Original Article

Anti-PD-1 plus anti-angiogenesis combined with chemotherapy in patients with HER2-negative advanced or metastatic gastric cancer: a multi-institutional retrospective study

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Background: Immunotherapy plus chemotherapy have been confirmed to be effective in treating advanced or metastatic gastric cancer (GC). Anti- programmed death-1 (PD-1) plus antiangiogenic agents have shown promising activity and tolerant toxicity in subsequent therapy of late-stage gastric cancer. The aim of this study was to assess the efficacy and safety of anti-PD-1 plus anti-angiogenic agents and chemotherapy in advanced or metastatic GC and to explore the potential biomarkers associated with response.

Methods: We retrospectively reviewed thirty human epidermal growth factor receptor 2 (HER2)-negative advanced or metastatic GC patients who received PD-1 plus anti-angiogenic drugs and chemotherapy. Conversion therapy was defined when the patients could undergo resection post combination therapy. Clinical data were retrieved from medical records. We conducted exploratory biomarker analysis of baseline gene mutations and tumor mutation burden (TMB) using the next-generation sequencing (NGS), PD-L1 by immunohistochemistry (IHC), and the tumor immune microenvironment (TIME) by multiplex immunofluorescence.

Results: A total of 30 patients received anti-PD-1plus anti-angiogenic drugs and chemotherapy during the study period. The objective response rate (ORR) was 76.7% [95% confidence interval (CI): 57.7–90.1%] and disease control rate (DCR) was 86.7% (95% CI: 69.3–96.2%). A total of 11 patients (36.7%) achieved conversion therapy and underwent surgery. The R0 resection rate was 90.9%. Of the 11 patients, 9 (81.8%) responded to the treatment, 1 with a pathological complete response (pCR) and 8 with a major pathological response (MPR). No adverse events of grade 3 or higher occurred. Neither PD-L1 expression nor TMB was significantly correlated with treatment response. Analysis of TIME revealed that the fraction of $CD8^+$ T cell in the invasive margin was higher in responders than non-responders before treatment. TAM2 in the tumor center and $CD8^+$ T cell in the invasive margin was significantly increased after combination therapy, which suggested that combination therapy promoted infiltration of $CD8^+$ T cells, thereby exerting an antitumor effect.

Conclusions: Immunotherapy plus anti-angiogenic drugs and chemotherapy is a promising treatment strategy for advanced or metastatic GC patients. Tumor infiltration $CD8^+$ T cells may serve as potential predictive biomarker.

Keywords: Advanced or metastatic gastric cancer; immunotherapy; anti-angiogenesis; chemotherapy; tumor immune microenvironment (TIME)

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Introduction

Gastric cancer (GC) is the fifth most common malignant tumor with poor prognosis, and the third leading cause of cancer-related deaths (1). Systemic chemotherapy of fluoropyrimidine and platinum-based combination regimens remains the standard first-line therapy for unresectable advanced or metastatic human epidermal growth factor receptor 2 (HER2) overexpression-negative gastric adenocarcinoma (2). For patients with programmed death ligand-1 (PD-L1) positive expression [Combined Positive Score (CPS) ≥5], nivolumab could be added to chemotherapy, which has been shown to prolong the median overall survival (mOS) from approximately 11.1 to 14.4 months (3). The Food and Drug Authority (FDA) approved pembrolizumab for microsatellite instability high/mismatch repair deficient (MSI-H/dMMR) (4-6) or tumor mutation burden (TMB) high (≥10 mutations/megabase) (7) metastatic gastric or gastroesophageal junction (G/GEJ) adenocarcinoma after ≥2 prior lines of therapy. However, patients with positivity for these three common biomarkers account for only a small portion of GC cases. Therefore, the development of novel combination therapies for advanced or metastatic GC patients with biomarkers-negative is urgently required.

The CheckMate-649 and ORIENT-16 studies have confirmed that first-line therapy with the combination of immunotherapy and chemotherapy lead to more significant progression-free survival (PFS) and overall survival (OS) benefits than chemotherapy alone among patients with advanced G/GEJ adenocarcinoma (3,8). Antiangiogenic agents have been shown to prolong OS by inhibiting the growth of new blood vessels and were approved for subsequent-line treatment of advanced GC (9,10). It was reported that anti-angiogenesis inhibitors could target tumor microenvironment (TME) components and synergize with immune checkpoint blockades by promoting $CD8^+$ T cells infiltration and activation (11). The phase Ib trial REGONIVO initiated a novel treatment pattern of anti-programmed death-1 (PD-1) agents plus anti-angiogenic drugs as subsequent-line treatment in 25 patients with GC and achieved a promising objective response rate (ORR) of 44% (12). In the EPOC1706 study, combination of pembrolizumab and lenvatinib resulted in a 69% ORR as first- or second-line treatment in 29 advanced or metastatic G/GEJ adenocarcinoma cases (13). Recently, there were several preliminary explorations about the application of anti-PD-1 agent plus anti-angiogenic drug and chemotherapy in second-line (14) and pre-operative (15) therapy of advanced GC. A phase 2, single-arm, prospective study assessed the efficacy and safety of the combination therapy of camrelizumab, apatinib, and S-1 in patients with G/GEJ adenocarcinoma as second-line treatment. Some 7 of 24 patients had objective response. The median progression-free survival (mPFS) was 6.5 months and the mOS was not reached. No serious treatment-related adverse events or treatment-related deaths was reported (14). Another phase II trial explored the application of camrelizumab, apatinib, and chemotherapy as neoadjuvant/conversion therapy in stage T4a/bN + M0 GC patients. Complete and major pathological response (pCR and MPR) rates were 15.8% and 26.3%, respectively. Grade 3 or higher adverse events occurred in 2 out of 25 patients (15). Although these studies have explored the application of immunotherapy plus anti-angiogenic drugs and chemotherapy in treating advanced or metastatic GC, and indicated its efficacy and safety in

Highlight box

Key findings

- Anti-PD-1 plus anti-angiogenesis and chemotherapy is an effective treatment strategy for HER2-negative, unresectable advanced or metastatic gastric cancer (GC).

What is known and what is new?

- Chemotherapy and immunotherapy combined with chemotherapy are two mainstream first-line treatments for HER2-negative advanced or metastatic GC.
- Compared with immunotherapy plus chemotherapy, the addition of an anti-angiogenesis agent improved ORR with a tolerable toxicity in HER2-negative advanced or metastatic GC.

What is the implication, and what should change now?

- This treatment strategy provides a new option for first-line and subsequent-line treatment of HER2-negative advanced or metastatic GC and is worth further exploration in a randomized control trial. The tumor immune microenvironment offers the possibility for screening the beneficiary population.

second-line treatment and neoadjuvant/conversion therapy, there is still lack of sufficient evidence about efficacy in the first-line setting. In this study, we retrospectively analyzed the efficacy and safety of immunotherapy in combination with anti-angiogenic agents and chemotherapy in 30 advanced or metastatic GC patients who were systemic treatment-naïve or had received treatment previously. Meanwhile, the association of combination regimens' efficacy and the tumor immune microenvironment (TIME) was also investigated. We present the following article in accordance with the STROBE reporting checklist (available at https://jgo.amegroups.com/article/view/10.21037/jgo-23-73/rc).

Methods

Patients and study design

From 13 August 2019 and 14 June, 2022, advanced or metastatic GC patients from the First Affiliated Hospital of Nanjing Medical University and Suzhou Municipal Hospital who received anti-PD-1 inhibitors combined with anti-angiogenic drugs and chemotherapy as first- or subsequent-line therapy were retrospectively screened. The main selection criteria were: (I) histologic confirmation of gastric adenocarcinoma; (II) unresectable advanced or metastatic disease; (III) age between 18–75 years; (IV) Eastern Cooperative Oncology Group scale performance status 0–1; (V) provision of written informed consent. Follow-up computed tomography (CT) imaging was conducted every 2 months for at least 4 months. Patient demographics, clinical data, survival data, and treatment history were retrieved from medical records. The study was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University and Suzhou Municipal Hospital (No. KL901343) and conducted in accordance with the Declaration of Helsinki (as revised in 2013) and the International Conference on Harmonization Good Clinical Practice guidelines. The patients provided written informed consent to participate in this study.

We conducted exploratory biomarker analysis of baseline gene mutations and TMB using the next-generation sequencing (NGS), PD-L1 using immunohistochemistry (IHC), and TIME using multiplex immunofluorescence (mIF), with the aim of discovering novel biomarker of response to anti-PD-1 combined therapy in GC patients.

NGS and TMB determination

NGS was performed in a Clinical Laboratory Improvement Amendments (CLIA)-approved laboratory (3D Medicines Inc., Shanghai, China) using tumor tissue as described previously (16), and the NGS panel targeted the exons of 733 (Table S1) to select the cancer-related genes. The TMB was defined as the number of somatic single nucleotide variations (SNVs) and insertions/deletions (indels) per megabase of coding genome sequenced. SNVs included synonymous and non-synonymous mutations, stop gain/loss, and splicing variants. Indels contained both frameshift and non-frameshift insertions and deletions. Non-coding alterations were excluded from TMB calculation.

