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Original Article

The Optimal Tumor Mutational Burden Cutoff Value as a Novel Marker for Predicting the Efficacy of Programmed Cell Death-1 Checkpoint Inhibitors in Advanced Gastric Cancer

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ABSTRACT

Purpose: The optimal tumor mutational burden (TMB) value for predicting treatment response to programmed cell death-1 (PD-1) checkpoint inhibitors in advanced gastric cancer (AGC) remains unclear. We aimed to investigate the optimal TMB cutoff value that could predict the efficacy of PD-1 checkpoint inhibitors in AGC.
Materials and Methods: Patients with AGC who received pembrolizumab or nivolumab between October 1, 2020, and July 27, 2021, at Samsung Medical Center in Korea were retrospectively analyzed. The TMB levels were measured using a next-generation sequencing assay. Based on receiver operating characteristic curve analysis, the TMB cutoff value was determined.
Results: A total 53 patients were analyzed. The TMB cutoff value for predicting the overall response rate (ORR) to PD-1 checkpoint inhibitors was defined as 13.31 mutations per megabase (mt/Mb) with 56% sensitivity and 95% specificity. Based on this definition, 7 (13.2%) patients were TMB-high (TMB-H). The ORR differed between the TMB-low (TMB-L) and TMB-H (8.7% vs. 71.4%, P=0.001). The progression-free survival and overall survival (OS) for 53 patients were 1.93 (95% confidence interval [CI], 1.600–2.268) and 4.26 months (95% CI, 2.992–5.532). The median OS was longer in the TMB-H (20.8 months; 95% CI, 2.292–39.281) than in the TMB-L (3.31 months; 95% CI, 1.604–5.019; P=0.049).
Conclusions: The TMB cutoff value for predicting treatment response in AGC patients who received PD-1 checkpoint inhibitor monotherapy as salvage treatment was 13.31 mt/Mb. When applying the programmed death ligand-1 status to TMB-H, patients who would benefit from PD-1 checkpoint inhibitors can be selected.

Keywords: Gastric cancer; Immune checkpoint inhibitor; PD-1 inhibitor

INTRODUCTION

Programmed cell death-1 (PD-1) checkpoint inhibitors have proven to be effective in the treatment of locally advanced unresectable or metastatic gastric cancer (advanced gastric cancer

Journal of Gastric Cancer

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Conflict of Interest
No potential conflict of interest relevant to this article was reported.

Author Contributions
Conceptualization: K.S.T.; Data curation: J.Y., J.S.Y.; Formal analysis: J.J.Y.; Funding acquisition: K.S.T.; Investigation: K.S.T.; Methodology: L.S.H.; Project administration: J.J.Y.; Resources: L.J.; Software: J.J.Y.; Supervision: K.W.K.; Validation: K.W.K.; Visualization: J.J.Y.; Writing - original draft: J.J.Y.; Writing - review & editing: J.Y., J.S.Y.

[AGC]). Nivolumab combined with capecitabine or 5-fluorouracil plus oxaliplatin (XELOX or FOLFOX) is currently indicated as the first-line treatment for programmed death ligand-1 (PD-L1) combined positive score (CPS) ≥5 and human epidermal growth factor receptor 2-negative AGC [1,2]. In Korea, pembrolizumab was approved as the second-line or subsequent treatment for microsatellite instability-high (MSI-H) or mismatch repair-deficient (dMMR) AGC based on the KEYNOTE-158 trial [3,4]. Nivolumab monotherapy is also recommended as a third-line therapy based on a randomized phase 3 ATTRACTION-2 trial [2,5].

However, the predictive markers for the efficacy of PD-1 checkpoint inhibitor monotherapy in AGC have not yet been sufficiently established [6-8]. There is no clear standard for identifying patients who are likely to benefit from PD-1 checkpoint inhibitors does not exist. Although PD-L1 and MSI are considered novel markers for PD-1 checkpoint inhibitors, their roles have not yet been determined [9-11].

