A Pilot Study of the PD-1 Targeting Agent AMP-224 Used With Low-Dose Cyclophosphamide and Stereotactic Body Radiation Therapy in Patients With Metastatic Colorectal Cancer

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Abstract

This study aimed to investigate the benefit of combining immunotherapy (AMP-224) with radiation for patients with metastatic colorectal cancer. Fifteen patients were enrolled. No objective response was observed although 3 patients (20%) had stable disease. A signal of immune modulation was noted.

Background: The prognosis of metastatic colorectal cancer (mCRC) is poor. We assessed the feasibility, safety, and efficacy of the anti-programmed cell death 1 fusion protein AMP-224 in combination with low-dose cyclophosphamide and stereotactic body radiation (SBRT) treatment in patients with mCRC refractory to standard chemotherapy. **Patients and Methods:** Fifteen patients were enrolled. Six received SBRT 8 Gy on day 0 (dose level 1), whereas 9 received 8 Gy on days −2 to day 0. All received cyclophosphamide 200 mg/m^2 intravenously (I.V.) on day 0. On day 1, both groups received AMP-224 10 mg/kg I.V., repeated every 2 weeks for a total of 6 doses. Primary end points were feasibility and safety. **Results:** Ten (67%) patients completed 6 doses of AMP-224; 5 patients (33%) discontinued treatment because of disease progression. No dose-limiting toxicity was observed; 9 patients (60%) experienced treatment-related adverse events, all Grade 1 or 2. No objective response was noted; 3 patients (20%) had stable disease. Median progression-free survival and overall survival were 2.8 months (95% confidence interval [CI], 1.2-2.8 months) and 6.0 months (95% CI, 2.8-9.6 months), respectively. M2 macrophage polarization was present in the pretreatment tumor biopsy samples, but not post-treatment samples. **Conclusion:** AMP-224 in combination with SBRT and low-dose cyclophosphamide was well tolerated, however, no significant clinical benefit was observed in patients with mCRC.

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Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related death in the United States, with approximately 150,000 new cases expected to be diagnosed in 2019.[1] Up to 25% of CRC patients have overt metastases at diagnosis, and 25% to 35% of early stage patients will develop metastases in the course of their disease.[2] Chemotherapy remains the standard treatment modality offered to patients with metastatic CRC (mCRC). Using a fluoropyrimidine backbone with folinic acid with either oxaliplatin or irinotecan is a standard first-line approach. Epidermal growth factor receptor (EGFR) inhibitors (cetuximab or panitumumab) for wild type Kirsten rat sarcoma virus (*KRAS*) or an antiangiogenetic agent such as the vascular endothelial growth factor inhibitor bevacizumab are commonly incorporated and have been shown to improve efficacy and survival.[3] At progression, the combination of a fluoropyrimidine and folinic acid with ziv-aflibercept or ramucirumab are often used.[4-6] For patients who progress beyond first- and second-line treatments, regorafenib and TAS-102 (trifluridine/tipiracil) have also been approved by Food and Drug Administration (FDA), resulting in median overall survival (OS) of 6.4 months and 5.3 months, respectively.[7,8]

The progress achieved with immune checkpoint inhibition using anticytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), anti-programmed death 1 (PD-1), and anti-programmed death ligand 1 (PD-L1) immune checkpoint inhibitors in melanoma and other solid tumors[9-11] generated interest in immunotherapy for CRC, especially in the metastatic setting. The relevance of the immunotherapeutic approach was augmented by findings correlating improved survival in CRC with increased tumor-infiltrating CD8-positive ($CD8^+$) T cells.[12] Of particular interest were strategies to enhance the antitumor immune response. These strategies included combination with radiation therapy to increase the immunogenicity of the tumor.[13,14] Recently, the PD-1 inhibitors pembrolizumab and nivolumab were approved by the FDA with or without anti-CTLA-4 for tumors with high microsatellite instability (MSI-H), with objective response rates of 40% to 69% in this population.[15-17] There were no objective responses in the microsatellite-stable population.[15]

AMP-224 is an anti-PD-1 recombinant fusion protein composed of the extracellular domain of the human programmed cell death 1 ligand 2 (PD-L2) fused to the Fc domain of human immunoglobulin G1, which binds to PD-1 on the cell surface of T cells. Contrary to anti—PD-1 monoclonal antibodies, AMP-224 has a nonblocking mechanism of action, because it binds to PD-1 high ($PD\text{-}1^{HI}$) $PD\text{-}L1^-$ T cells (chronically stimulated/exhausted T cells), but not to $PD\text{-}1^+$ $PD\text{-}L1^+$ T cells (normally activated T cells). In preclinical models in mice, AMP-224 was active as a single agent. When combined with low-dose cyclophosphamide, used to decrease the inhibitor effect of T regulatory cells, an increased activity of AMP-224 was seen.[18,19] In a subsequent phase I trial that used the combination AMP-224 and low-dose cyclophosphamide, a reduction in $PD\text{-}1^{HI}$ cells and an emergence of a functional T-cell response was seen in patient responders.[20] In addition, an increase in peripheral antitumor immunity after radiation treatment has been documented. The underlying mechanism is thought to be related to radiation-induced tumor necrosis causing an increase in tumor antigens available for cross presentation.[14] Stereotactic body radiation therapy has been shown to be safe and effective in patients with unresectable liver metastasis.[21] Various dosing and schedules have been tested. However, an ideal effective dose has not been established.[22-25] In this trial, 2 doses of radiation were administered, either 8 Gy for 1 dose on day 0 (dose level 1 [DL1]) or 8 Gy for 3 doses on day −2 to day 0 (dose level 2 [DL2]). This schedule was on the basis of the safety and tolerability of both arms shown in a previous report.[26] Although radiation alone seems inefficient to produce a systemic immune effect, recent evidence suggests the combination of radiation and checkpoint blockade can lead to improved antitumor activity.[27-30] On the basis of these findings, it was hypothesized that the combination of AMP-224, low-dose cyclophosphamide, and SBRT in mCRC would generate potential benefit by modulating antitumor immunity.

