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ORIGINAL ARTICLE

Epigenomic promoter alterations predict for benefit from immune checkpoint inhibition in metastatic gastric cancer

R. Sundar[1,2,3†], K. K. Huang[2†], A. Qamra[2], K.-M. Kim[4], S. T. Kim[5], W. K. Kang[5], A. L. K. Tan[2], J. Lee[5*] & P. Tan[2,6,7,8*]

[1]Department of Haematology-Oncology, National University Health System, Singapore; [2]Cancer and Stem Cell Biology Program, Duke-NUS Medical School, Singapore; [3]Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore; [4]Department of Pathology and Translational Genomics; [5]Division of Hematology-Oncology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea; [6]Biomedical Research Council, Agency for Science, Technology and Research, Singapore; [7]Cancer Science Institute of Singapore, National University of Singapore, Singapore; [8]SingHealth/Duke-NUS Institute of Precision Medicine, National Heart Centre Singapore, Singapore, Singapore

Correspondence to: Prof. Patrick Tan, Duke-NUS Medical School, 8 College Road, Singapore 169857, Singapore. Tel: +65-6516-1783; Fax: +65-6221-2402; E-mail: gmstanp@duke-nus.edu.sg
Prof. Jeeyun Lee, Division of Hematology-Oncology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135–710, South Korea. Tel: +82-2-3410-1779; Fax: +82-2-3410-1754; E-mail: jyunlee@skku.edu
[†]Both authors contributed equally as first authors.

Background: Utilization of alternative transcription start sites through alterations in epigenetic promoter regions causes reduced expression of immunogenic N-terminal peptides, which may facilitate immune evasion in early gastric cancer. We hypothesized that tumors with high alternate promoter utilization would be resistant to immune checkpoint inhibition in metastatic gastric cancer.

Patients and methods: Two cohorts of patients with metastatic gastric cancer treated with immunotherapy were analyzed. The first cohort ($N = 24$) included patients treated with either nivolumab or pembrolizumab. Alternate promoter utilization was measured using the NanoString® (NanoString Technologies, Seattle, WA, USA) platform on archival tissue samples. The second cohort was a phase II clinical trial of patients uniformly treated with pembrolizumab ($N = 37$). Fresh tumor biopsies were obtained, and transcriptomic analysis was carried out on RNAseq data. Alternate promoter utilization was correlated to T-cell cytolytic activity, objective response rate and survival.

Results: In the first cohort 8 of 24 (33%) tumors were identified to have high alternate promoter utilization (AP_{high}), and this was used to define the AP_{high} tertile of the second cohort (13 AP_{high} of 37). AP_{high} tumors exhibited decreased markers of T-cell cytolytic activity and lower response rates (8% versus 42%, $P = 0.03$). Median progression-free survival was lower in the AP_{high} group (55 versus 180 days, $P = 0.0076$). In multivariate analysis, alternative promoter utilization was an independent predictor of immunotherapy survival [hazard ratio 0.29, 95% confidence interval 0.099–0.85, $P = 0.024$). Analyzing tumoral evolution through paired pre-treatment and post-treatment biopsies, we observed consistent shifts in alternative promoter utilization rate associated with clinical response.

Conclusion: A substantial proportion of metastatic gastric cancers utilize alternate promoters as a mechanism of immune evasion, and these tumors may be resistant to anti-PD1 immune checkpoint inhibition. Alternate promoter utilization is thus a potential mechanism of resistance to immune checkpoint inhibition, and a novel predictive biomarker for immunotherapy.

Trial Registration: ClinicalTrials.gov Identifier: NCT#02589496

Key words: epigenetic alternate promoter, immune checkpoint inhibition, pembrolizumab, immunotherapy, gastric cancer

Introduction

Gastric cancer is a leading cause of death and patients with metastatic disease have a survival that remains below 2 years despite current therapies. The Cancer Genome Atlas (TCGA) group recently described four subtypes for gastric cancer—high microsatellite instability (MSI), Epstein–Barr virus positive (EBV), genomically stable (GS) and chromosomally unstable (CIN) [1]. CIN and GS subtypes constitute a majority of tumors, with EBV and MSI being relatively rare. These classifications, however, have yet to be incorporated into clinical practice, and currently the only biomarkers of clinical value in metastatic gastric cancer are Her2, MSI and PD-L1 status [2–4]. Among treatment modalities, immune checkpoint inhibition (ICI) has made significant breakthroughs in several tumor types including gastric cancer. Pembrolizumab and nivolumab are monoclonal antibodies that bind to and block interactions between the immune checkpoint programmed cell death protein - 1 (PD-1) and its ligands (e.g. PD-L1, PD-L2). These ICI drugs are currently approved for patients who have failed at least two prior lines of systemic chemotherapy in metastatic gastric cancer [5, 6].