PD-L1 staining and TIME

PD-L1 expression was detected using the PD-L1 IHC 22C3 pharmDx assay (Agilent Technologies, Santa Clara, CA, USA) and was assessed by combined positive score (CPS), where CPS ≥1 was considered as positive. The mIF staining was performed using PANO 7-plex IHC kit (Panovue, Beijing, China), according to the manufacturer's instructions as described previously (17). Briefly, CD8 marker was used to identify T cells. The natural killer (NK) cells were divided into CD56dim (weak staining) and CD56bright (strong staining) according to the intensity of membrane staining by CD56 antibody. Tumor-associated macrophages (TAMs) were identified by CD68 and HLA-DR and were divided into TAM1 ($CD68^+$ and $HLA\text{-}DR^+$) and TAM2 ($CD68^+$ and $HLA\text{-}DR^-$). S100 staining was used to define the tumor center and the invasive margin. The stained slides were scanned and built a single stack image subsequently by the Mantra System (PerkinElmer, Waltham, MA, USA). The reconstruction of images was performed using inForm image analysis software (PerkinElmer) for multispectral unmixing to remove autofluorescence.

Assessment

Treatment-related adverse events (TRAEs) were reported according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 5.0. CT images obtained before and after therapy were used to assess the radiographic response of the primary tumor according to Response Evaluation Criteria in Solid Tumors version (RECIST) 1.1. Pathological regression was performed on surgical specimens stained with hematoxylin and eosin (H&E). Tumors with ≤10% residual viable tumor cells were considered to have achieved MPR, and no residual tumor

Table 1 Patient characteristics

Characteristics	Patients (n=30)
Age, years, median [range]	58 [32–73]
Sex, n (%)	
Male	17 (56.7)
Female	13 (43.3)
Tumor differentiation, n (%)	
Poor	20 (66.7)
Moderate	6 (20.0)
Unknown	4 (13.3)
Lauren classification, n (%)	
Intestinal type	5 (16.7)
Diffuse type	12 (40.0)
Mixed	5(16.7)
Unknown	8 (26.7)
PD-L1 CPS, n (%)	
<1%	23 (76.7)
≥1%	5(16.7)
Unknown	2 (6.7)
Disease status, n (%)	
Metastatic	26 (86.7)
Locally advanced/recurrence	4 (13.3)
Metastases, n (%)	
None	1 (3.3)
One	8 (26.7)
Two or more	21 (70.0)
MSI status, n (%)	
MSS	29 (96.7)
MSI-H	0 (0.0)
Unknown	1 (3.3)
TMB*, Muts/Mb, median [range]	6.3 [2.1–24.0]

*, TMB could be assessed in 25 patients. PD-L1, programmed death ligand 1; CPS, combined positive score; TMB, tumor mutation burden; MSI, microsatellite instability; MSS, microsatellite stable.

was defined as having pCR. All imaging and pathological dates were reviewed by 2 independent radiologists or pathologists.

Statistical analyses

Categorical variables were compared using Fisher's exact test and continuous variables using unpaired *t*-test or paired *t*-test. Exploratory analysis of the association between clinical response and PD-L1 expression, TMB, or TIME was conducted. For all analyses, a P value <0.05 (two-sided) was considered statistically significant, and a confidence interval of 95% (95% CI) was used. All analyses and graph generation were performed by SPSS 25 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

Results

Patient characteristics and efficacy

Between 13 August 2019 and 14 June, 2022, 30 patients with HER2-negative advanced or metastatic GA received the regimens of anti-PD-1 agent plus anti-angiogenic drugs in combination with chemotherapy. The baseline participant characteristics are summarized in *Table 1*. Of these patients, 17 (56.7%) were males and the median age was 58 years (range, 32–73 years). 21 of 30 (70%) patients were PD-L1 negative and 7 of 30 (23.3%) patients were PD-L1 positive. Except for 5 patients, all cases underwent NGS testing. Each patient carried at least 1 variant except for patient #11 and patient #24 (Table S2). The detailed regimens and response to the combination therapy of thirty patients were described in Table S3. The ORR was 76.7% (95% CI: 57.7–90.1%) with 3 complete responses (CR; 10.0 %). There were 3 cases (10.0%) of stable disease (SD) and 4 case (13.3%) of progressive disease (PD). The disease control rate (DCR) was 86.7% (95% CI: 69.3–96.2%). Adverse events occurred in all patients, but none of events was grade 3 or higher (Table S3).

Clinical course to conversion therapy

A flow diagram of the patients' treatment course is shown in *Figure 1*. A total of 11 patients (36.7%) achieved conversion therapy, including 1 who was assessed for SD. Thus, the conversion rate was 36.7% in this cohort. R0 resection was performed in 10 (90.9%) cases and R2 resection in 1 case.

More importantly, patient #3 obtained pCR (*Figure 2*). The patient was a 58-year-old female diagnosed with poorly differentiated gastric adenocarcinoma with left supraclavicular lymph node, abdominal aortic lymph node, and left ovarian metastasis. She received 6 cycles

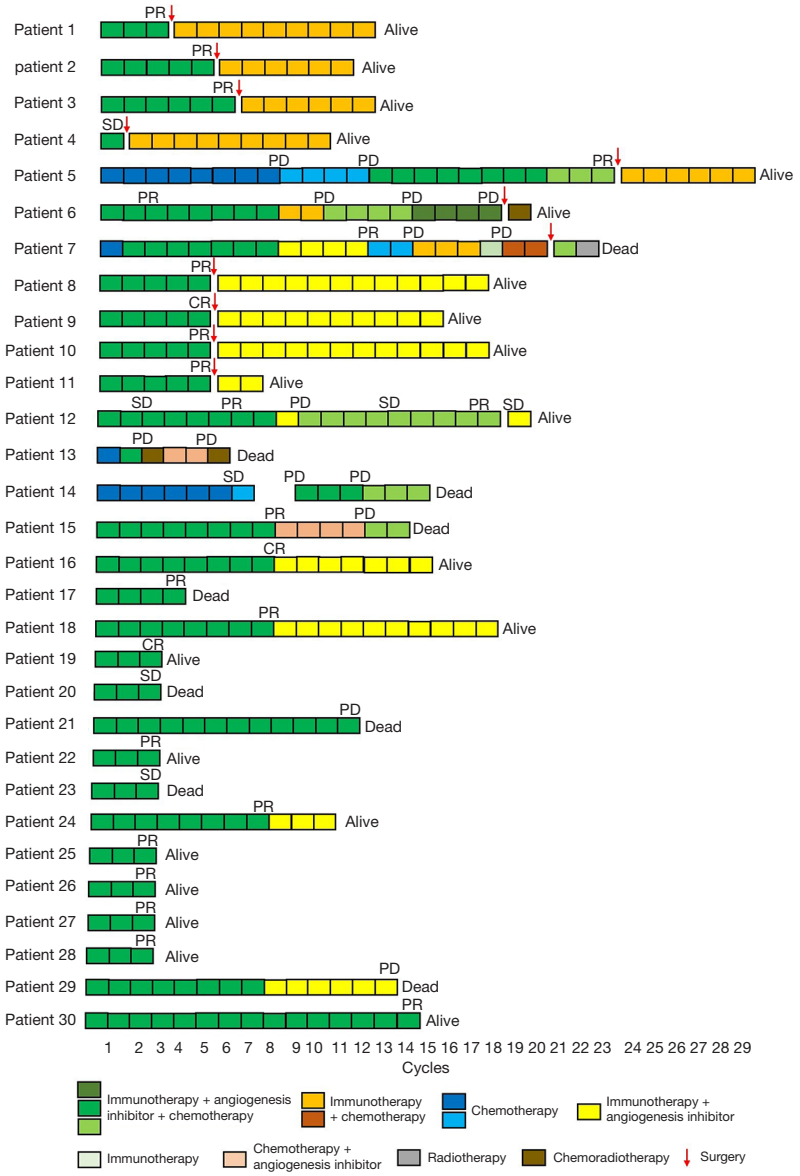

Figure 1 A flow diagram of the patients' treatment course. SD, stable disease; PD, progressive disease; PR, partial response; CR, complete response.

of pembrolizumab plus lenvatinib and CAPEOX. The CT examination showed that the primary tumor had disappeared and the tumor in left ovary was significantly reduced. Radical distal gastrectomy was subsequently performed. Pembrolizumab plus paclitaxel and capecitabine maintenance therapy was administered for 6 cycles postoperatively. In addition, 8 cases had an MPR in the primary tumor (Figures S1-S3). No difference was observed in the number of cells and in the fraction of immune cells in center or invasive margin between MPR and non-MPR ($P>0.05$, Figure S4A,S4B).