We present the results of a single-center pilot trial in which we aimed to evaluate the feasibility, safety, and efficacy of the anti—PD-1 fusion protein AMP-224 in combination with low-dose cyclophosphamide and SBRT in patients with mCRC refractory to standard chemotherapy.

Patients and Methods

Study Design and Patients

This was a single-center pilot study conducted at the National Cancer Institute (NCI) Clinical Center in the United States. Eligible patients were at least 18 years old with a histologically confirmed diagnosis of mCRC. Patients must have had documented radiographic progression or intolerance to previous irinotecan and oxaliplatin-containing chemotherapeutic regimens; those with *KRAS* wild type tumors must have also progressed or were intolerant to anti-EGFR treatment. Patients who were enrolled were not amenable to potentially curable resection. Patients must also have had 1 focus of metastatic disease in the liver that was amenable to SBRT as well as at least 1 measurable lesion outside the radiation field as defined according to the Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST); Eastern Cooperative Oncology Group performance status score of 0 to 1; a predicted life expectancy of >3 months, and adequate organ function. The study was approved by the NCI institutional review board. All patients provided written informed consent before enrollment. All patients were discussed at a multidisciplinary tumor board, which included evaluation by a radiation oncologist to determine the site of radiation. Microsatellite instability (MSI) status was not required, because the trial was designed and initiated before knowledge associating MSI-H with response to anti—PD-1 immune checkpoint blockade was published and known. The trial was performed in accordance with the Declaration of Helsinki.

Treatment

Six patients were enrolled at DL1 and received SBRT 8 Gy and cyclophosphamide 200 mg/m^2 of body surface area (BSA) intravenously (I.V.) on day 0, followed by AMP-224 10 mg/kg I.V. on day 1 and then every 14 days for a total of 6 doses. The next 9 patients received SBRT 8 Gy on days −2 to 0 and cyclophosphamide 200 mg/m^2 of BSA I.V. on day 0 (DL2), followed by AMP-224 10 mg/kg I.V. on day 1 and then every 14 days for a total of 6 doses. For both dose levels, an option to continue treatment with AMP-224 was considered in responding patients until disease progression. The lesion subjected to irradiation was chosen by the consulting radiation oncologist. Imaging studies were done using contrast-enhanced

computed tomography (CT) or magnetic resonance imaging (when CT was contraindicated) at baseline, 2 weeks after completion of treatment; and every 8 weeks thereafter. Tumor biopsies from the liver lesions were performed optionally before the beginning of the study treatment (baseline) and at day 29 (post-treatment). Patients were assessed for toxicity before each treatment cycle.

End Points and Assessments

The primary objective was to determine the feasibility and safety of AMP-224 in combination with SBRT and chemotherapy in patients with advanced, unresectable CRC who had disease progression during or after oxaliplatin and irinotecan-containing chemotherapy. Secondary objectives included evaluation of the response rate (assessed using RECIST guidelines[31] by the investigator, in lesions not subjected to SBRT), progression-free survival (PFS; defined as time from first day of treatment to first documented disease progression or death), and OS (defined as time from the first day of treatment to death from any cause) after the trial treatment. The population evaluable for response was defined as all patients who had received at least 1 dose of therapy and had at least 1 postbaseline tumor response assessment.

Safety and toxicity were monitored and managed accordingly. The safety population was defined as all patients who received at least 1 dose of AMP-224. The assessment period for dose-limiting toxicities (DLTs) was the first 4 weeks of the study. All adverse events (AEs) that occurred within 30 days of the last dose of treatment were reported according to the NCI Common Terminology Criteria for Adverse Events version 4.0.[32]

RNA Sequencing

Fresh-frozen paraffin-embedded tumor samples from the core biopsies were processed for RNA isolation. Libraries for RNA sequencing were prepared using Illumina TruSeq Stranded Total RNA Library Prep and were pooled and sequenced on Illumina HiSeq3000/4000 using 150-base pair paired-end protocol following the manufacturer's protocol. The obtained short sequence reads were aligned to the human hg38 genome using STAR[33] and processed using RSEM[34] to compute raw and normalized counts per gene and in all samples.

Bioinformatics

RNA sequencing data were analyzed for differential gene expression with DESeq2.[35] Differentially expressed genes with unadjusted P value $< .05$ were used for Gene Set Enrichment Analysis (GSEA; available at: http://software.broadinstitute.org/gsea/msigdb/index.jsp), compared against the canonical pathways gene sets, the Kyoto Encyclopedia of Genes and Genomes gene sets, the Reactome Pathway Knowledgebase gene sets, the oncogenic signatures gene sets, and the immune signatures gene sets.[36] To study the changes in the $CD8^+$ tumor infiltrating lymphocytes after treatment, we used the RNA expression data of the tumor samples from pre- and post-treatment biopsies, and used the CIBERSORT algorithm (available at: https://cibersort.stanford.edu) to enumerate the immune cells.[37]

Trial Design and Statistical Analysis

The primary objectives of the protocol were to determine if it is feasible to safely administer 6 doses of AMP-224 in combination with radiation to patients with mCRC and to explore effects of treatment on immunologic parameters. All patients enrolled in the trial were considered as 1 group. For the feasibility outcome, a goal of 11 of 15 evaluable patients able to safely receive 6 doses of AMP-224 was determined to be consistent with an 80% goal of administration and consequently considered a successful outcome for the trial. We estimated 95% confidence intervals (CIs) for response rates using the Wilson method.[38] OS and PFS beginning at cycle 1, day 1 of treatment were estimated using the Kaplan–Meier method,[39] and presented along with medians and 95% CIs for PFS and OS. Data were analyzed with R[40] using the Rstudio environment[41]; the Hmisc package[42] was used for CIs. SAS version 9.4 (SAS Institute, Cary, NC) was used to perform the survival analyses.