A recent phase II study of single-agent pembrolizumab in metastatic gastric cancer described remarkable and durable response in the MSI and EBV subtypes [7]. In MSI tumors, ICI activity is associated with high tumor mutation load and neoantigen formation [8], with response rates for MSI gastric cancer ranging from 57% to 86% [3, 4, 7]. EBV subtype tumors are associated with tumoral immune-cell infiltration and high expression of PD-L1 in a majority of tumors [9], and in the phase II study described above, 100% of EBV-positive gastric cancers responded to pembrolizumab [7]. Although the sample size was small ($n = 6$), and further validation studies are required, it is thus possible that testing for EBV status may be incorporated into clinical workflows in the future to select patients for immunotherapy. Notably, while not as sensitive to ICI as EBV and MSI tumors, responses have also been reported in the CIN/GS subtypes, suggesting other mechanisms of response and primary resistance to ICI which are currently unexplored. Biomarker selection of gastric cancer for ICI is thus an area of urgent unmet clinical need, in particular for the CIN/GS subtypes.

Epigenetic activation of promoter elements allows for regulation of genomic transcription and plays a role in normal biologic function. Usage of alternate promoters allows initiation of transcription at different transcription start sites (TSS), resulting in production of altered mRNA transcripts and protein isoforms. Recently, somatic recruitment of alternate promoters in gastric cancer has been described as a mechanism of tumor immune-editing [10]. Specifically, reduced production of high-affinity major histocompatibility complex class I binding gastric cancer peptides through loss of immunogenic N-terminal peptides has been proposed as a mechanism of immune evasion, allowing nascent tumor formation. However, because this study was limited to early-stage gastric cancer (surgical resection samples), the applicability of these findings to metastatic (late-stage) gastric cancer remains unclear. Further, we hypothesized that metastatic gastric cancers with high alternate promoter utilization would be resistant to anti-PD-1 ICI.

Here, in two independent metastatic gastric cancer patient cohorts treated with ICIs, we monitored alternative promoter utilization using two different technology platforms (Nanostring and RNA-sequencing). We confirmed that similar relationships between alternative promoter utilization and intra-tumor immunity exist in metastatic gastric cancers. Moreover, by analyzing gastric tumors corresponding to the aforementioned phase II trial [7], we show that tumors with high alternate promoter utilization, and thereby lower predicted immunogenicity, are more resistant to ICI in a manner independent of previous ICI biomarkers such as mutational burden and PD-L1 CPS scores. Increased alternative promoter utilization may thus represent a novel biomarker of ICI response in metastatic gastric cancer.

Methods

REMARK criteria for validation of tumor biomarkers was followed in this study [11].

Clinical cohorts

Discovery cohort. Consecutive patients with metastatic gastric cancer treated with nivolumab or pembrolizumab treatment at Samsung Medical Centre, Seoul, Korea, were included in this cohort. ICIs were administered as salvage treatment in patients who failed to at least one cytotoxic regimen. Nivolumab 3 mg/kg was administered as a 1-hour infusion every 2 weeks and pembrolizumab 200 mg was administered as a 30-minute i.v. infusion every 3 weeks until disease progression or unacceptable toxicity. Ethics approval was obtained, and all patients provided written informed consent before archival tumor tissue specimens from primary tumors were collected and prospectively followed up for survival data.