Recent advances in whole-exome sequencing (WES) and next-generation sequencing (NGS) of multiple genes have defined the tumor biology of AGC [12-14]. Furthermore, molecular targeted therapies and immunotherapies, including PD-1 checkpoint inhibitors, have been developed based on the NGS findings [14-17]. In addition, a high level of tumor mutational burden (TMB), the total number of non-synonymous mutations in the tumor-coding regions, is a novel predictive marker for immunotherapy or a prognostic marker in various tumor types [18-20]. Some somatic mutations in tumor DNA are associated with tumor neoantigens, which are targets of the immune system [21-23]. Therefore, tumors with a high TMB are likely to have more neoantigens and respond better to immunotherapies that activate T cell immunity. Although TMB is regarded as a biomarker for the response to immunotherapy in several solid tumors [24-26], the optimal value for defining TMB-high (TMB-H) is insufficient for AGC.

Therefore, in the present study, the optimal TMB cutoff value that could predict the efficacy of PD-1 checkpoint inhibitor monotherapy as a salvage treatment for AGC was determined.

MATERIALS AND METHODS

Patients

This retrospective study included patients with AGC who received pembrolizumab or nivolumab between October 1, 2020, and July 27, 2021, at Samsung Medical Center in Korea. All patients underwent a tissue biopsy, and tissues were used for NGS diagnostic platform, including TMB, with the Illumina's TruSight™ Oncology 500 (TSO 500) assay (Illumina Inc., San Diego, CA, USA). Clinicopathological characteristics were collected from electronic medical records. PD-L1 was detected using the immunohistochemical assay PD-L1 antibody 22C3 pharmDx. Tumors with a CPS of ≥1 were defined as positive PD-L1 expression. Epstein-Barr virus was detected using in situ hybridization. The Institutional Review Board (IRB) of Samsung Seoul Medical Center approved this study (IRB No. 2022-12-068), which was conducted in accordance with the Declaration of Helsinki.

NGS and TMB calculation

The tumor samples were obtained at the time of diagnosis of advanced or metastatic AGC, and tissues were formalin-fixed paraffin-embedded. The TSO 500 Kit was used for DNA library preparation and enrichment following the manufacturer's instructions. Post-

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jgc Journal of Gastric Cancer

enrichment libraries were quantified, pooled, and sequenced using TSO 500 (Illumina Inc.). The quality of TSO 500 sequencing runs was assessed using an Illumina Sequencing Analysis Viewer. Sequencing data were analyzed using TSO 500 Local App version 1.3.0.39. The TSO 500 is a comprehensive tumor profiling assay that measures biomarkers, including single nucleotide variants, copy number variants, indels, fusions, splice variants, TMB, and MSI. The TMB scores are reported as mutations per megabase (mt/Mb) sequenced and TMB levels.

Defining the TMB cutoff value

The TMB was measured using an NGS-based assay and reported as mt/Mb sequenced. Receiver operating characteristic (ROC) curve analysis was used to measure the diagnostic ability of TMB to predict the overall response rate (ORR) of anti-PD-1 therapy. The optimal TMB cutoff value was determined as the point at which Youden's index was the maximum, defined as follows [27-29].

$$\text{Youden's Index J} = \text{Sensitivity} + \text{Specificity} - 1.$$

Statistical analysis

Treatment response to anti-PD-1 therapy was evaluated radiologically based on the Response Evaluation Criteria in Solid Tumors guidelines, version 1.1.

Descriptive statistics were used to summarize the patient and tumor characteristics and treatment histories. Associations between TMB status and tumor response, including the ORR and disease control rate (DCR), were analyzed using t-test, and categorical variables were analyzed using the χ^2 test and Fisher's exact test. The progression-free survival (PFS) and overall survival (OS) were analyzed using the Kaplan-Meier method, with medians and corresponding 95% confidence intervals (CIs). The log-rank test was used to compare the differences in PFS and OS between the TMB-H and TMB-low (TMB-L) groups. The P-values of <0.05 were considered to indicate statistical significance. All statistical analyses were performed using SPSS version 25 (IBM Corp., Armonk, NY, USA).

RESULTS

Patient characteristics

A total of 53 patients with AGC with quantifiable TMB were analyzed in this study. The median age at diagnosis was 56 years, and 60.4% of the patients were female. Overall, patients were treated with pembrolizumab (n=21, 39.6%) or nivolumab (n=32, 60.4%). The median TMB of patients was 6.3 mt/Mb and ranged from 0.8–107.1 mt/Mb. Four (7.5%) patients harbored MSI-H tumors, and PD-L1 expression was detected in 28 of 39 (71.8%) patients (**Table 1**).