Results

Population

Between November 2014 and August 2016, 17 patients were assessed for eligibility and 15 patients were included. Six patients were assigned to the DL1 arm and 9 patients to the DL2 arm (Figure 1). All 15 patients were treated with at least 1 dose of AMP-224 and were included in the analysis. The baseline characteristics of the participants are listed in Table 1. Of the 15 patients, 93% had metastatic cancer at diagnosis, and 80% had undergone a metastasectomy. The median number of previous chemotherapy lines was 3 (range, 2-5). Four (27%) patients had microsatellite stable (MSS) tumors tested using immunohistochemistry, which was not available for the remaining 11 patients. *KRAS* status was available in 12 (80%) of patients, of whom 8 (53%) had *KRAS*-mutated tumors.

Feasibility and Safety

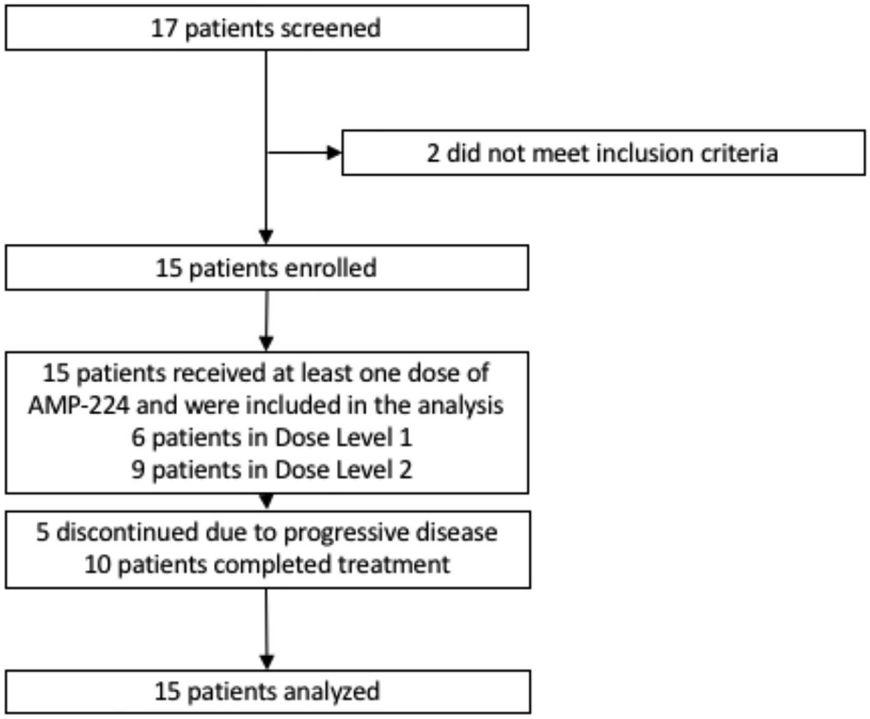
As of data cutoff on March 7, 2018, no participant was still receiving the treatment. Ten (67%) patients received all 6 doses of AMP-224, and 1 patient (7%) continued treatment with AMP-224 for an additional 4 cycles before disease progression. Treatment was discontinued before completion of the planned 6 doses of AMP-224 in 5 (33%) patients because of progressive disease. There was no treatment discontinuation because of AEs. No DLT was encountered.

The safety population consisted of 15 patients. All patients experienced at least 1 AE, with 9 (60%) experiencing treatment-related AEs, all of which were of Grade 1 or 2. Treatment-related toxicities are summarized in Table 2, which excludes toxicities directly attributable to the SBRT or the biopsy (eg, pain) according to standard of care experience. Infusion reactions were the most common treatment-related AEs, occurring in 6 (40%) patients, which were managed accordingly.

Efficacy

No complete or partial response was recorded. Stable disease was recorded in 3 (20%) of 15 participants (95% CI, 7-45; Table 3), all in the DL2 group, with the longest lasting 12.7 months (range, 4.3-12.7 months). The disease control rate was 20%. Five (33%) of 15 patients had progression of disease before completion of the trial treatment, including 3 who developed new brain metastases. Median PFS and OS were 2.8 months (Figure 2A; 95% CI, 1.2-2.8 months) and 6.0 months

Figure 1 Consolidated Standards of Reporting Trials Diagram



(Figure 2B; 95% CI, 2.8-9.6 months), respectively. No subgroup analyses were performed.

Enumeration of Immune Cells

Ten paired pre- and post-treatment tumor biopsies were available from 5 patients. After quality control analysis, 1 sample had a low count of mapped reads and was excluded with its pair, leaving 8 paired samples for further analysis, derived from 1 patient with stable disease as the best response and 3 patients with progressive disease as the best response. Differential gene expression analysis results are presented in Supplemental Figure 1 in the online version. Immune infiltrate CIBERSORT (https://cibersort.stanford.edu) analysis showed P values $< .05$ for 3 of 4 pretreatment samples; in 2 of these 3 samples, there was a predominance of M2 macrophages and comparatively little T-cell response (see Supplemental Figure 2 in the online version). All post-treatment samples had P values $> .05$, which are summarized in Figure 3 and are reported descriptively and considered exploratory.

Pathway Analysis

Of the 1043 genes differentially expressed (see Supplemental Table 1 in the online version), genes with P values $< .05$ that were submitted to the Webserver, 893 genes were included in the GSEA. Of the 50 top overlapping gene sets (see Supplemental Table 2 in the online version), there was a predominance of the immune signature gene sets, with almost all of the 50 overlapping gene sets from that collection.

Discussion

In this single-center pilot study, we explored the feasibility, safety, and efficacy of AMP-224 treatment in combination with low-dose cyclophosphamide and SBRT in patients with mCRC in whom standard treatments had failed.

In our study, the treatment was well tolerated. All patients experienced at least 1 AE, and 9 (60%) experienced treatment-related AEs, all of which were Grade 1 or 2. No toxicity-related deaths and Grade 3 or 4 AEs were observed. The overall toxicity profile for AMP-224 used in combination with low-dose cyclophosphamide and SBRT in our study was mild to moderate. The results of our study failed to meet the feasibility cutoff of 11 patients completing treatment, which formed part of the primary end point of the study: 10 of 15 patients received all 6 AMP-224 infusions. Disease progression was responsible for treatment noncompletion in the remaining patients. There was no difference in response or toxicity seen between the 2 arms. Because of the small patient population, it is difficult to determine if the radiation dosing schedule affected the changes in immune response seen in the tumor samples.