Pembrolizumab trial cohort. Patients with histologically proven metastatic and/or recurrent gastric adenocarcinoma that had failure of at least one line of chemotherapy that included platinum/fluoropyrimidine were enrolled in this study. The trial was conducted in accordance with the Declaration of Helsinki and the Guidelines for Good Clinical Practice (ClinicalTrials.gov identifier: NCT#02589496). The trial protocol was approved by the Institutional Review Board of Samsung Medical Center (Seoul, Korea) and all patients provided written informed consent before enrollment. Pembrolizumab 200 mg was administered as a 30-minute i.v. infusion every 3 weeks until documented disease progression, unacceptable toxicity, or up to 24 months. Tumor responses were evaluated every two cycles according to RECIST 1.1 criteria. Details have been reported previously [7].

Nanostring analysis

NanoString nCounter Reporter CodeSets (NanoString Technologies, Seattle, WA, USA) were designed for 80 recurrent somatic alternate promoter-related genes, as well as immune-related genes corresponding to intra-tumoral cytolytic activity (CYT) [12], cytokines and immune checkpoints. At least two probes were designed for each gene to measure the expression of canonical and alternate promoter-driven transcripts. A canonical probe at the 5′ transcript marked by unaltered H3K4me3 and an alternate probe at the 5′ transcript of the somatic promoter [10]. Data analysis was carried out using the vendor-provided nCounter software (nSolver, NanoString Technologies, Seattle, WA, USA). Raw counts were normalized using the geometric mean of the internal positive control probes included in each CodeSet.

RNA sequencing

Tumor tissues were obtained between day −42 and day 1 before initiation of study treatment. If tumor content was estimated as more than 40% after thorough pathological review, tumor DNA and RNA were extracted

from freshly obtained tissues using a QIAamp Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. We measured concentrations and 260/280 and 260/230 nm ratios with an ND1000 spectrophotometer (Nanodrop Technologies, Thermo-Fisher Scientific, MA, USA) and then further quantified DNA/RNA using a Qubit fluorometer (Life Technologies, CA, USA).

RNA transcriptomic analysis

RNAseq data were aligned to GENCODE v19 transcript annotation using TopHat and FPKM abundance measure were generated using Cufflinks. Transcripts were then merged across all samples and normalized using Cuffnorm. To analyze alternative promoter-associated expression, RNAseq reads were mapped against the genomic location previously identified by epigenomic profiling [10]. RNAseq mapping to these epigenome-defined promoter regions were then quantified, normalized by promoter length and by library size. Finally, fold changes in expression at each promoter site were computed between each tumor and the median expression level across all tumor samples.

PDL1 immunohistochemistry analysis, MSI status, EBV status, TCGA subtyping and tumor mutational burden were based on classifications used for the phase II cohort reported previously[7] and briefly summarized in supplementary Methods, available at *Annals of Oncology* online.

Statistical analysis

Associations of clinicopathologic features to histologic sub-classification was carried out using Fisher's exact test. Progression-free survival (PFS) was calculated from the time of first dose of pembrolizumab or nivolumab to the time of disease progression or death, and overall survival (OS) was calculated from time of first dose of pembrolizumab or nivolumab to time of death. Kaplan–Meier (KM) curves and log rank test were used for survival analysis. The hazard ratio (HR) and its 95% confidence interval (CI) were evaluated for each analysis using Cox proportional hazards regression model. All analyses were done using R (3.4.1). In the validation cohort, samples with alternate promoter usage score greater than the 66th percentile were defined as high alternate promoter (AP_{high}) and remaining low alternate promoter (AP_{low}).

Results

Alternate promoter utilization in metastatic gastric cancer

Our first cohort consisted of 24 metastatic gastric cancer patients treated with nivolumab and pembrolizumab (29 subjects were initially included, with 24 tumor samples passing quality control for sufficient tissue for Nanostring analysis). We used a customized Nanostring panel to measure transcripts associated with either the canonical or alternate promoter, as previously described [10]. Differentially expressed alternative promoters were defined as a promoter site showing $<0.25\times$-fold change (for lost somatic promoters) or $>4\times$-fold change in expression level (for gained somatic promoters) over the median across all samples. Using this algorithm, we found a third of the tumors (8/24) displayed high alternate promoter utilization in more than 10% of the sites ($>8/80$). We defined this group as AP_{high} while the rest were defined as AP_{low} (supplementary Figure S1A, available at *Annals of Oncology* online).