Definition of the TMB cutoff value

Based on the ROC curve and Youden's index analyses, the area under the ROC curve was 0.7285, indicating excellent discrimination. As shown in **Fig. 1**, the optimal TMB cutoff value that showed the maximum sensitivity and specificity for predicting the ORR of immunotherapy in AGC was ≥13.31 mt/Mb. Based on this cutoff value, the sensitivity and specificity were 55.6% and 95.5%, respectively. Based on this definition of the TMB cutoff value, 46 (86.8%) patients were TMB-L, and 7 (13.2%) were TMB-H. The incidence rates of MSI and TMB are shown in **Table 2**.

https://doi.org/10.5230/jgc.2023.23.e29

jgc Journal of Gastric Cancer

Table 1. Patients' baseline characteristics

Characteristics	Total (n=53)
Age (yr)	56 (28–84)
Sex	
Male	21 (39.6)
Female	32 (60.4)
ECOG performance status	
0–1	37 (69.9)
≥2	16 (30.2)
MSI status	
MSS	49 (92.5)
MSI-H	4 (7.5)
EBV (n=43)	
Negative	37 (86.0)
Positive	6 (14.0)
PD-L1 (IHC 22C3) (n=39)	
Negative	11 (28.2)
Positive	28 (71.8)
C-erbB-2 (n=50)	
Negative	47 (94.0)
Positive	3 (6.0)
PD-1 checkpoint inhibitor	
Nivolumab	32 (60.4)
Pembrolizumab	21 (39.6)
No. of chemotherapies before immunotherapy	
2	44 (83.0)
3	9 (17.0)

Values are presented as median (range) or number (%).
ECOG = Eastern Cooperative Oncology Group; MSI = microsatellite instability; MSS = microsatellite stable; MSI-H = microsatellite instability-high; EBV = Epstein-Barr virus; PD-L1 = programmed death ligand-1; IHC = immunohistochemistry; PD-1 = programmed cell death-1.

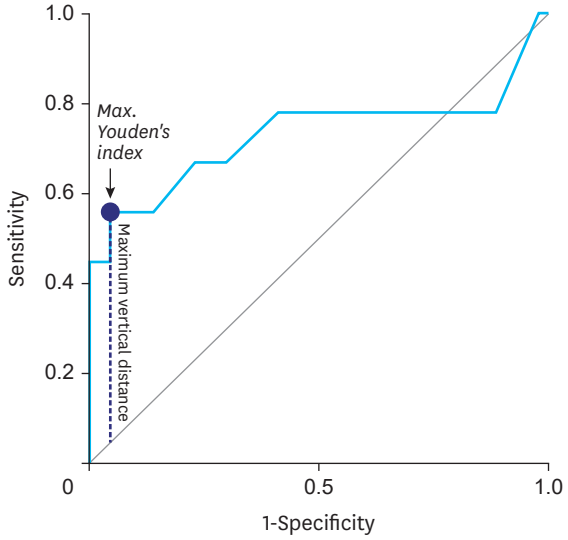

Fig. 1. Receiver operating characteristic curve for predicting the overall response rate of immunotherapy in advanced gastric cancer. The tumor mutational burden cut-off value with maximum sensitivity and specificity is 13.31 mt/Mb, representing sensitivity of 55.6% and specificity of 95.5%.

Table 2. Incidence of MSI status and TMB

No. of patients	MSS	MSI-H	Total	Value
TMB-L	46 (100)	0 (0)	46 (100)	P-value=0.000
TMB-H	3 (42.9)	4 (57.1)	7 (100)	OR, 0.429 (95% CI, 0.182–1.008)

Values are presented as number (%). The P-value was calculated using Fisher's exact test. The 95% CI of the OR included 1.
MSI = microsatellite instability; TMB = tumor mutational burden; MSS = microsatellite stable; H = high; L = low; OR = odds ratio; CI = confidence interval.