No objective response was observed, although disease stabilization was documented in 3 patients (3/15, 20%). In this heavily pretreated population of patients, the median PFS (mPFS) and OS were 2.8 months and 6.0 months, respectively. These results are comparable with previous placebo-controlled studies of third-line treatment in mCRC. Specifically, regorafenib resulted in a mPFS of 2.0 months in the CORRECT trial[7] and 3.2 months in the CONCUR trial,[43] whereas the TAS-102 resulted in a mPFS of 2.0

Table 1 Baseline Characteristics of Participants (n = 15)

Characteristic	Value
Sex	
Female	6 (40)
Male	9 (60)
Age, Years	66 (32-78)
Race A/B/W	1 (7)/3 (20)/11 (73)
ECOG PS	
0	6 (40)
1	9 (60)
Microsatellite Status	
MSS	4 (27)
NA	11 (73)
KRAS	
WT	4 (27)
MT	8 (53)
NA	3 (20)
M1 at Diagnosis	14 (93)
Years With M1	3 (1-5)
Metastasectomy	12 (80)
Number of Previous Regimens	
2	6 (40)
3	2 (13)
≥4	6 (40)

Data are presented as n (%) or median (range).
Abbreviations: A = Asian; B = black; ECOG PS = Eastern Cooperative Oncology Group performance status; M1 = metastasis; MSS = microsatellite stable; MT = mutated; W = white; WT = wild type.

months.[8] The placebo arm in all 3 studies was uniformly reported as having a median PFS of 1.7 months. In terms of OS, for the active treatment arms the median OS was 6.4 months in the CORRECT trial, 8.8 months in the CONCUR trial, and 7.1 months in the TAS-102 trial.[7,8,43] The validity of such a comparison, however, is limited by the differences in the characteristics of the treated patients, such as the number of previous regimens received by the patients. Meanwhile, AMP-224 targets to PD-L2 and executes inhibition of PD-1/PD-L2 binding. It is not clear if the blockade of PD-1/PD-L2 by anti−PD-L2 is not striking enough to inhibit the PD-1 signaling pathway to reflect clinical benefit compared with anti−PD-L1. However, there is evidence that indicates the molecular mechanism difference of PD-1 interaction with PD-L1 and PD-L2.[44]

Table 2 Treatment-Related Adverse Events

Adverse Event	Grade 1	Grade 2	Grade 3	Grade 4
Dizziness	1	0	0	0
Hypokalemia	1	0	0	0
Hypothyroidism	1	0	0	0
Infusion-Related Reaction	2	6	0	0
Lymphocyte Count Increased	0	1	0	0
Nausea	1	0	0	0
Pain	1	0	0	0
Rash, Papulopustular	1	0	0	0
Pruritus	2	0	0	0
Vomiting	1	0	0	0
White Blood Cell Count Decreased	1	0	0	0

Patients are counted once for each applicable specific adverse event and could have more than 1 treatment-related event.

Table 3 Tumor Response (n = 15)

Response	n (%; 95% CI[a])
PD	13 (87)
CR	-
PR	-
SD	3 (20; 7-45)
Disease Control Rate	3/15 (20)

[a]Wilson binomial confidence intervals.

After initiating this trial, the association of MSI-H status with objective response to the anti−PD-1 immune checkpoint inhibitors nivolumab and pembrolizumab in CRC was published.[15,17] In our study, information on the microsatellite instability status of the patients was available in only 4 (27%) participants, and all 4 were MSS (1 of these patients had disease stabilization). It is possible that there might be improved efficacy of the treatment if administered to MSI-H patients, similar to treatment with nivolumab and pembrolizumab, however, we have no data from this study to support such a hypothesis.

Results of the immune infiltrate cell enumeration via CIBERSORT (https://cibersort.stanford.edu) deconvolution analysis of bulk RNA sequencing data revealed a trend toward M2 tumor-associated macrophage (TAM) polarization in the pretreatment biopsy tumor samples, which was not evident in the post-treatment samples. Rather, in the post-treatment samples there was a trend toward M0 (uncommitted) macrophage predominance.

This reversal in TAM polarization might be the result of M2 TAM reprogramming,[45] in turn effected by the direct action of AMP-224 on the TAMs. PD-1 is expressed in high levels by TAMs, especially the M2 population,[46] and PD-L2 competes with PD-L1 for PD-1 binding[44] but has a higher affinity for PD-1 than PD-L1.[47] Although the endogenous expression of PD-L2 is more restricted than PD-L1,[48] the presence of AMP-224 with its PD-L2 moiety would tip that balance and result in the predominance of PD-1/PD-L1 binding on TAMs. Another mechanism of immune regulation of the microenvironment might involve additional TAM-related pathways. PD-L2, but not PD-L1, is also a ligand for repulsive guidance molecule b, a bone morphogenetic protein coreceptor[49] shown to be highly expressed in macrophages.[50] In the lung, this interaction has been shown to be required for respiratory tolerance by modifying local T-cell responses.[49]

It is known that M2 TAMs are tumor-promoting[37] and their presence in higher numbers in CRC tumor samples has been associated with a poor prognosis.[43] The cytokines produced by M2 TAMs, such as interferon-γ and transforming growth factor (TGF)-β, result in the upregulation of PD-L1 and tumor escape. Consequently, TAM-associated molecules such as the TGF-β inhibitor galunisertib, or INCAGN01876 (a glucocorticoid-

Figure 2 Kaplan–Meier Estimates of (A) Progression-Free Survival (PFS) and (B) Overall Survival (OS)