Measurement of cytolytic T-cell activity has previously been described by studying expression of *CD8A* (CD8+ tumor infiltrating lymphocytes), granzyme A (*GZMA*) and perforin 1 (*PRF1*) [13, 14]. The AP_{low} group demonstrated significantly increased expression of *GZMA* ($P=0.025$), *PRF1* ($P=0.011$) and *CD8A* ($P=0.059$) when compared with the AP_{high} group suggesting increased cytotoxic T-cell activity in the AP_{low} group (supplementary Figure S1B, available at *Annals of Oncology* online). These findings are concordant with earlier results described in early gastric cancer [10], demonstrating that alternate promoter utilization in metastatic gastric cancers is also inversely related to antitumor immunity. Notably, despite the small sample size, heterogenous treatment regimens, and non-trial-based nature of this cohort, there was a trend for patients with AP_{high} tumors to have worse PFS compared with patients with AP_{low} tumors (129 versus 389 days, HR 1.96, 95% CI 0.55–6.93, $P=0.29$, supplementary Figure S1C, available at *Annals of Oncology* online). Based on these findings, we thus proceeded to further test this hypothesis in a separate cohort of uniformly treated patients.

Alternate promoter utilization as a predictor of response and survival with pembrolizumab treatment

For the second cohort, we used transcriptomic data from the phase II study described earlier [7]. Transcriptomic data from pretreatment biopsy samples and matched clinical data was available for 37 subjects and used for analysis. The median age was 57 years, 73% were male ($N=27$), four (11%) were EBV-positive and four were MSI (11%) with the rest defined as CIN or GS TCGA subtype. Complete or partial responses to therapy was seen in 11 subjects (30%) (supplementary Table S1, available at *Annals of Oncology* online). Using 2732 somatic alternate promoter sites previously identified in gastric cancer [10], differentially expressed alternative promoters were defined similar to the first cohort ($<0.25\times$-fold change for known somatically lost promoters or $>4\times$-fold change for known somatically gained promoters). Notably, we have previously described good concordance between RNAseq and Nanostring platforms for assessment of alternate promoter utilization [10]. The sum of differentially expressed sites in each sample was calculated to define an alternate promoter usage score (Figure 1A). Scores ranged from 37 to 426 (median 136) (Figure 1B). Using data from the first cohort to guide cut-off points, the AP_{high} group was defined as samples $>66\%$ centile ($n=13$), while AP_{low} constituted the remaining samples.

The AP_{high} group had no statistically significant differences in clinicopathological characteristics compared with the AP_{low} group for age, gender or histological subtype. No differences were also detected between the two groups between TCGA subtypes, mutational load and PDL1 CPS scores (supplementary Table S2, available at *Annals of Oncology* online). The AP_{low} group demonstrated significantly increased expression of *CD8A* ($P=0.0037$), *GZMA* ($P=0.0055$) and *PRF1* ($P=0.016$) when compared with the AP_{high} group suggesting increased cytotoxic T-cell activity in the AP_{low} group (Figure 1C). Objective response rate, defined as either partial response (PR) or complete response (CR) to therapy, was higher in the AP_{low} group than in the AP_{high} group (10/24 versus 1/13, $P=0.03$) (Figure 1D). Of note, in the AP_{high} group, the only response was in an MSI subtype tumor. Median PFS was 55 days in the AP_{high} group compared