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Comparison of the efficacy of ICIs based on the TMB status

The ORR for all enrolled patients was 17% (95% CI, 8.1%–29.8%). Patients with TMB-H showed a better tumor response than those with TMB-L. The ORR to PD-1 checkpoint inhibitors in patients with TMB-H was 71.4% (5 of 7 with partial response; 95% CI, 29.0–96.3), and the ORR in patients with TMB-L was 8.7% (4 of 46 with partial response; 95% CI, 2.4–20.8; P=0.001; **Table 3**, **Fig. 2**). The DCR was not statistically significantly different between patients with TMB-L and TMB-H (30.4% [95% CI, 29.0–96.3] vs. 71.4% [95% CI, 17.7–45.8]; P=0.084; **Table 3**). In the PD-L1 CPS ≥1 group, TMB-H showed a higher ORR of 66.7% (4 of 6; 95% CI, 22.3–95.7) than TMB-L with 13.6% (3 of 22; 95% CI, 2.9–34.9; P=0.021; **Table 4**). However, when patients with a PD-L1 CPS of ≥5 are analyzed separately, the ORR was superior in TMB-H numerically, but there was no statistically significant difference (18.2% [95% CI, 2.3–51.8] vs. 66.7% [95% CI, 9.4–99.2]; P=0.538; **Table 5**).

The PFS and OS in 53 patients were 1.93 (95% CI, 1.600–2.268) and 4.26 (95% CI, 2.992–5.532) months, respectively. The median PFS in patients with TMB-H was 18.6 months (95% CI, 4.636–32.479), and that in patients with TMB-L was 1.9 months (95% CI, 1.499–2.370; P=0.040; **Fig. 3A**). The OS was longer in patients with TMB-H (median, 20.8 months; 95% CI, 2.292–39.281) than in patients with TMB-L (median, 3.3 months; 95% CI, 1.604–5.019; P=0.049; **Fig. 3B**).

Table 3. Comparison of programmed cell death-1 checkpoint inhibitor efficacy in patients with advanced gastric cancer based on TMB

Characteristics	TMB <13.31 mt/Mb (n=46)	TMB ≥13.31 mt/Mb (n=7)	P-value*
ORR	8.7 (2.4–20.8)	71.4 (29.0–96.3)	0.001†
DCR	30.4 (17.7–45.8)	71.4 (29.0–96.3)	0.084
Best response			
Complete response	0 (0.0)	0 (0.0)	
Partial response	4 (8.7)	5 (71.4)	
Stable disease	10 (21.7)	0 (0.0)	
Progressive disease	32 (69.6)	2 (28.6)	

Values are presented as number (%) or % (95% confidence interval). Responses were assessed according to the revised Response Evaluation Criteria in Solid Tumors guidelines (version 1.1) by the *European Journal of Cancer*. The 95% CI was calculated using the Clopper-Pearson method.
TMB = tumor mutational burden; ORR = overall response rate; DCR = disease control rate.
*These P-values were calculated using Fisher's exact test.
†The P-value was calculated using the Pearson χ^2 test.