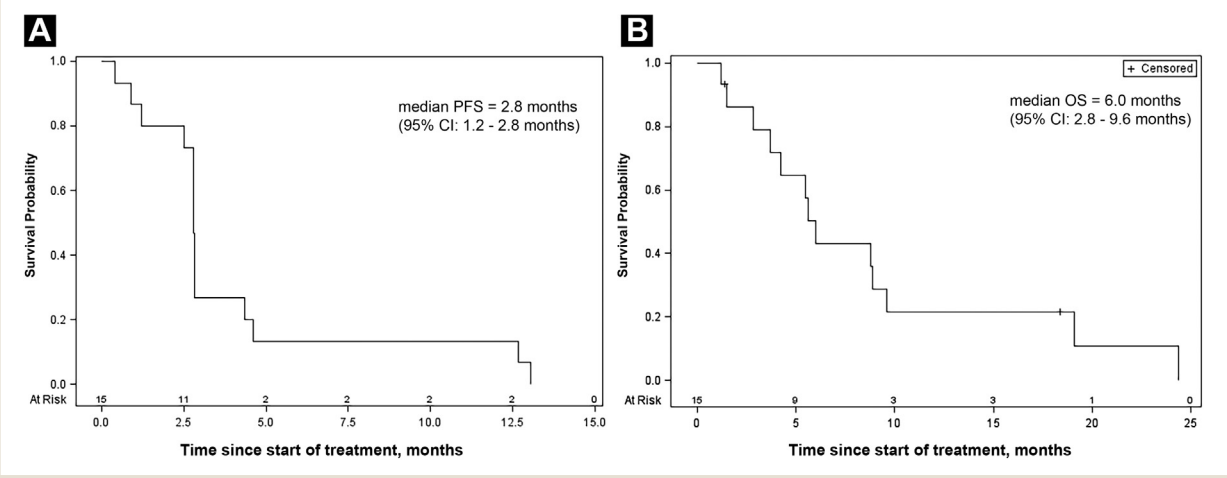
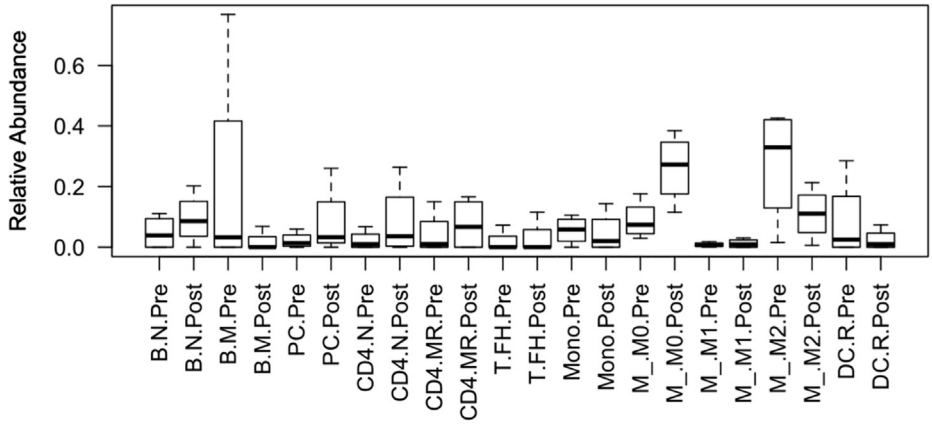


Figure 3 Results of the CIBERSORT (https://cibersort.stanford.edu) Immune Cell Population Comparisons. (A) Table of the Relative Abundancies of Immune Infiltrate Populations Across All Samples, for the 2 Most Abundant Population Categories (Shown in Bold) for Each Sample. (B) Box Plots of the Relative Abundances of Immune Infiltrate Populations in Pre and Post

A

Sample	B-N	B-M	PC	CD4-N	CD4-MR	T-FH	Mono	Mφ-M0	Mφ-M1	Mφ-M2	DC-R
Pt1-Pre	0.077	0.000	0.059	0.000	**0.150**	0.000	0.078	0.029	0.008	**0.415**	0.000
Pt2-Post	**0.202**	0.000	0.000	0.065	0.166	0.000	0.000	**0.308**	0.000	0.090	0.000
Pt1-Pre	0.000	0.065	0.007	0.068	0.019	0.000	**0.105**	0.088	0.001	**0.425**	0.049
Pt2-Post	0.000	0.069	**0.260**	**0.264**	0.000	0.000	0.040	0.115	0.018	0.130	0.000
Pt3-Pre	0.111	0.000	0.020	0.019	0.000	0.000	0.038	0.176	0.018	**0.243**	**0.285**
Pt3-Post	0.099	0.000	0.027	0.006	0.000	0.115	0.000	**0.384**	0.000	**0.212**	0.019
Pt4-Pre	0.000	**0.768**	0.000	0.000	0.000	**0.072**	0.000	0.059	0.005	0.015	0.000
Pt4-Post	0.072	0.000	0.039	0.000	0.133	0.000	**0.143**	**0.236**	0.030	0.006	0.073



Abbreviations: B-M = B cells, memory; B-N = B cells, naive; CD4-MR = CD4 T cells, memory, resting; CD4-N = CD4 T cells, naive; DC-R = dendritic cells, resting; Mono = monocytes; Mφ-M0 = macrophages, M0; Mφ-M1 = macrophages, M1; Mφ-M2 = macrophages, M2; PC = plasma cells; Post = post-treatment biopsy sample; Pre = pretreatment biopsy sample; T-FH = T cells, follicular helper.

induced tumor necrosis factor-related protein (GITR) inhibitor; a member of the tumor necrosis factor receptor superfamily), are being pursued as targets in combination therapies with immune checkpoint blockade.[51] Another mechanism by which M2 TAMs have been implicated in resistance to anti−PD-1 monoclonal antibody therapies is by macrophage-mediated anti−PD-1 antibody removal from the PD-1^{+} CD8 T cells.[52] It is unknown whether the latter mechanism is also active in the case of AMP-224, which acts like a high-affinity decoy ligand for PD-1. It might also be argued that the reversal of protumor macrophage polarity in the tumor microenvironment might be positioning AMP-224 as an interesting agent suitable for exploration in combination with immune blockade by anti-PD-1/PD-L1 monoclonal antibodies.