Characteristics and combination therapy results of the patients undergoing and not undergoing conversion therapy

In the patient characteristics at baseline, no significant differences were observed in terms of age, sex, tumor differentiation, Lauren classification, disease status, number of metastases, PD-L1 CPS, microsatellite instability (MSI) status and TMB between the conversion therapy group and

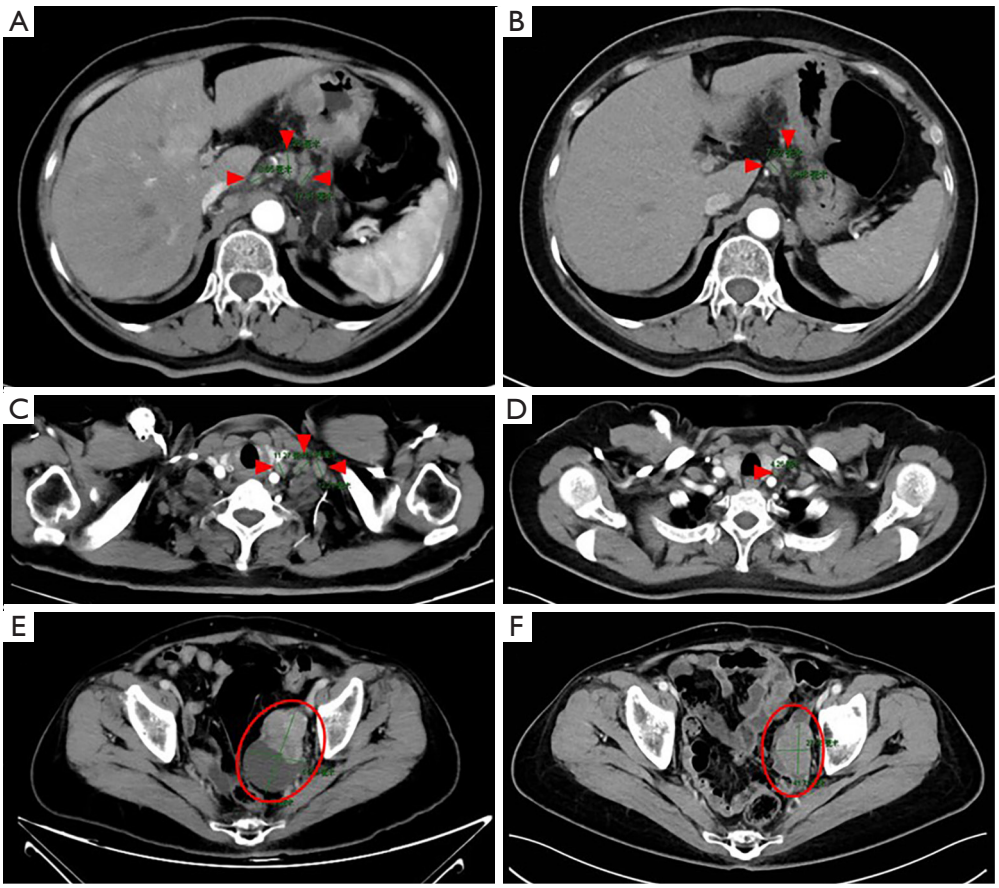

Figure 2 CT images of patient #3 before and after treatment of combination regimens. (A) The paraaortic lymph nodes were marked by red arrow heads before treatment and the longest diameter of lymph nodes was 17.25, 8.05, and 12.31 mm, respectively; (B) after three cycles of combination treatment, the sizes of paraaortic lymph nodes marked by red arrow heads reduced to 7.52 mm, 6.48 mm, and unmeasurable; (C) the left supraclavicular lymph nodes were marked by red arrow heads before treatment and the longest diameter of lymph nodes was 11.27, 13.04, 12.21 mm, respectively; (D) after three cycles of combination treatment, the sizes of left supraclavicular lymph nodes marked by red arrow head reduced to 4.26 mm and unmeasurable. The left ovarian metastatic tumor marked by red ellipse circle shrank dramatically from 74.32 mm × 51.95 mm (E) to 41.71 mm × 27.96 mm (F) after 3 cycles of treatment. CT, computed tomography.

non-conversion therapy group (*Table 2*). Among those who underwent conversion therapy, 90.9% patients displayed a major response, which was better than in those who did not receive conversion therapy (68.4%) (P=0.215).

Genomic and immunologic correlates of response to combination therapy

In addition, we divided 30 patients into responders (n=22) and non-responders (n=8) based on their response to the combination therapy. PD-L1 expression was positive in 6 of 21 responders and 1 of 7 non-responders among the 28 tumors which could be evaluated, suggesting that the expression of PD-L1 was not associated with efficacy of combination therapy (P=0.639, Fisher's exact test) (Figure S5A). At the same time, we also analyzed the correlation between TMB and the efficacy of combination therapy, and found that there was no statistically significant difference in TMB between responders and non-responders (t=0.787, P=0.439) (Figure S5B).

Moreover, the tumor specimens of 14 patients were subjected to mIF analysis to investigate their TIME. The densities of $CD8^+$ T cells, TAMs (M1 and M2), and NK cells (CD56bright and CD56dim) were quantified. Except for the fractions of $CD8^+$ T cells in the invasive margin (t=2.672, P=0.02), no significant difference was observed in

Table 2 Results of conversion cases in comparison to non-conversion cases regarding the characteristics and combination therapy

Characteristics	Conversion therapy (N=11)	Non-conversion therapy (N=19)	P value
Age, years, median [range]	58 [35–69]	57 [32–73]	–
Sex, n (%)			>0.9999
Male	6 (54.5)	11 (57.9)	
Female	5 (45.5)	8 (42.1)	
Tumor differentiation, n (%)			0.7409
Poor	8 (72.7)	13 (68.4)	
Moderate	3 (27.3)	5 (26.3)	
Unknown	0 (0.0)	1 (5.3)	
Lauren classification, n (%)			0.2201
Intestinal	2 (18.2)	3 (15.8)	
Diffuse	2 (18.2)	10 (52.6)	
Mixed	2 (18.2)	3 (15.8)	
Unknown	5 (45.4)	3 (15.8)	
PD-L1 CPS, n (%)			0.8315
<1%	8 (72.7)	13 (68.4)	
≥1%	2 (18.2)	5 (26.3)	
Unknown	1 (9.1)	1 (5.3)	
Disease status, n (%)			0.268
Metastatic	11 (100.0)	15 (78.9)	
Locally advanced/recurrence	0 (0.0)	4 (21.1)	
Metastases, n (%)			0.1754
None	1 (9.1)	0 (0.0)	
One	3 (27.3)	2 (10.5)	
Two or more	7 (63.6)	17 (89.5)	
MSI status, n (%)			0.439
MSS	11 (100.0)	18 (94.7)	
Unknown	0 (0.0)	1 (5.3)	
TMB*, Muts/Mb, median (range)	6.15 (2.23–24.02)	6.3 (2.1–16.7)	–
Response, n (%)			0.215
Partial response	10 (90.9)	13 (68.4)	
Stable disease	1 (9.1)	2 (10.5)	
Progressive disease	0 (0.0)	4 (21.1)	

*, TMB could be assessed in 25 patients. PD-L1, programmed death ligand 1; CPS, combined positive score; TMB, tumor mutation burden; MSI, microsatellite instability; MSS, microsatellite stable.

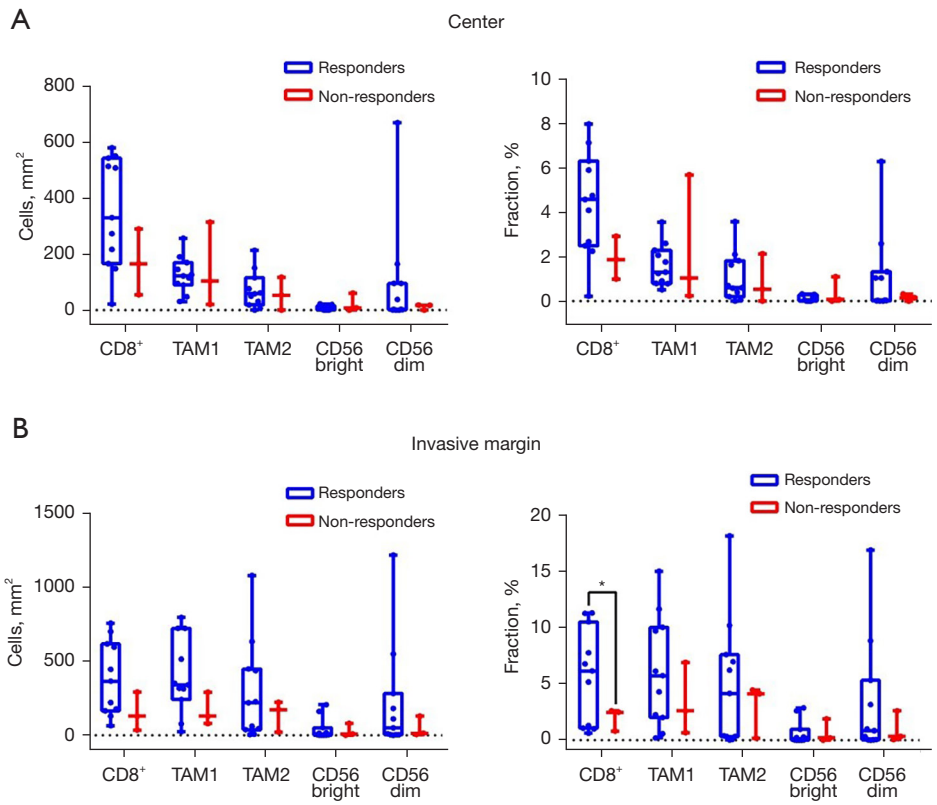

Figure 3 Immune cells in center or invasive margin between responders (N=11) and non-responders (N=3). (A) Comparisons of immune cells in tumor center between responders and non-responders; (B) comparisons of immune cells in invasive margin between responders and non-responders. *P<0.05.

the densities and fractions of TAMs and NK cells between responders and non-responders before treatment (*Figure 3*). We found that the abundance of immune cells which play a positive role in anti-tumor immunity, such as $CD8^+$ T, TAM1, and CD56dim NK cells, was always higher in responders than non-responders at baseline (*Figure 3*). In the tumor center, the density and fraction of TAM2 were significantly increased after combination therapy (t=3.945, P=0.006, and t=3.359, P=0.012) (*Figure 4A*). In the invasive margin, the density and fraction of $CD8^+$ T cells were higher after combination therapy (t=2.049, P=0.063, and t=2.671, P=0.02, *Figure 4B*). Although the density and fraction of TAM1 did not change significantly before and after the combination therapy, TAM1/TAM2 increased 4.8-fold (from 4.8 to 9.6) after combination therapy, suggesting that the increment of TAM1 was greater than that of TAM2 during this process (*Figure 4B*). These results suggest that combination therapy may promote infiltration of $CD8^+$ T cells and the transformation of TAM2 into TAM1 in the center and invasive margin, thereby exerting an antitumor effect.