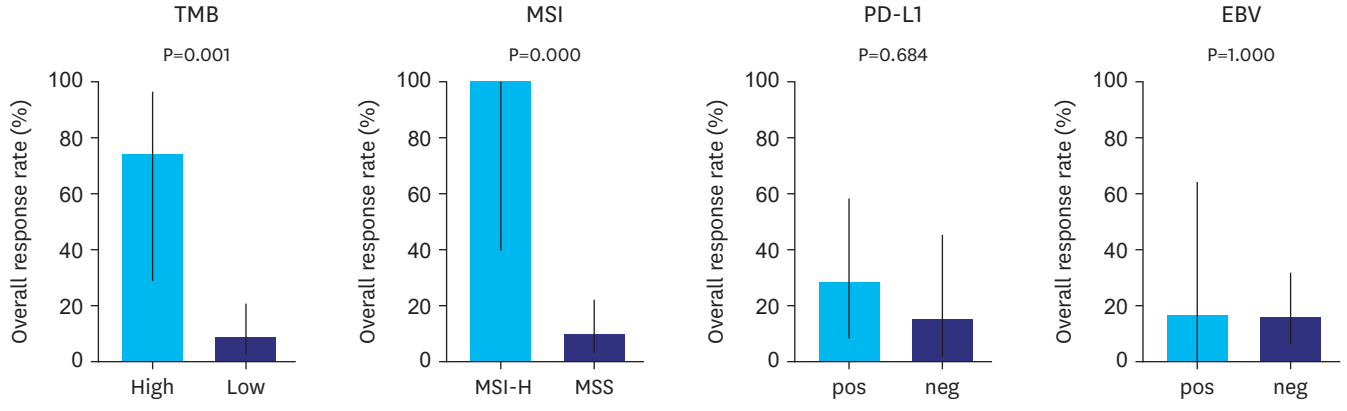

Fig. 2. Objective response rate in relation to TMB, MSI status, PD-L1 expression, and EBV status. TMB-high 71.9% (95% CI, 29.0–96.3) versus TMB-low 8.7% (95% CI, 2.4–20.8; P=0.001), MSI-H 100% (95% CI, 39.8–100) versus MSS 10.2% (95% CI, 3.4–22.2; P=0.000), PD-L1-positive 25% (95% CI, 10.7–44.9) versus PD-L1-negative 0% (95% CI, 0.0–28.5; P=0.159), and EBV-positive 16.7% (95% CI, 0.4–64.1) versus EBV-negative 16.2% (95% CI, 6.2–32; P=1.000).
TMB = tumor mutational burden; MSI = microsatellite instability; PD-L1 = programmed death ligand-1; EBV = Epstein-Barr virus; CI = confidence interval; MSI-H = microsatellite instability-high; MSS = microsatellite stable; pos = positive; neg = negative.

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Table 4. Comparison of programmed cell death-1 checkpoint inhibitors efficacy based on TMB in patients with advanced gastric cancer whose PD-L1 CPS score was ≥1

Characteristics	PD-L1 CPS ≥1		P-value*
	TMB <13.31 mt/Mb (n=22)	TMB ≥13.31 mt/Mb (n=6)	
ORR	13.6 (2.9–34.9)	66.7 (22.3–95.7)	0.021†
DCR	31.8 (13.9–54.9)	66.7 (22.3–95.7)	0.174
Best response			
Complete response	0 (0.0)	0 (0.0)	
Partial response	3 (13.6)	4 (66.7)	
Stable disease	4 (18.2)	0 (0.0)	
Progressive disease	15 (68.2)	2 (33.4)	

Values are presented as number (%) or % (95% confidence interval). Responses were assessed according to the revised Response Evaluation Criteria in Solid Tumors guidelines (version 1.1) by the *European Journal of Cancer*.
CPS = combined positive score; DCR = disease control rate; ORR = overall response rate; PD-L1 = programmed death ligand-1; TMB = tumor mutational burden.
*These P-values were calculated using Fisher's exact test.
†The P-value was calculated using the Pearson χ^2 test.

Table 5. Comparison of programmed cell death-1 checkpoint inhibitors efficacy based on TMB in patients with advanced gastric cancer whose PD-L1 CPS score was ≥5

Characteristics	PD-L1 CPS ≥5		P-value*
	TMB <13.31 mt/Mb (n=11)	TMB ≥13.31 mt/Mb (n=3)	
ORR	18.2 (2.3–51.8)	66.7 (9.4–99.2)	0.176
DCR	36.4 (10.9–69.2)	66.7 (9.4–99.2)	0.538
Best response			
Complete response	0 (0.0)	0 (0.0)	
Partial response	2 (18.2)	2 (66.7)	
Stable disease	2 (18.2)	0 (0.0)	
Progressive disease	7 (63.6)	1 (33.3)	

Values are presented as number (%) or % (95% confidence interval). Responses were assessed according to the revised Response Evaluation Criteria in Solid Tumors guidelines (version 1.1) by the *European Journal of Cancer*.
CPS = combined positive score; DCR = disease control rate; ORR = overall response rate; PD-L1 = programmed death ligand-1; TMB = tumor mutational burden.
*These P-values were calculated using Fisher's exact test.

DISCUSSION

The ROC curve analysis showed that the TMB cutoff value for predicting tumor response in patients with AGC who received PD-1 checkpoint inhibitors was 13.31 mt/Mb. Furthermore, when applying TMB-H, in addition to PD-L1 expression, patients who would benefit more from PD-1 checkpoint inhibitors could be selected. These findings may be helpful for the use of PD-1 checkpoint inhibitors in routine clinical practice.

The reported optimal threshold for defining TMB-H differed with various studies, tumor types, pre-analytic variables, bioinformatics, and test methods [18,20,24-26,30]. In addition, most studies were mainly conducted in patients with lung cancer and melanoma and relatively few in AGC [30]. In the present study, the TMB of 53 patients was evaluated using the same NGS method to decrease heterogeneity. The NGS diagnostic platform, Illumina's TSO 500 assay, has a diagnostic value similar to that of WES [31].