Furthermore, the pathway analysis of the pre- and post-treatment differential gene expression testing results using GSEA revealed overrepresentation of overlap with gene sets from the immunologic signatures gene set collection. Indicative overlapping gene sets include 4 sets specifically involving T regulatory lymphocytes, as well as gene sets that are upregulated in CD4 thymocytes versus thymic stromal cells, the set of upregulated genes after interleukin-4 treatment of macrophages and monocytes, genes downregulated in a comparison of untreated CD8 lymphocytes versus CD8 lymphocytes treated with leukocyte costimulatory blockade antibodies, and genes downregulated in mature natural killer (NK) cells versus intermediate NK cells. These findings highlight that a significant part of the changes that happen in the tumor microenvironment after the trial treatment are immune-related. Our results suggest that the combination of AMP-224 with a low dose of cyclophosphamide and SBRT at the dose and schedule administered in this trial exhibits some effect on antitumor immunity modulation. However, the magnitude of this immunomodulatory effect does not result in clinical benefit.

Conclusion

The results of this trial showed that the combination of the PD-1 antagonist AMP-224 with low-dose cyclophosphamide and SBRT in patients with mCRC is safe but did not provide evidence that is feasible or that it possesses antitumor efficacy. Further exploration of alternative combinations of AMP-224 is warranted.

Clinical Practice Points

- In this study we investigated the benefit of using AMP-224 immunotherapy with radiation in 15 patients with mCRC.
- No objective response was observed although 20% of the patients had stable disease.
- AMP-224 in combination with SBRT and low-dose cyclophosphamide was well tolerated, however, no significant clinical benefit was observed in patients with mCRC.

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Disclosure

The authors have stated that they have no conflicts of interest.

Supplemental Data

Supplemental figures and tables accompanying this article can be found in the online version at https://doi.org/10.1016/j.clcc.2019.06.004.

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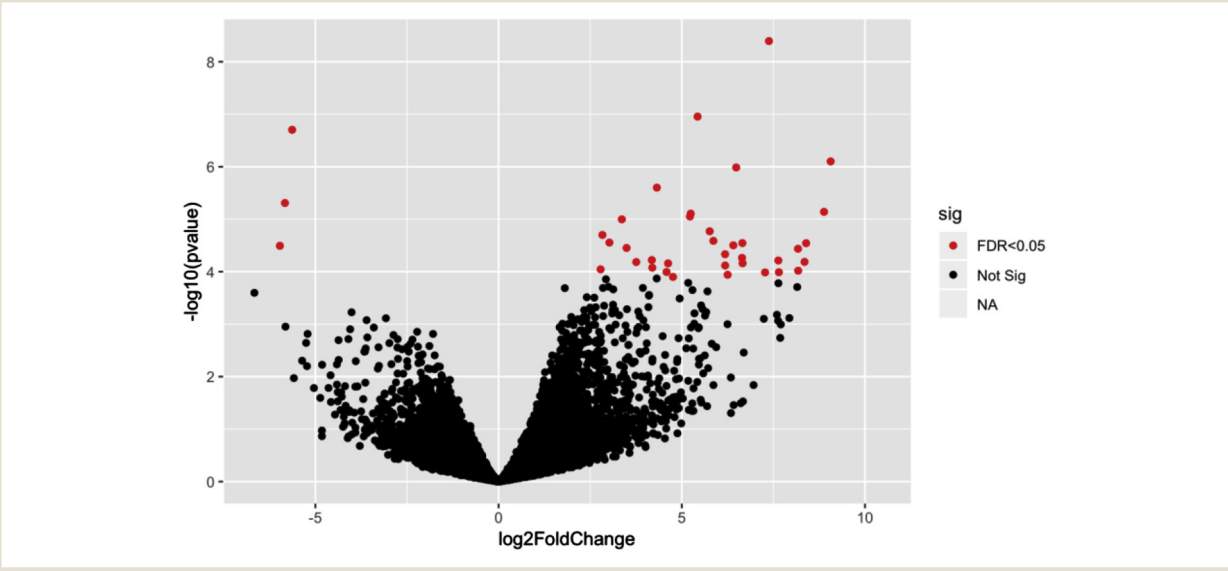
Supplemental Figure 1 Volcano Plot of the RNA Sequencing Differential Gene Expression Results



Abbreviations: FDR = false discovery rate; sig = significant.

Supplemental Figure 2 CIBERSORT (https://cibersort.stanford.edu) Results

Input Sample	B cells naive	B cells memory	Plasma cells	T cells CD8	T cells CD4 naive	T cells CD4 memory resting	T cells CD4 memory activated	T cells follicular helper	T cells regulatory (Tregs)	T cells gamma delta	NK cells resting	NK cells activated	Monocytes	Macrophages M0	Macrophages M1	Macrophages M2	Dendritic cells resting	Dendritic cells activated	Mast cells resting	Mast cells activated	Eosinophils	Neutrophils	P-value	Pearson Correlation	RMSE
590PRE	0.077	0	0.059	0	0	0.15	0.041	0	0	0	0.085	0	0.078	0.029	0.008	0.415	0	0	0.017	0	0	0.039	0.040	0.191	1.008
590POST	0.202	0	0	0	0.065	0.166	0.001	0	0	0	0.041	0	0	0.308	0	0.09	0	0.035	0	0.045	0	0.046	0.380	0.019	1.097
074PRE	0	0.065	0.007	0	0.068	0.019	0.011	0	0	0	0.021	0	0.105	0.088	0.001	0.425	0.049	0	0	0.055	0	0.086	0.010	0.357	0.942
074POST	0	0.069	0.26	0.041	0.264	0	0	0	0	0	0.035	0.008	0.04	0.115	0.018	0.13	0	0	0	0.021	0	0	1.000	-0.043	1.128
2B2PRE	0.111	0	0.02	0	0.019	0	0	0	0	0.014	0	0	0.038	0.176	0.018	0.243	0.285	0	0.037	0	0	0.039	0.070	0.142	1.046
2B2POST	0.099	0	0.027	0	0.006	0	0	0.115	0	0.026	0	0.001	0	0.384	0	0.212	0.019	0	0	0.063	0.037	0.009	0.070	0.161	1.051
361PRE	0	0.768	0	0.003	0	0	0	0.072	0	0	0.066	0	0	0.059	0.005	0.015	0	0	0	0.011	0	0	0.000	0.650	0.764
361POST	0.072	0	0.039	0.011	0	0.133	0	0	0.055	0	0.08	0.06	0.143	0.236	0.03	0.006	0.073	0	0	0.053	0.01	0	0.080	0.128	1.035

Abbreviations: NK = natural killer; RMSE = root mean squared error.