In addition, 2 of 14 patients underwent TIME analysis at 3 different time points (pre-treatment, post-treatment, and progression). Both of them showed the same variation trend that $CD8^+$ T cell had increased infiltration when the patients responded to the combination treatment and the fraction of $CD8^+$ T cell was decreased when the patients had progressive disease in both the tumor (Figure S6A) and invasive margin (Figure S6B). ThemIF images of the two patients were displayed in Figure S6C.

Discussion

Almost half of the global new GC cases annually are found in China and half of the Chinese patients are diagnosed at an advanced stage. For patients with HER2-negative advanced or metastatic GC, standard doublet chemotherapy has shown limited efficacy and their prognosis has remained poor. Here we reported the combination therapy of anti-PD-1 agent plus angiogenesis inhibitor and chemotherapy

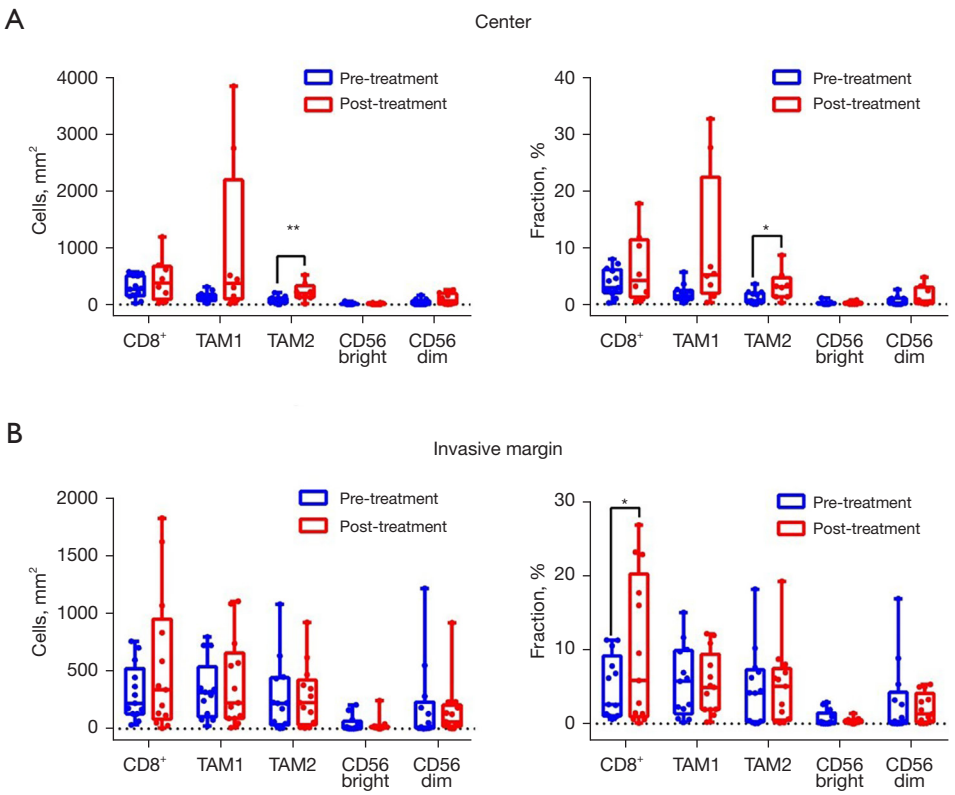

Figure 4 Immune cells in center or invasive margin before and after combination therapy. (A) Comparison of immune cells in tumor center before and after treatment (N=13). Since 5 samples did not pass the quality control which suggested the tumor cell content was less than 20%, there were only 8 samples after treatment. (B) Comparisons of immune cells in invasive margin before and after treatment (N=13). *P<0.05; **P<0.01.

had encouraging anti-tumor activity for patients with advanced and metastatic GC in the first-line or subsequent-line setting. The ORR was 76.7%, DCR was 86.7%, and 36.7% patients had surgical resection after combination therapy. Furthermore, for most patients, the TRAEs were manageable. To our knowledge, the efficacy was superior to that of other combination regimens reported previously.

Several previous studies had explored different regimens as first-line setting for G/GEJ adenocarcinoma. The ORR of nivolumab plus chemotherapy and sintilimab in combination with chemotherapy achieved 58% (3) and 58.2% (8), respectively, in all randomized patients. Another multicenter, open-label, phase II trial reported an ORR of 58.3% with camrelizumab plus CAPOX followed by camrelizumab plus apatinib as first-line therapy for advanced G/GEJ adenocarcinoma (18). In our study, the combination therapy of immunotherapy plus angiogenesis inhibitors and chemotherapy improved ORR to 76.7% compared with doublet therapy or triplet sequential therapy and was well tolerated by most patients.

The results of 2 phase II single-arm trials have displayed the effects of anti-PD-1 agent plus angiogenesis inhibitor and chemotherapy in neoadjuvant/conversion therapy, with R0 resection rates of 82.6% (15) and 94.4% (the surgical conversion rate was 47.2%) (19), respectively. In our study, 36.7% (11/30) of patients underwent surgical resection after combination therapy and the R0 resection rate was 90.9%. The timing of the surgery is very important, and functional and psychological aspects must be taken into consideration in each case. Our study showed that for patients with rapidly shrinking tumors after 3–6 cycles of treatment, surgical resection might be selected, followed by 6–9 cycles of maintenance therapy. For patients whose tumors shrank relatively slowly, more cycles of treatment or different regimens would be given until the tumors shrank to a resectable size. Once progressive disease was found by gastroscopy and CT images during treatment, which suggested that the patient had been resistant to the

combination treatment, clinicians would prepare for surgery immediately if the tumors were deemed resectable.

PD-L1, MSI/dMMR, and TMB are the most validated and FDA-approved positive predictive biomarkers for immune checkpoint inhibitor (ICI) responses. Among evaluable patients in our study, all patients were microsatellite stable (MSS) (N=29), only 7 patients were PD-L1 expression-positive (N=28), and 6 were TMB-high (TMB-H) with the top quartile as the cutoff (N=25). This meant most of the patients received combination treatment were biomarkers-negative. In previous study, patients with PD-L1 expression-positive had better survival outcomes when they received mono-immunotherapy or immunotherapy plus chemotherapy (3). Conversely, another study showed poor correlation of PD-L1 expression with pathological response to neoadjuvant immunochemotherapy (20). The frequency of PD-L1 expression positivity was considerably lower than in previous GC trials. No clear correlation between PD-L1 and efficacy outcomes was found in the limited number of patients. TMB had been reported to be correlated with enhanced clinical response to mono-immunotherapy in several solid tumors (21,22) and TMB-H patients responded significantly better than TMB-low (TMB-L) patients (23). TMB could only be assessed in 25 patients in this study and there was no statistical difference of TMB between responders and non-responders. This suggests that more patients might have better survival outcomes from combination therapy regardless of these 3 biomarkers.

Although 3 common biomarkers were not associated with efficacy of the combination in our study, TIME analysis brought new insights. Previous studies had confirmed that the characteristics of TIME were associated with response to ICIs and prognosis in several solid tumors. In the exploratory NICHE study, $CD8^+PD\text{-}1^+$ T cell infiltration was found to be predictive of response to neoadjuvant ICIs treatment in pMMR colon cancer. Responders had higher density of $CD8^+PD\text{-}1^+$ T cell than non-responders before treatment (P=0.049). Assessment of post-treatment changes in pMMR tumors revealed a significant increase in $CD8^+$ T cell and $CD68^+$ immune infiltration (24). Fumet *et al.* addressed the role of $CD8^+$ TILs and PD-L1 expression to predict response to nivolumab in a cohort of 85 NSCLC patients treated with nivolumab in second line or beyond. A high expression of $CD8^+$ TILs measured with IHC and messenger RNA (mRNA) was significantly associated with PFS (25). Another study revealed that increased $CD4^+FOXP3^+$ T-cell density in the GC tumor correlated with prolonged survival. High densities of $CD4^+FOXP3^+$ T cells and $CD8^+$ T cells (high-high) independently predicted prolonged patient survival (26). Besides T cells, high TAMs infiltration was reported to be associated with poor prognosis in GC (27). Our results were consistent with the previous reports. Pre- to post-treatment changes in $CD8^+$ T cell in invasive margin showed significantly increased infiltration after treatment. A similar trend in the center of $CD8^+$ T cell was also observed, but there was no statistical difference. In this study, we found that primary tumors and distant metastases of some patients showed different responses to the combination therapy. This may be related to tumor heterogeneity and tumor microenvironment heterogeneity of primary and metastatic lesions in the same patient.

