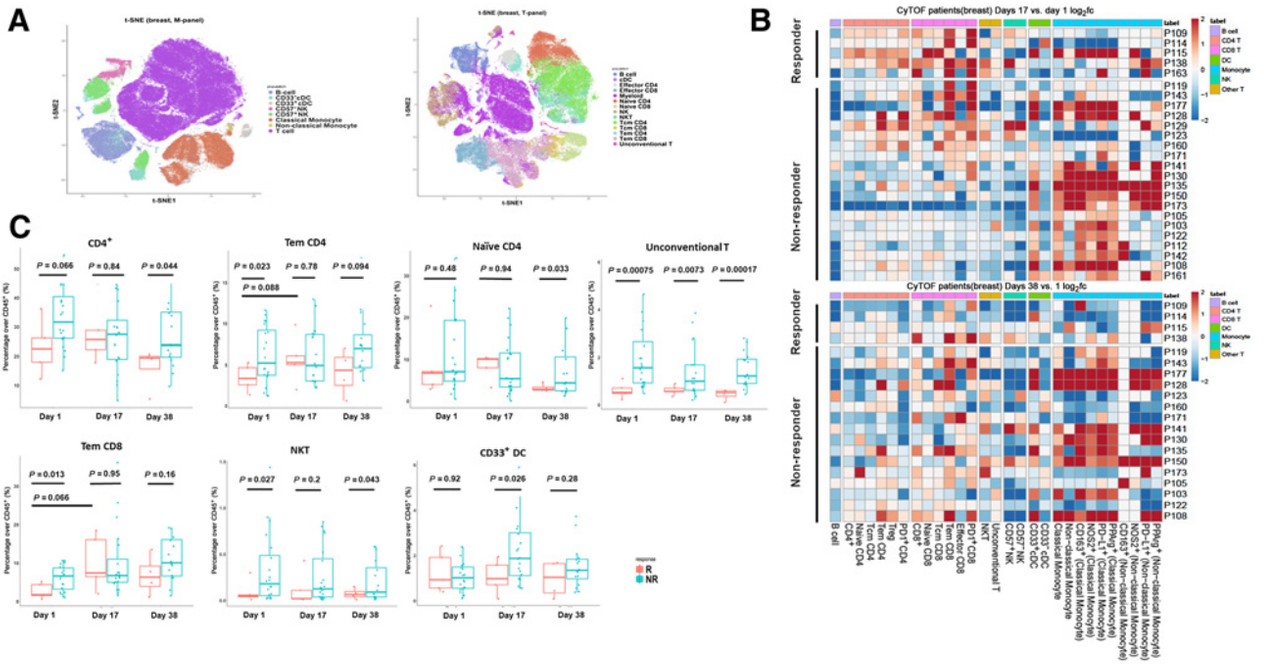

Figure 2.

CyTOF analysis of paired peripheral blood mononuclear cells on days 1, 17, and 38. **A,** tSNE plots showing cell population clusters. **B,** Heatmap of log_2 fold change between days 17 and 1 of responders and non-responders (top) and log_2 fold change between days 38 and 1 of responders and non-responders (bottom). **C,** Comparison of different cell populations between responders (red box) and non-responders (green box) at days 1, 17, and 38. Tem, effector memory T-cell. The *P* value was calculated by unpaired *t* test with Welch's correction for responder and non-responder comparison, and paired *t* test for fold change comparison at different days in responders.

CPS score as assessed by the PD-L1 IHC 22C3 PharmDx kit was later added as another measurement after FDA approval of pembrolizumab in combination with chemotherapy in untreated mTNBC based on CPS. This led to the unavailability of CPS in almost one third of patients. (iii) Although the correlative biomarker analyses suggest that an increase of CD8 T cells and myeloid cells may serve as useful predictive markers for responders and non-responders, the results need to be interpreted with caution in view of the small sample size of this study. Larger studies are needed to validate these initial findings.

Conclusion

Even though the study did not meet its statistical significance, the durable responses in heavily pretreated patients with mTNBC suggest that the efficacy of ICB monotherapy may be enhanced by harnessing the abscopal effect of SBRT and intratumoral ADV/HSV-tk plus valacyclovir gene therapy, even in those with low PD-L1 level and liver metastases. Early detection of increased effector and effector memory CD8 T cells and myeloid cells correlates with response and non-response, respectively.

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Authors' Contributions

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Note

Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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