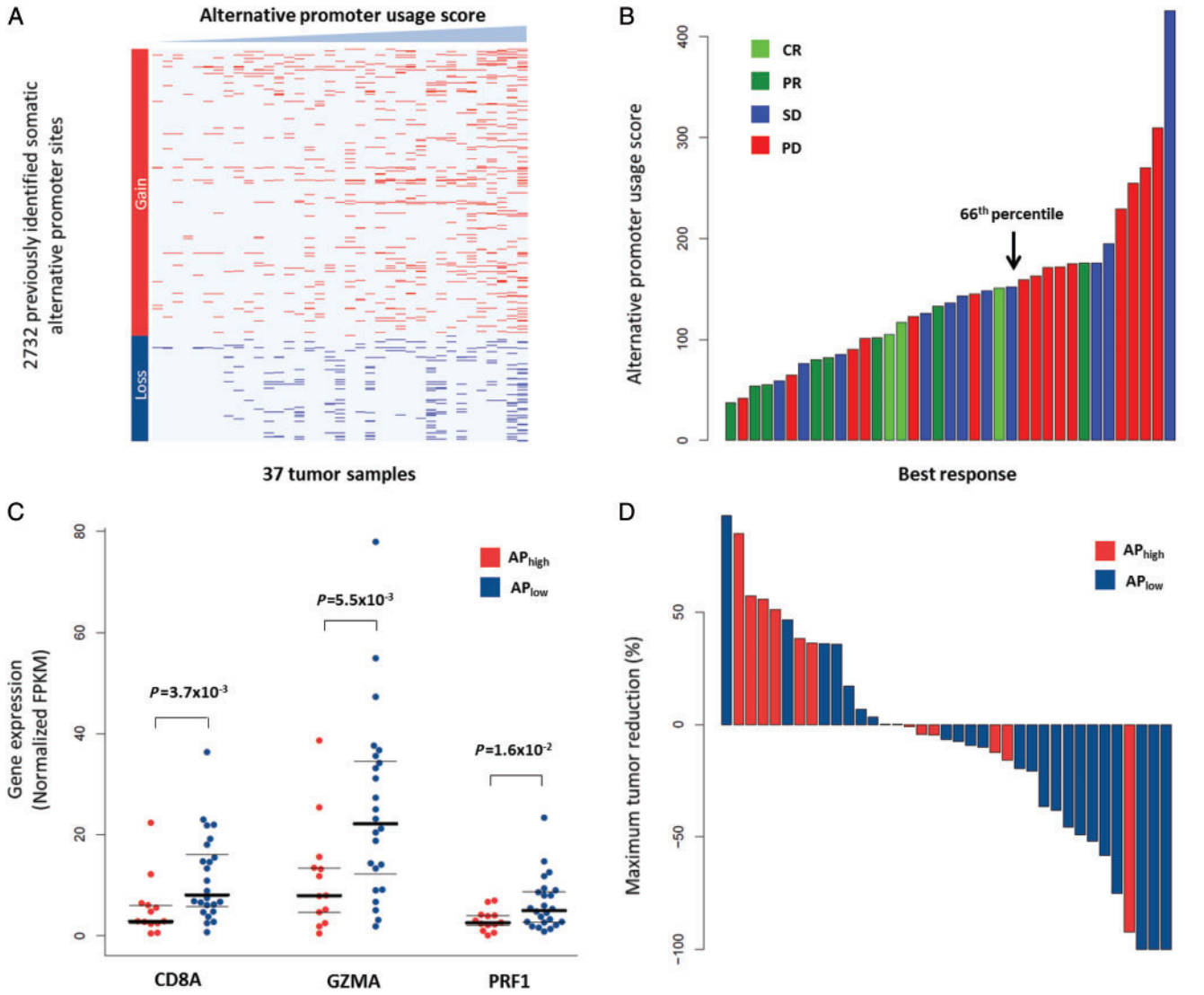

Figure 1. Alternate promoter utilization in gastric cancer (pembrolizumab trial cohort, $n = 37$). (A) Heatmap of alternate promoter utilization. Transcript with higher than fourfold expression level compared with the median level in all tumor and mapping to the previously identified gain alternative promoter site were considered as gained alternative promoter (marked red in the heatmap). Transcript with lower than fourfold expression level compared with the median level in all tumor and mapping to the previously identified lost alternative promoter site were considered as lost alternative promoter (marked blue in the heatmap). (B) Alternative promoter utilization score is calculated as the sum of gained and lost alternative promoter in each sample. High alternate promoter utilization was defined as those >66th centile. (C) Association between high alternate promoter (AP_{high}) group and low alternate promoter (AP_{low}) group with T-cell immune correlates. AP_{high} group are in red, whereas those in AP_{low} group are in blue. Depicted are the expression of T-cell markers CD8A ($P = 0.0037$) and the T-cell cytolytic markers GZMA ($P = 0.0055$) and PRF1 ($P = 0.016$). AP_{high} group shows lower expression of immune markers. (D) Waterfall plot of response to pembrolizumab according to AP_{high} (red) and AP_{low} (blue) subgroups. *Y* axis represents percentage of maximum tumor reduction assessed according to RECIST 1.1 criteria.