Based on preliminary data, the combination of immunotherapy plus anti-angiogenesis and chemotherapy may be a promising option in patients with advanced or metastatic GC as a first-line therapy. However, there are still some limitations to our study, including those inherent limitation in the retrospective design. Not all patients included in this study were on first-line treatment, leading to bias in the results of assessments such as ORR. However, we observed that the benefits of this regimen were superior to those of other combinations. Due to the limited number of patients, PD-L1 and TMB could not effectively screen beneficiaries, but the results of TIME confirmed that $CD8^+$ T cells were significantly increased in patients responding to combination therapy. Such results should be confirmed in large cohorts.

Conclusions

Our study suggested the potential of anti-PD-1 agents in combination with angiogenesis inhibitors and chemotherapy as a first-line and subsequent-line treatment in patients with advanced or metastatic GC, which showed better anti-tumor activity than combination of immunotherapy plus chemotherapy or immunotherapy plus anti-angiogenesis drugs accompanying a manageable safety profile. Based on these preliminary results, a confirmatory randomized controlled trial will be launched, and more precise biomarkers analysis will also be elucidated in the large cohort study.

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Footnote

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Supplementary

Table S1 List of genes of the 733-gene panel

ABL1	*CDX2*	*FGFR4*	*MLH1*	*PTEN*	*VEGFA*	*JMJD1C*	*TRIM37*	*BCL11A*	*EZR*	*TBL1XR1*	*PLXNB1*	*LIG1*	*RNF168*	*POLD3*
ACVR2A	*CHD2*	*FH*	*MLLT3*	*PTK6*	*VHL*	*LMO1*	*TSHR*	*BCL11B*	*FAT4*	*TCF7L2*	*SPRED1*	*LIG3*	*RNF4*	*POLD4*
AFF3	*CHEK1*	*FHIT*	*MPL*	*PTPRD*	*NSD3*	*LZTR1*	*UROD*	*BCORL1*	*FUBP1*	*TCL1A*	*ERF*	*LIG4*	*RNF8*	*POLE2*
AKT1	*CHEK2*	*FLCN*	*MRE11A*	*RAC1*	*ZNF479*	*MAX*	*WAS*	*BIRC3*	*FUS*	*TET1*	*RPS6KA3*	*MAD2L2*	*RPA1*	*POLE4*
AKT2	*CHIC2*	*FLT1*	*MSH2*	*RAD50*	*ZNRF3*	*MEN1*	*WRN*	*BRD4*	*GAS7*	*TFE3*	*GSK3B*	*MBD4*	*RPA2*	*PPP4R1*
AKT3	*CIC*	*FLT3*	*MSH3*	*RAD51*	*ABCB11*	*MTAP*	*WT1*	*CACNA1D*	*H3F3A*	*TNFAIP3*	*NOTCH3*	*MDC1*	*RPA3*	*PPP4R3A*
ALK	*CIITA*	*FLT4*	*MSH6*	*RAD51C*	*APOBEC3B*	*MUTYH*	*XPA*	*CALR*	*HIF1A*	*USP8*	*NOTCH4*	*MGMT*	*RPA4*	*PPP4R3B*
ANK1	*CRBN*	*FOXA1*	*MTOR*	*RAF1*	*AXIN2*	*NBN*	*XPC*	*CAMTA1*	*HIP1*	*WIF1*	*ALKBH2*	*MLH3*	*RRM2B*	*PPP4R4*
APC	*CRLF2*	*FRS2*	*MYC*	*RARA*	*BARD1*	*NHP2*	*XRCC2*	*CANT1*	*HNRNPA2B1*	*XPO1*	*ALKBH3*	*MMS19*	*SETMAR*	*RAD9B*
AR	*CRNKL1*	*G6PD*	*MYCN*	*RB1*	*BMPR1A*	*NME1*	*HOXB13*	*CARD11*	*HOXA11*	*ZFHX3*	*APEX1*	*MNAT1*	*SEM1*	*RBX1*
ARAF	*CRTC3*	*GATA3*	*MYD88*	*RET*	*BUB1B*	*NOP10*	*BCL2L1*	*KNL1*	*IL6ST*	*ACVR1B*	*APEX2*	*MPG*	*SHPRH*	*RFC1*
AREG	*CSF1R*	*GLI2*	*NF1*	*RGS7*	*CDC73*	*NTHL1*	*BCL6*	*CASP8*	*KDM6A*	*ARID1B*	*CENPS*	*MSH4*	*SMUG1*	*RFC2*
ARHGAP5	*CSF3R*	*GNA11*	*NF2*	*RICTOR*	*CDKN1C*	*PHOX2B*	*CDK8*	*CBFA2T3*	*KEAP1*	*DNMT1*	*APLF*	*MUS81*	*SPO11*	*RFC3*
ARID1A	*CTNNB1*	*GNAQ*	*NFE2L2*	*RNF43*	*CEBPA*	*PMS1*	*FOXP1*	*CBFB*	*KLF4*	*FOXL2*	*APTX*	*NEIL1*	*TDG*	*RFC4*
ARNT	*CTNND2*	*GNAS*	*NFIB*	*ROS1*	*COL7A1*	*POLH*	*GRIN2A*	*CBLB*	*LCK*	*GATA1*	*ATRIP*	*NEIL2*	*TDP1*	*TELO2*
ASXL1	*CUL3*	*HDAC2*	*NKX2-1*	*RPTOR*	*CTR9*	*POLQ*	*IKBKE*	*CCDC6*	*LEF1*	*HIST1H3B*	*FAAP100*	*NEIL3*	*TDP2*	*TIMELESS*
ATM	*CYSLTR2*	*HEY1*	*NOTCH1*	*RUNX1*	*CXCR4*	*POT1*	*MEF2B*	*CCNB1IP1*	*LIFR*	*KDM5C*	*FAAP24*	*NHEJ1*	*TOP3A*	*TMEM189*
ATR	*DDR2*	*HGF*	*NOTCH2*	*SDC4*	*CYLD*	*PRDM9*	*NFKBIA*	*CD79A*	*MAPK1*	*MAP3K1*	*FAAP20*	*NUDT1*	*TOP3B*	*WDR48*
AURKA	*DICER1*	*HOOK3*	*NPM1*	*SDHC*	*DDB2*	*PRF1*	*PIK3CD*	*CD79B*	*MED12*	*KMT2C*	*MPLKIP*	*NABP2*	*TOPBP1*	*GFI1*
AXL	*DNMT3A*	*HRAS*	*NRAS*	*SERPINB3*	*DIS3L2*	*PRKAR1A*	*SRC*	*CDH11*	*NAB2*	*NCOR1*	*CCNH*	*OGG1*	*TP53BP1*	*CYP17A1*
B2M	*DPYD*	*IDH1*	*NRG1*	*SETD2*	*DKC1*	*PRSS1*	*BTG1*	*CHD4*	*NCOR2*	*PHF6*	*CDK7*	*PARP1*	*TREX1*	*ELF3*
BAP1	*EGFR*	*IDH2*	*NTRK1*	*SF3B1*	*DOCK8*	*PTPN11*	*DIS3*	*CLIP1*	*NDRG1*	*PPP2R1A*	*CETN2*	*PARP2*	*TREX2*	*SGK1*
BAZ1A	*EPHA2*	*IGF1R*	*NTRK2*	*SH2B3*	*DROSHA*	*PTPN13*	*EED*	*CLTCL1*	*NONO*	*PRDM1*	*CHAF1A*	*PARP3*	*UBE2A*	*GSTT1*
BCL2	*EPHA3*	*IGF2*	*NTRK3*	*SLC29A1*	*ELANE*	*RAD51B*	*GNA13*	*CNBP*	*PAX3*	*SOCS1*	*CLK2*	*PCNA*	*UBE2B*	*AEN*
BCOR	*ERBB2*	*IL7R*	*PAK1*	*SMAD4*	*EPCAM*	*RAD51D*	*NT5C2*	*CNOT3*	*PAX7*	*SOX9*	*DCLRE1A*	*PNKP*	*UBE2N*	*CCNO*
BLM	*ERBB3*	*INPP4B*	*PALB2*	*SMARCA1*	*ERCC3*	*RECQL*	*PPP2R2A*	*CREB3L1*	*PAX8*	*TRAF7*	*DCLRE1B*	*POLB*	*UBE2T*	*CENPX*
BMP5	*ERBB4*	*ITGAV*	*PAX5*	*SMARCA4*	*ERCC5*	*RECQL4*	*NSD2*	*CREB3L2*	*PER1*	*IKZF1*	*DCLRE1C*	*POLI*	*UBE2V2*	*CUL4A*
BRAF	*ERCC1*	*JAK1*	*PBRM1*	*SMARCB1*	*ETV6*	*RFWD3*	*EPHA7*	*CREBBP*	*PICALM*	*MYCL*	*DDB1*	*POLK*	*UNG*	*CUL5*
BRCA1	*ERCC2*	*JAK2*	*PDCD1LG2*	*SMO*	*EXT1*	*RHBDF2*	*GLI1*	*CRTC1*	*PIM1*	*NCOA3*	*DMC1*	*POLL*	*USP1*	*DNTT*
BRCA2	*ERCC4*	*JAK3*	*PDGFB*	*SRGAP3*	*EXT2*	*SBDS*	*MYB*	*CTCF*	*POU2AF1*	*CDK2*	*DUT*	*POLM*	*XAB2*	*ELOA*
BRIP1	*ERCC6*	*JUN*	*PDGFRA*	*SRSF2*	*FAH*	*SDHA*	*NRG3*	*CUX1*	*POU5F1*	*LATS1*	*EME1*	*POLN*	*XRCC1*	*HUS1B*
BTK	*EREG*	*KCNJ5*	*PDGFRB*	*STAG2*	*FANCD2*	*SDHAF2*	*NUP93*	*DAXX*	*PPP6C*	*LATS2*	*EME2*	*PRKDC*	*XRCC3*	*PER2*
CARS	*ESR1*	*KDR*	*PDPK1*	*STK11*	*FANCE*	*SDHB*	*PTK2*	*DDIT3*	*PRDM16*	*YAP1*	*ENDOV*	*PRPF19*	*XRCC4*	*PER3*
CBL	*EWSR1*	*KIT*	*PIK3CA*	*SUZ12*	*FANCF*	*SDHD*	*RXRA*	*DDX10*	*PREX2*	*TEAD2*	*ERCC8*	*RAD1*	*XRCC5*	*MSH5*
CCND1	*EZH2*	*KMT2A*	*PIK3CB*	*SYK*	*FANCI*	*SERPINA1*	*SMARCA2*	*DDX3X*	*PRKACA*	*MGA*	*EXO1*	*RAD18*	*XRCC6*	*PARP4*
CCND2	*FAM135B*	*KMT2D*	*PIK3R1*	*TBX3*	*FANCL*	*SETBP1*	*TYK2*	*DDX5*	*PTPRT*	*HES1*	*FAN1*	*RAD23A*	*ABRAXAS1*	*POLE3*
CCND3	*FAM47C*	*KRAS*	*PIK3R2*	*TCF3*	*FANCM*	*SH2D1A*	*ZNF750*	*DDX6*	*QKI*	*KDM5A*	*FANCB*	*RAD23B*	*FRK*	*PPP4R2*
CCNE1	*FANCA*	*LASP1*	*PLCG2*	*TERT*	*FAS*	*SHOC2*	*ABI1*	*DNM2*	*RAD21*	*SPEN*	*GEN1*	*RAD52*	*BIRC5*	*SLX1A*
CD274	*FANCC*	*LMNA*	*PML*	*TET2*	*FEN1*	*SLC25A13*	*ACKR3*	*EBF1*	*RANBP2*	*THBS2*	*GTF2H1*	*RAD54B*	*EMSY*	*RAD54L2*
CDH1	*FANCG*	*LRP1B*	*PMS2*	*TMEM127*	*GALNT12*	*SLX4*	*ACSL3*	*EIF3E*	*RAP1GDS1*	*CUL1*	*GTF2H3*	*RAD54L*	*CRKL*	*RFC5*