However, the clinical significance of the TMB in AGC remains unclear. Wang et al. [32] selected a cutoff of the top 20% TMB (12 mt/Mb) assessed using WES and toripalimab, a humanized PD-1 antibody. Patients with TMB-H showed improved clinical outcome. In a subset analysis of the ATTRACTION-2 phase III trial, TMB-H and TMB-L were classified as having a median value of 8.2 mt/Mb using an NGS panel, and the 2 groups showed no difference in the efficacy of nivolumab as third- or later-line therapy for AGC [11]. Mishima

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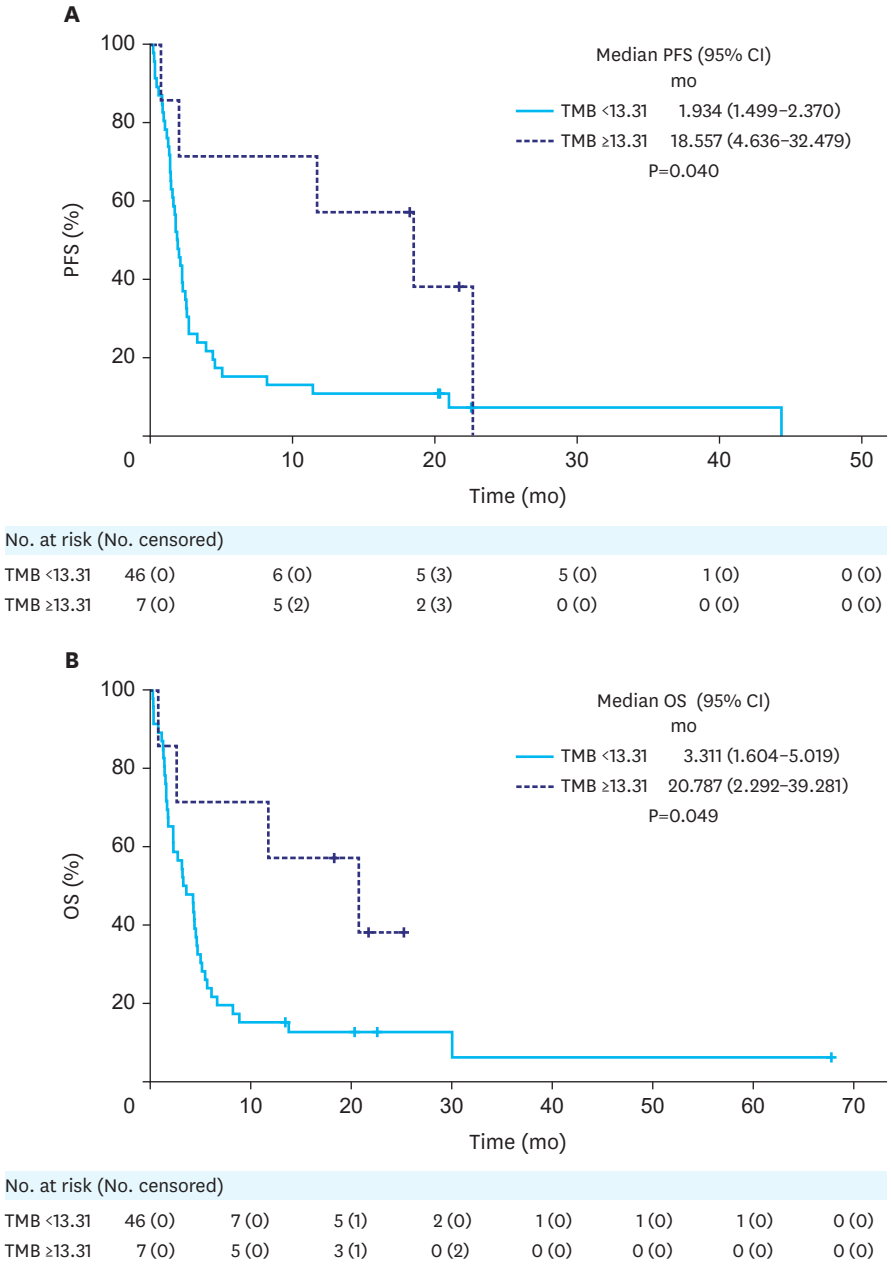