with 180 days in the AP_{low} group (log rank $P = 0.0076$) (Figure 2A and B). The AP_{low} group had 17% EBV ($n = 4$) and 12% MSI ($n = 3$) TCGA subtype samples, while the AP_{high} group had only 8% MSI ($n = 1$) and no EBV samples (Figure 2C). As previously shown, PFS between the various TCGA subtypes were different ($P = 0.0026$) (supplementary Figure S2, available at *Annals of Oncology* online), with the MSI and EBV subtypes having a significantly longer survival [491 days (MSI/EBV) versus 80 days (CIN/GS)]. Notably, among the CIN/GS subtype, PFS was also statistically significantly different between the AP_{low} and AP_{high} groups [48 days (CIN/GS AP_{high}) versus 161 days (CIN/GS AP_{low}), $P = 0.0019$] (Figure 2D). OS data were not mature at the time of reporting of this article, but with the existing data, a trend toward improved survival was seen in the AP_{low} group (340 versus 292 days, $P = 0.16$). Multivariate analysis of clinicopathologic and alternate promoter utilization revealed high alternate promoter utilization as an independent predictive factor for PFS with pembrolizumab [HR 0.29, 95% CI 0.099–0.85, $P = 0.024$) (supplementary Table S3, available at *Annals of Oncology* online).