Table S1 *(Continued)*

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Table S1 *(Continued)*

CDH10	*FAT1*	*MAP2K1*	*POLD1*	*TMPRSS2*	*GATA2*	*SOS1*	*ACVR1*	*EIF4A2*	*RBM10*	*HDAC1*	*GTF2H4*	*RAD9A*	*EPHB1*	*HMGA2*
CDK12	*FBXW7*	*MAP2K2*	*POLE*	*TOP2A*	*GBA*	*SPOP*	*AFF4*	*ELF4*	*RHOA*	*MLST8*	*GTF2H5*	*RBBP8*	*GLI3*	*TSPAN31*
CDK4	*FES*	*MAP2K4*	*POLG*	*TP53*	*GJB2*	*SPRTN*	*AMER1*	*ELK4*	*RHOH*	*PIK3R3*	*H2AFX*	*RDM1*	*IRS2*	*MYOD1*
CDK6	*FGF19*	*MCL1*	*PPARG*	*TPMT*	*GPC3*	*SRY*	*ARID2*	*ELL*	*RNF213*	*RHEB*	*HELQ*	*RECQL5*	*RUNX1T1*	*CHD1*
CDKN1A	*FGF3*	*MDM2*	*PPM1D*	*TSC1*	*GREM1*	*STAT3*	*ATP1A1*	*EP300*	*SFPQ*	*RPS6KB1*	*HFM1*	*REV1*	*SLIT2*	*ZBTB16*
CDKN1B	*FGF4*	*MDM4*	*PRCC*	*TSC2*	*HFE*	*SUFU*	*ATP2B3*	*EPAS1*	*SLC34A2*	*GRB2*	*HLTF*	*REV3L*	*SOX2*	*PCDH9*
CDKN2A	*FGFR1*	*MECOM*	*PRKCH*	*U2AF1*	*HMBS*	*TGFBR1*	*ATRX*	*EPS15*	*SLC45A3*	*RIT1*	*HMGB1*	*RIF1*	*SPTA1*	*PLXNA1*
CDKN2B	*FGFR2*	*MET*	*PSIP1*	*UGT1A1*	*HNF1A*	*TGFBR2*	*AXIN1*	*ERC1*	*SMAD2*	*RASA1*	*HUS1*	*RMI1*	*ZNF217*	
CDKN2C	*FGFR3*	*MITF*	*PTCH1*	*USP6*	*ITK*	*TP63*	*BCL10*	*ETNK1*	*SMAD3*	*ERRFI1*	*UVSSA*	*RMI2*	*ZNF703*	

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Table S2 Clinicophysiological and molecular characteristics of each patient

Patient #	Gender	Age	Site of metastases	Gene variation	TMB (Muts/Mb)	PD-L1 expression
1	Male	58	None	*TP53, EGFR, MYC*	8.4	Negative
2	Male	58	LN, liver, spleen	*TP53, CDK6, CHEK2*	6	Positive
3	Female	58	LN, ovary	*ARID1A, CCND1, CDKN1B, ERBB3, FGFR2, FRS2, TP53*	10.1	Negative
4	Female	50	Ovary	*PIK3CA*	5.0	Negative
5	Female	41	Ovary	*ARID1A, TP53*	3.9	Negative
6	Male	58	Bladder, ureter	*CDH1, EGFR, ERBB3, NTRK1, POLD1, PREX2, TP53*	9.2	Negative
7	Female	35	Pelvic cavity	*CDH1, SMAD4, TP53*	3.2	Negative
8	Male	68	LN	*LRP1B, AR, GRM3, JAK3, MAP3K1, RICTOR, ROS1, TP53*	6.3	Positive
9*	Male	67	Liver, para-abdominal aorta, liver stomach space	*Unknown*	Unknown	Unknown
10	Male	69	LN	*ACVR2A, APC, ARID1A, CTCF, MSH3, RNF43, SETD2, CCND1, FGF19, FGF4, FGF3, CDK8*	24.02	Negative
11	Female	40	LN	*NA*	2.23	Negative
12	Male	59	LN, liver	*TP53*	16.7	Negative
13	Female	34	Meninges	*CCNE1, CDH1, DOT1L, PRKAR1A, TP53, BUB1B, PRSS1*	5.3	Negative
14	Male	52	Peritoneum, pelvic cavity	*TP53*	7.8	Positive
15*	Male	60	LN, liver	*Unknown*	Unknown	Unknown
16	Female	55	Liver	*TP53, IL7R, RICTOR, PREX2, MYC, PTK2, KMT2A, CDK8, FLT3, FLT1, IRS2, CCNE1, SRC, AURKA, GNAS, PTK6, AR, BTK, BCORL1*	5.03	Negative
17	Male	56	Liver, peritoneum	*TP53, MET*	2.13	Negative
18*	Female	53	Liver, LN, peritoneum	*Unknown*	Unknown	Negative
19	Female	57	LN, anastomotic, porta, mesentery	*TP53, LATS1, ERBB4, PTPRO, CTNNA2, APC, HIST1H3B, PLCB1, MLL3*	7.68	Negative
20	Female	32	Bone	*ARID1A, KRAS, RNF43, CDH1, RHOA, KMT2A, KMT2B, PIK3C3, STAG2*	8.2	Negative
21*	Female	56	Peritoneum	*Unknown*	Unknown	Negative
22	Male	46	Liver	*ERBB2, TP53, BAP1, BCORL1, PHF6, RARA, SMARCE1, TOP2A, XIAP*	6.3	Negative
23	Male	61	Lung	*TP53, CDK4, MDM2*	4.96	Negative
24	Male	48	Peritoneum	*NA*	4.5	Negative
25*	Male	73	LN, peritoneum	*Unknown*	Unknown	Positive
26	Female	57	LN, liver	*TP53, RICTOR, ERBB2, RARA, TOP2A, STAT3, RNF43*	11.17	Positive
27	Male	64	Liver	*KRAS, BLK, CCND2, FGF23, FGF6, GATA4, RAD51P1*	11.5	Positive
28	Male	67	Abdominal cavity	*CDK4, ERBB3, MDM2, BRAF, CDK2, FRS2, GRM3, HMGA2, NAB2, STAT6, TSPAN31*	2.1	Negative
29	Male	63	Liver	*ERBB2, TP53, RARA*	6.6	Negative
30	Female	68	Abdominal aorta, LN	*ERBB2, TP53, APC, GNA13, MYC, SOX9, GATA3, TET2*	15	Positive

*, patient without gene variation and TMB results. LN, lymph node; TMB, tumor mutation burden; LN, lymph node.