Supplemental Table 1 RNA Sequencing Results

Gene	Log2 Fold Change	*P*	*P* Adjusted
INO80B-WBP1	9.06234174	7.93E-07	.00295195
CDK3	8.88100423	7.24E-06	.01294001
CARNS1	8.39296443	2.86E-05	.02507583
GRIP2	8.34991882	6.50E-05	.03567611
FSCN2	8.17569096	9.58E-05	.04269671
HNF1A-AS1	8.17100392	3.66E-05	.02595469
SH3GL1P2	7.64977783	.00010229	.04269671
RTL1	7.63465938	6.12E-05	.03567611
STAG3L5P-PVRIG2P-PILRB	7.37917997	4.04E-09	6.01E-05
NEURL1	7.27272034	.00010322	.04269671
CCDC138	6.65882532	6.92E-05	.03567611
RHBDL1	6.65292606	2.85E-05	.02507583
CD99P1	6.64345278	5.46E-05	.03534586
ZC2HC1A	6.48440297	1.03E-06	.00307605
DNAH2	6.40606545	3.14E-05	.02518528
ANK1	6.25053307	.00011449	.04608078
KRT8P39	6.18302159	7.62E-05	.03783272
LRRC56	6.18112879	4.65E-05	.03147028
TTLL10	5.86232375	2.58E-05	.02507583
SLC9A3-AS1	5.76108041	1.70E-05	.02106863
GABRE	5.43097168	1.11E-07	.00082529
KLHL17	5.24363239	7.82E-06	.01294001
DDX12P	5.22311021	8.88E-06	.01322373
TNFRSF25	4.7592585	.00012596	.04936131
CROCCP3	4.62539191	6.95E-05	.03567611
PDZD2	4.58416307	.00010116	.04269671
SOX5	4.31975175	2.50E-06	.0062122
PIGL	4.19506498	8.40E-05	.04034052
MSH5	4.18110435	6.01E-05	.03567611
ANO9	3.75652262	6.54E-05	.03567611
GABPB1-AS1	3.49258117	3.54E-05	.02595469
PLXNA3	3.36371548	1.01E-05	.01361571
RNU1-3	3.02421579	2.79E-05	.02507583
RHPN1	2.83394696	1.99E-05	.02283778
CYP3A5	2.78486271	9.02E-05	.04198792
PLD4	−5.6333142	1.98E-07	.00098289
PPP1R16B	−5.8305017	4.91E-06	.01044382
SLAMF7	−5.9684224	3.21E-05	.02518528

Genes with statistically significant (adjusted) differential expression in the pre- and post-treatment biopsies, ranked per log2 fold change.