Fig. 3. Kaplan-Meier estimates of time to PFS and OS based on TMB 13.31 mt/Mb. The median follow-up duration was 4.26 months (range, 0.3–67.8 months). (A) PFS for TMB-high and TMB-low. The median PFS for TMB-high patients was 18.6 months (95% CI, 4.636–32.479) and TMB-low patients was 1.9 months (95% CI, 1.499–2.370; P=0.040). (B) OS for TMB-high and TMB-low. The median OS for TMB-high patients was 20.8 months (95% CI, 2.292–39.281) and TMB-low patients was 3.3 months (95% CI, 1.604–5.019; P=0.049).
OS = overall survival; TMB = tumor mutational burden; PFS = progression-free survival; CI = confidence interval.

et al. [33] defined TMB-H as >10 mt/Mb based on an NGS panel in AGC and reported that TMB-H was not associated with response to nivolumab. In the present study, the cutoff value for TMB-H was determined to be 13.31 mt/Mb. Patients with TMB-H showed better ORR (71.4% vs. 8.7%, P=0.001), PFS (18.6 vs. 1.9 months, P=0.040), and OS (20.8 vs. 3.3 months, P=0.049) than patients with TMB-L. Compared with previous studies, a relatively larger number of patients were included in the present study, and the cutoff value was identified using the ROC curve analysis by calculating sensitivity and specificity.

https://doi.org/10.5230/jgc.2023.23.e29

jgc Journal of Gastric Cancer

The patients with TMB-H and positive PD-L1 expression had a higher ORR (66.7%; 95% CI, 22.3–95.7), which was in agreement with a previous study [32]. TMB status and PD-L1 expression are independent biomarkers for predicting the efficacy of PD-1 checkpoint inhibitors [18,30,34]. In gastric cancer, a cutoff value of PD-L1 CPS ≥5 is associated with an increased efficacy of PD-1 checkpoint inhibitors in combination with oxaliplatin-based chemotherapies [1,2,35]. However, in PD-1 checkpoint inhibitor monotherapy as a salvage treatment, the effect of PD-L1 is not clear. In the present study, defining the PD-L1 positive as a CPS of ≥1, the PD-L1-positive and TMB-H group exhibited better efficacy than the PD-L1-positive and TMB-L group. This means that among the patients for whom PD-1 checkpoint inhibitors were not indicated because the PD-L1 CPS was <5, additional patients who were expected to be effective could be found using TMB. Furthermore, the limitation of low sensitivity when the cutoff value of TMB is set to 13.31 mt/Mb could be corrected when used with PD-L1.

A higher mutational load is associated with higher neoantigens in tumor immunity, resulting in the recognition of the tumor as foreign and causing an immune response. Thus, hypermutated tumors, such as MSI-H or dMMR tumors, show a better response to PD-1 checkpoint inhibitors. MSI-H is associated with high TMB, which accounts for the majority of MSI-H tumors [36]. In the present study, all MSI-H tumors were TMB-H; however, only 57% (4 of 7) of the TMB-H tumors were MSI-H. In addition, one of 3 patients with both TMB-H and microsatellite stable showed a partial response as the best response and 11.8 months of PFS. Therefore, TMB should be assessed independently of other biomarkers such as MSI status when considering PD-1 checkpoint inhibitors.

The present study has several limitations. First, this was a retrospective study conducted at a single center. There were more female than male patients, which is different from previous reports, suggesting a potential for selection bias. Second, the type (i.e., pembrolizumab or nivolumab) and lines of PD-1 checkpoint inhibitors, as well as sequential treatments, were heterogeneous. Third, only patients who received PD-1 checkpoint inhibitors as monotherapy were included. Finally, because the study cohort was small, additional analyses, such as ROC curve analysis using a combination of PD-L1 and TMB, were not conducted.

In conclusion, in the present study, the TMB cutoff value for predicting the tumor response in patients with AGC who received PD-1 checkpoint inhibitor monotherapy as a salvage treatment was 13.31 mt/Mb based on the ROC curve analysis. Furthermore, when PD-L1 status is combined with TMB-H, patients who could benefit from PD-1 checkpoint inhibitors can be selected. These results may be helpful in the use of PD-1 checkpoint inhibitors in routine clinical practice.

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