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Table S3 The regimens, duration of therapy, response data and adverse events

Patient #	Lines of therapy	Anti-PD-1	Anti-angiogenesis	Chemotherapy	Duration of therapy (cycles)	Clinical response	Pathological response	PFS (months)	OS (months)	Adverse events
1	First-line	Pembrolizumab	Regorafenib	XELOX	3	PR	MPR	24	NR	Anaphylaxis
2	First-line	Pembrolizumab	Apatinib	Paclitaxel	5	PR	MPR	18	NR	Leucopenia
3	First-line	Pembrolizumab	Lenvatinib	XELOX	6	PR	pCR	16	NR	Febrile neutropenia
4	First-line	Tislelizumab	Lenvatinib	Paclitaxel + capecitabine	1	SD	Non-MPR	14	NR	Anaphylaxis
5	Third-line	Tislelizumab	Apatinib	XELOX	8	PR	Non-MPR	18	NR	Leucopenia
6	First-line	Pembrolizumab	Regorafenib	XELOX	8	PR	MPR	24	NR	Rash
7	First-line	Pembrolizumab	Regorafenib	XELOX	7	PR	MPR	24	24	Leucopenia
8	First-line	Pembrolizumab	Lenvatinib	Capecitabine	5	PR	MPR	12	NR	Anaphylaxis
9	First-line	Tislelizumab	Anlotinib	Oxaliplatin	5	CR	MPR	26	NR	Vomiting
10	First-line	Pembrolizumab	Lenvatinib	Oxaliplatin	5	PR	MPR	28	NR	Anorexia
11	First-line	Pembrolizumab	Lenvatinib	Oxaliplatin	5	PR	MPR	12	NR	Anemia
12	First-line	Toripalimab	Regorafenib	XELOX	8	PR	/	40	NR	Rash
13	First-line	Camrelizumab	Apatinib	Paclitaxel	1	PD	/	5	5	Leucopenia
14	Second-line	Penpulimab	Anlotinib	Paclitaxel	3	PD	/	10	12	Nausea
15	First-line	Pembrolizumab	Regorafenib	Paclitaxel + S-1	6	PR	/	12	14	Leucopenia
16	First-line	Tislelizumab	Lenvatinib	SOX	8	CR	/	36	NR	Vomiting
17	First-line	Sintilimab	Lenvatinib	XELOX	4	PR	/	10	12	Leucopenia
18	First-line	Camrelizumab	Apatinib	SOX	8	PR	/	16	NR	Anorexia
19	First-line	Sintilimab	Lenvatinib	XELOX	3	CR	/	16	NR	Asthenia
20	First-line	Tislelizumab	Lenvatinib	XELOX	3	SD	/	12	16	Anemia
21	First-line	Sintilimab	Apatinib	Lipusu + Tegafur	12	PD	/	8	10	Nausea
22	First-line	Sintilimab	Lenvatinib	XELOX	3	PR	/	14	NR	Leucopenia
23	First-line	Sintilimab	Lenvatinib	XELOX	3	SD	/	14	18	Nausea
24	First-line	Camrelizumab	Apatinib	TS	8	PR	/	36	NR	Vomiting
25	First-line	Sintilimab	Apatinib	Lipusu + Tegafur	3	PR	/	10	NR	Asthenia
26	First-line	Tislelizumab	Lenvatinib	XELOX	3	PR	/	14	NR	Nausea
27	First-line	Sintilimab	Lenvatinib	Irinotecan	3	PR	/	16	NR	Asthenia
28	First-line	Sintilimab	Lenvatinib	Oxaliplatin + capecitabine	3	PR	/	18	NR	Anemia
29	First-line	Tislelizumab	Apatinib	Lipusu + capecitabine	14	PD	/	12	12	Nausea
30	First-line	Pembrolizumab	Lenvatinib	Lipusu + tegafur	15	PR	/	14	NR	Vomiting

PFS, progression-free survival; OS, overall survival; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; MPR, major pathological response; pCR, pathological complete response; NR, not recorded.

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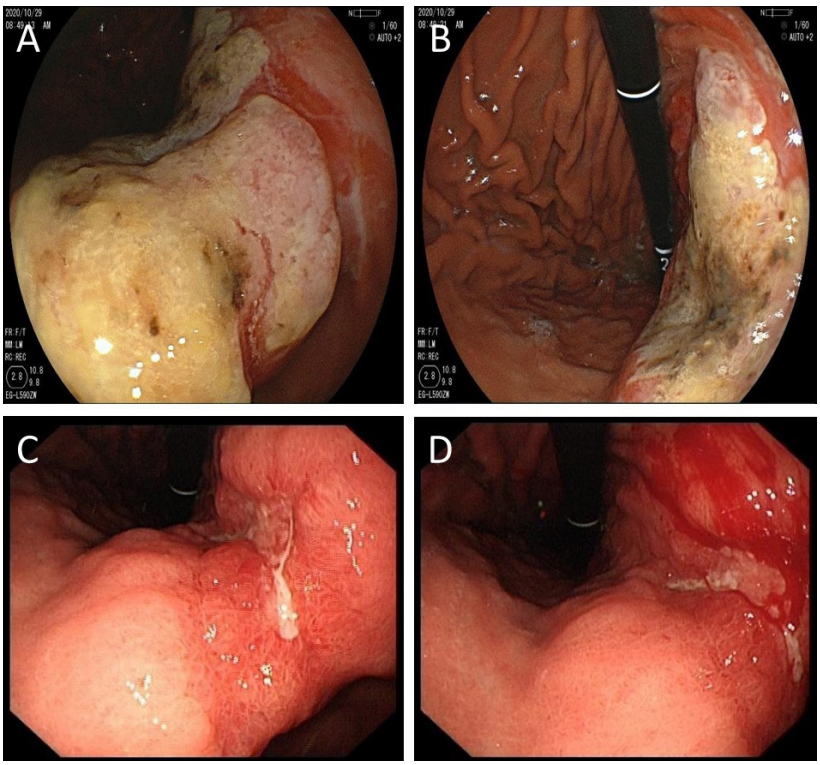

Figure S1 Gastroscopy findings of patient 1 before and after treatment of combination regimens. (A,B) Lesions in the lesser curvature of the stomach with surface erosion could be observed before treatment; (C,D) lesions disappear largely after 3 cycles of treatment.

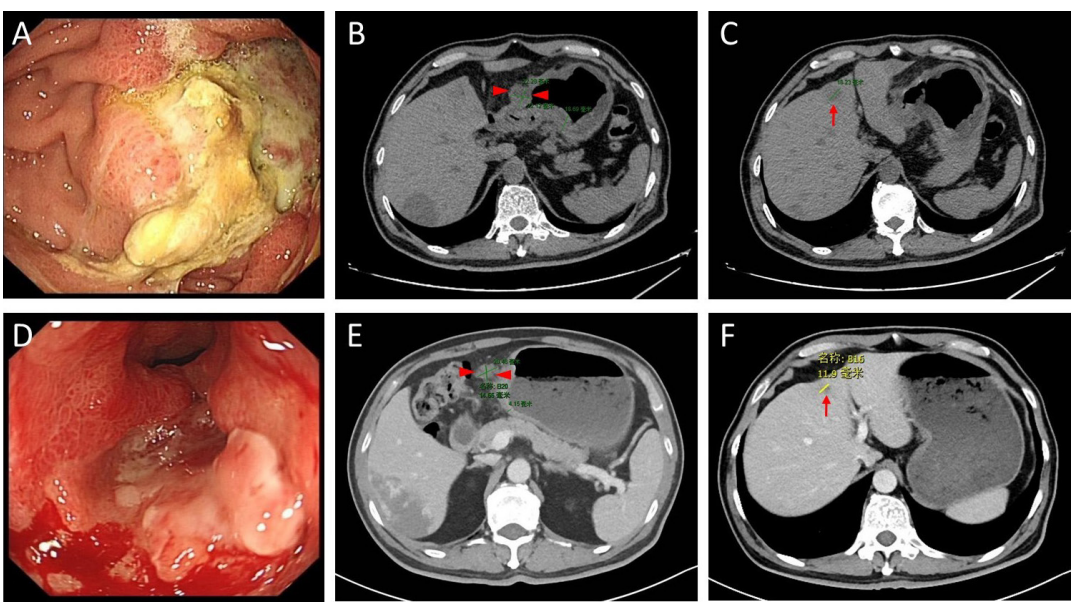

Figure S2 Gastroscopy and CT images of patient 2 before and after treatment of combination regimens. Cancerous ulcers in the body of the stomach (A), a 22.28 mm ×22.12 mm perigastric lymph node metastasis (red arrowheads), thickened gastric wall (B) and liver metastases (red arrow) (C) could be observed before treatment. After 5 cycles of treatment, cancerous ulcers disappeared largely (D), the perigastric lymph node metastasis shrank to 20.48 mm ×14.66 mm (E), and one case of liver metastasis shrank (F). CT, computed tomography.