Supplemental Table 2 RNA Sequencing Results

Description	Genes in Overlap, n	*P*	FDR q
Genes Upregulated in Comparison of CD4 (GeneID = 920) Thymocytes vs. Thymic Stromal Cells	26	2.77E-14	8.92E-11
Genes Upregulated in Bone Marrow-Derived Macrophages Treated With IL4 (GeneID = 3565): Wild Type vs. *STAT6* (GeneID = 6778) Knockout	26	2.77E-14	8.92E-11
Genes Downregulated in HMC-1 (Mast Leukemia) Cells: *CI-IB-MECA* (PubChem = 3035850) vs. Incubated With the Peptide ALL1 Followed by Stimulation With T-Cell Membranes	25	2.14E-13	4.60E-10
Genes Upregulated in Comparison of CD4 (GeneID = 920) CD8 Thymocytes vs. CD4 (GeneID = 920) Int CD8 Thymocytes	24	1.58E-12	1.70E-09
Genes Downregulated in Monocytes (12 Hours) vs. Macrophages (12 Hours) Treated With IL4 (GeneID = 3565)	24	1.58E-12	1.70E-09
Genes Upregulated in Comparison of Monocytes Cultured for 0 Days vs. Those Cultured for 7 Days	24	1.58E-12	1.70E-09
Genes Downregulated in Dendritic Cells: Anti-FcgRIIB vs. Inflammatory Cytokine Cocktail	20	5.79E-12	5.32E-09
Genes Downregulated in Monocytes: Untreated vs. Anti-FcgRIIB	21	7.77E-12	6.25E-09
Genes Upregulated in Thymus Subcapsular Cortical Region vs. the Whole Medulla	23	1.01E-11	6.76E-09
Genes Upregulated in Comparison of Macrophages Treated With IL25 (GeneID = 64806) vs. Neutrophils Treated With IL25 (GeneID = 64806)	23	1.12E-11	6.76E-09
Genes Upregulated in Comparison of PBMC From Healthy Donors vs. PBMC From Patients With Acute *Staphylococcus Aureus* Infection	22	1.15E-11	6.76E-09
Genes Downregulated in Comparison of Untreated CD8 T Cells vs. CD8 T Cells Treated With Leukocyte Co-Stimulatory Blockade Antibodies	22	7.49E-11	3.02E-08
Genes Upregulated in Mock Treatment During Adoptive Transfer Therapy of B16 Melanoma: Day 3 vs. Day 7	22	7.49E-11	3.02E-08
Genes Upregulated in CD8 T Cells: *KLRB1* High (GeneID = 3820) vs. *KLRB1* Int (GeneID = 3820)	22	7.49E-11	3.02E-08
Genes Downregulated in Monocyte-Derived Dendritic Cells: Untreated vs. Rosiglitazone (PubChem = 77999)	22	7.49E-11	3.02E-08
Genes Upregulated in Monocyte-Derived Dendritic Cells: Control vs. *TNF* (GeneID = 7124) Inhibitor Etanercept	22	7.49E-11	3.02E-08
Genes Involved in Transmembrane Transport of Small Molecules	31	2.25E-10	8.54E-08
Genes Downregulated in CD8 T Cells, Acute Infection With LCMV-Armstrong: Effectors at Day 8 vs. Memory at Day 30	21	4.36E-10	1.34E-07
Genes Downregulated in CD4 (GeneID = 920) T Cells: Tretinoin (PubChem = 444795) vs. Ro 41-5253 (PubChem = 5312120)	21	4.78E-10	1.34E-07
Genes Upregulated in CD4 T Conv: Control vs. Overexpression of *IKZF2* and *FOXP3* (GeneID = 22807;50943).	21	4.78E-10	1.34E-07
Genes Upregulated in Normal T Reg: *FOXO1* (GeneID = 2308) vs. Wild Type	21	4.78E-10	1.34E-07
Genes Upregulated in Allogeneic T Cells After Stimulation With Dendritic Cells From: Liver vs. Mesenteric Lymph Nodes	21	4.78E-10	1.34E-07
Genes Important for Mitotic Spindle Assembly	21	4.78E-10	1.34E-07
Genes Upregulated in Comparison of PBMC From Healthy Donors vs. PBMC From Patients With Acute *Escherichia coli* Infection	19	1.50E-09	3.85E-07
Genes Involved in Developmental Biology	29	1.51E-09	3.85E-07
Genes Downregulated in TIG3 Cells (Fibroblasts) Upon Knockdown of *EED* (Gene ID = 8726) Gene	20	1.56E-09	3.85E-07
Genes Upregulated in Bone Marrow-Derived Dendritic Cells Treated With Poly(IC): 3 Hours vs. 24 Hours	18	2.47E-09	4.79E-07
Genes Downregulated in Macrophages With *SOCS3* (GeneID = 9021) Knockout Treated With *IL6* (GeneID = 3569): 100 Minutes vs. 400 Minutes	20	2.66E-09	4.79E-07
Genes Upregulated in Day 7 Plasma Cells vs. Day 40 Memory B Cells	20	2.90E-09	4.79E-07
Genes Downregulated in Comparison of Mature NK Cells vs. Intermediate Mature NK Cells	20	2.90E-09	4.79E-07
Genes Downregulated in Comparison of Th1 Cells vs. Th17 Cells	20	2.90E-09	4.79E-07
Genes Upregulated in HMC-1 (Mast Leukemia) Cells: Untreated vs. CI-IB-MECA (PubChem = 3035850)	20	2.90E-09	4.79E-07
Genes Upregulated in CD8 T Cells: Control vs. Overexpressing *ID3* (GeneID = 3399)	20	2.90E-09	4.79E-07
Genes Downregulated in Polymorphonuclear Leukocytes 9 Hours After Infection by: *Staphylococcus Aureus* vs. *Anaplasma phagocytophilum*	20	2.90E-09	4.79E-07

Supplemental Table 2 Continued

Description	Genes in Overlap, n	*P*	FDR q
Genes Upregulated in *KLRB1* High (GeneID = 3820) T Cells: *CD8A* (GeneID = 925) vs. *CD8A CD8B* (GeneID = 925; 926)	20	2.90E-09	4.79E-07
Genes Downregulated in Comparison of Macrophages vs. Th2 Cells	20	2.90E-09	4.79E-07
Genes Downregulated in T Reg: *GATA1* (GeneID = 2623) Knockout vs. Wild Type	20	2.90E-09	4.79E-07
Genes Downregulated in T Reg: *XBP1* (GeneID = 7494) Knockout vs. Wild Type	20	2.90E-09	4.79E-07
Genes Upregulated in Resting CD8 T Cells: Wild Type vs. *MIR155* (GeneID = 406947) Knockout	20	2.90E-09	4.79E-07
Genes Downregulated in Memory CD8 T Cells: 2' vs. 3'	18	4.48E-09	7.20E-07
Genes Upregulated in CD4 T Conv: Control vs. Overexpression of *XBP1* (GeneID = 7494)	18	5.43E-09	8.52E-07
Genes Involved in Axon Guidance	22	5.62E-09	8.62E-07
Genes Downregulated in *CD4* (GeneID = 920) Th17 T Cells: Enriched vs. Negative	18	7.20E-09	1.08E-06
Genes Involved in Adaptive Immune System	33	1.01E-08	1.47E-06
Genes Involved in Immune System	46	1.26E-08	1.78E-06
Genes Upregulated in *CD4* (GeneID = 920) Th1 cells: Wild Type vs. *EGR2* (GeneID = 1959) Knockout	19	1.53E-08	1.78E-06
Genes Upregulated in Transitional B Lymphocytes From Cord Blood: *CR2* (GeneID = 1380) Low vs. *CR2* (GeneID = 1380) High	19	1.66E-08	1.78E-06
Genes Upregulated in Tumors Established by Injecting MC38 Cells (Colon Cancer): Control vs. CpG Oligodeoxynucleotide 1826	19	1.66E-08	1.78E-06
Genes Downregulated in HMC-1 (Mast Leukemia) Cells: CI-IB-MECA (PubChem = 3035850) vs. Incubated With the ALL1 Peptide Followed by Treatment With CI-IB-MECA (PubChem = 3035850)	19	1.66E-08	1.78E-06
Genes Upregulated in Comparison of T Reg LP vs. Homeo Convert (see Supplemental Table 1)	19	1.66E-08	1.78E-06

Gene set enrichment analysis results (top 50) for the set of differentially expressed genes.
Abbreviations: ALL1 = Leukemia, acute susceptibility to, 1; Conv = Conventional; CpG = cytosine-guanine islands; FDR = false discovery rate; HMC-1 = human mast cell line; IL = interleukin; Int = intermediate; NK = natural killer; PBMC = peripheral blood mononuclear cells; Poly(IC) = polyinosinic:polycytidylic acid; Reg = regulatory.