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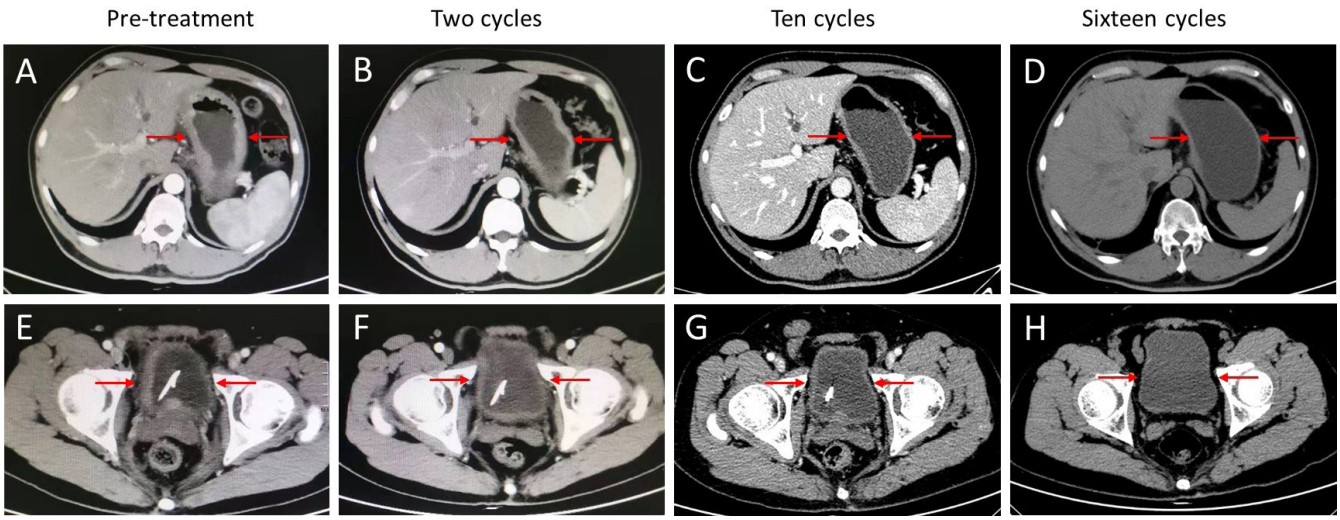

Figure S3 CT findings of patient 6 before and after treatment of combination regimens. Aberrant thickened stomach wall marked by red arrow (A) and bladder wall marked by red arrow (E) could be observed. After combination treatment, the thickened stomach wall (B-D) and bladder wall (F-H) gradually became thinner. CT, computed tomography.

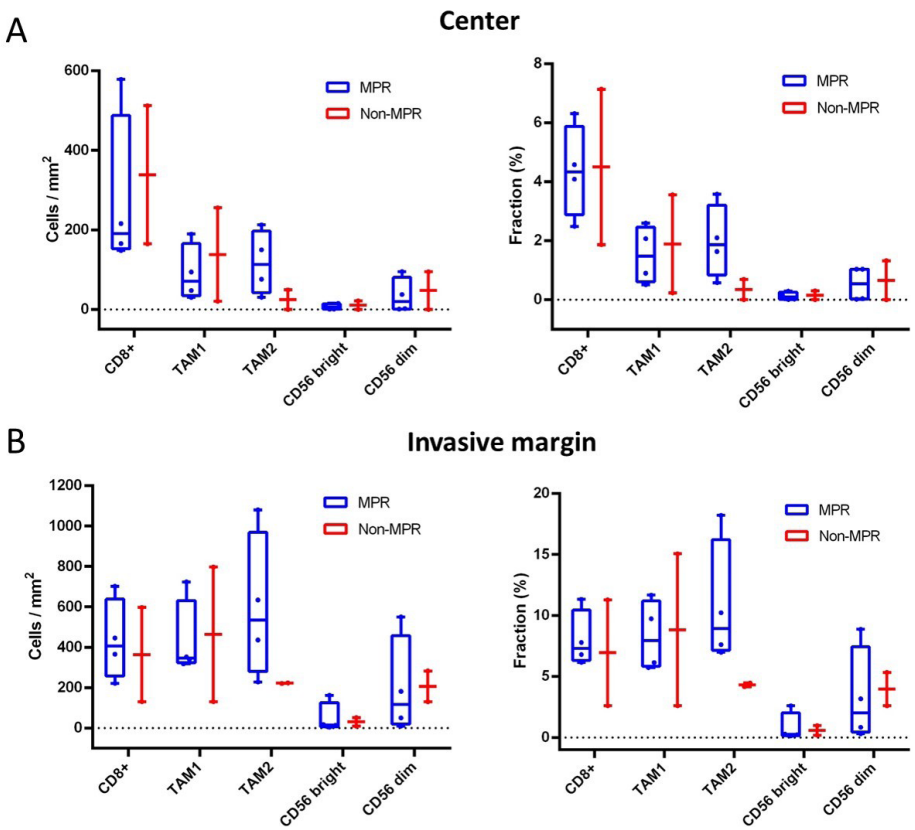

Figure S4 Immune cells in center or invasive margin between MPR and non-MPR. (A) Comparison of immune cells in tumor center between MPR and non-MPR; (B) Comparisons of immune cells in invasive margin between MPR and non-MPR. MPR, major pathological response.

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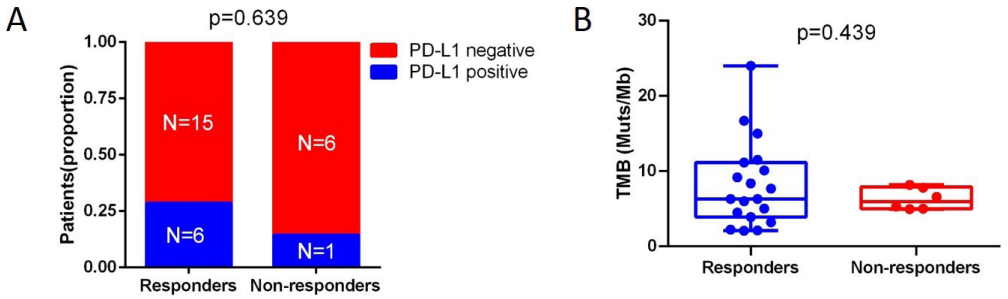

Figure S5 PD-L1 expression and TMB were not associated with the efficacy of combination therapy. (A) Dichotomized association between response to combination treatment and PD-L1 expression was analyzed (N=28, p=0.639); (B) poor correlation between response to combination treatment and TMB level was found (N=25, t=0.787, p=0.439). PD-L1, programmed death ligand 1; TMB, tumor mutation burden.

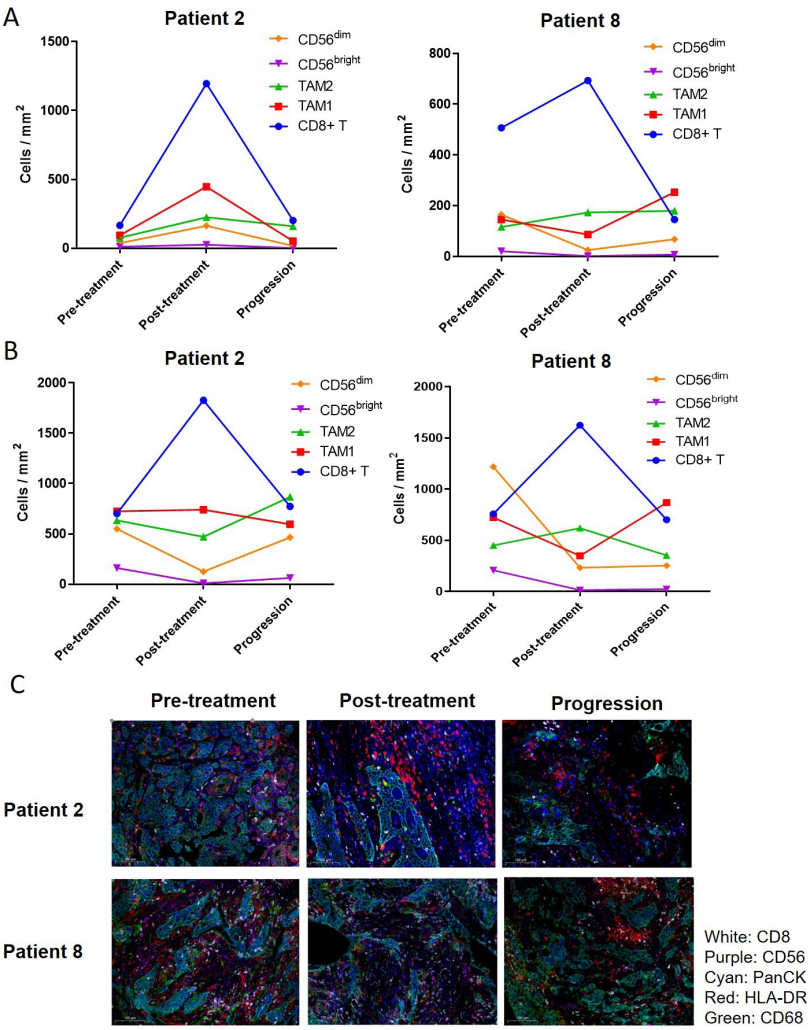

Figure S6 Variation of immune cells of two patients at three time points (pre-treatment, post-treatment, progression). The abundance of immune cells of two patients at three time points in center (A) and invasive margin (B). (C) The images of mIF of two patients at three time points (300× magnification). mIF, multiplex immunofluorescence.

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