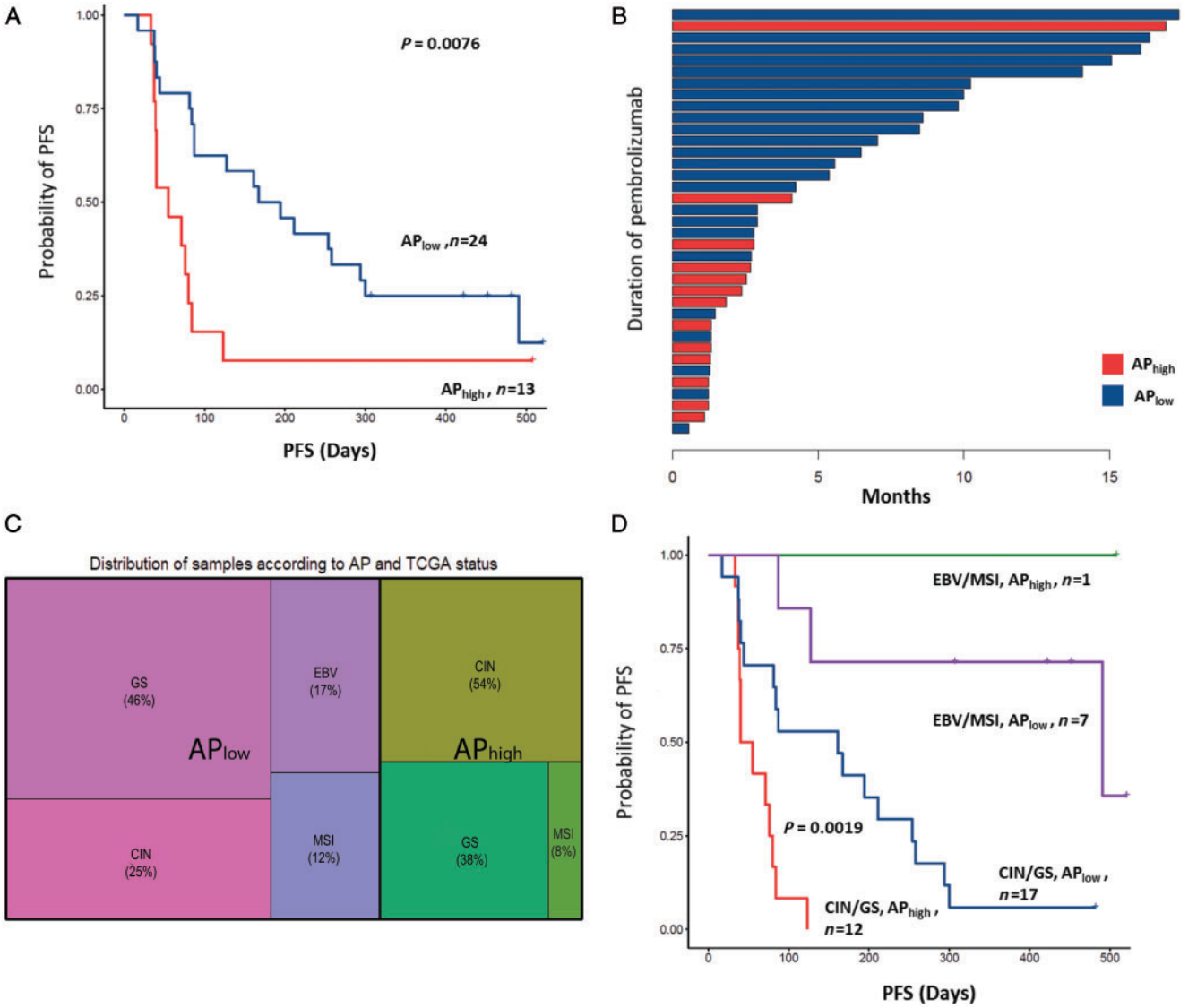

Figure 2. Survival curves based on alternate promoter utilization. (A) Kaplan–Meier curve of progression-free survival comparing high alternate promoter (AP_{high}) group versus low alternate promoter (AP_{low}) group. (B) Swimmer plot. *X* axis represents the duration of pembrolizumab therapy for each patient. AP_{high} (red) and AP_{low} (blue) subgroups depicted. (C) Distribution of TCGA subtypes among AP_{high} group and AP_{low} group. In the AP_{low} group, chromosomally unstable (CIN) ($n = 6$, 25%), genomically stable (GS) ($n = 11$, 46%), Epstein–Barr virus positive (EBV) ($n = 4$, 17%), microsatellite instability (MSI) ($n = 3$, 12%). In the AP_{high} group, CIN ($n = 7$, 54%), GS ($n = 5$, 38%), MSI ($n = 1$, 8%) and EBV ($n = 0$). (D) Kaplan–Meier curve of progression-free survival comparing TCGA subtypes split by AP_{high} group versus AP_{low} group.

Alternate promoter utilization evolution post-treatment with pembrolizumab

Paired biopsy samples were available for eight subjects from the second cohort, providing an opportunity to monitor tumor evolution as a consequence of ICI therapeutic pressure. Post-treatment biopsies were taken from the primary stomach tumor at the point of progression on pembrolizumab. Of these eight subjects, two had PR, with duration of response of 211 and 491 days (both AP_{low}), one had stable disease (SD), with duration of response for 167 days (AP_{low}), and five had progressive disease (PD) (AP_{high} $N = 3$; AP_{low} $N = 2$) as best response. Interestingly, we noted very consistent shifts in the directionality of alternative promoter utilization based on clinical responses. Specifically, tumors with PR and SD exhibited $\times 1.5$ or higher increase in alternate promoter usage score in the post-treatment biopsy samples compared with pre-treatment biopsy samples, while all five tumors with PD exhibited reductions in alternate promoter usage scores in the post-treatment biopsy sample (Fisher's exact test, $P = 0.018$, supplementary Figure S3, available at *Annals of Oncology* online). While the precise mechanism underlying these changes remains to be elucidated, these results further support a relationship between alternative promoter landscapes and ICI therapeutic pressure.

Discussion

Our data suggest that similar to early gastric cancer, alternative promoter utilization occurs in metastatic gastric cancer and may

function as a novel mechanism of resistance to ICI through epigenetic immune-editing. In this model, somatic recruitment of alternate promoter elements by gastric cancers leads to production of proteins with significantly reduced immunogenicity, leading to immune evasion. Supporting this model, high alternate promoter utilization is associated with lower cytotoxic T-cell activity, response rates and survival in gastric cancers treated with anti-PD-1 therapy. In our previous analysis of alternate promoters in primary resection samples of gastric cancer reported previously [10], we found that most of the alternate promoters observed in the primary gastric cancer cohorts (including the TCGA [1] and ACRG [15]) were also identifiable in gastric cancer cell lines. Genes associated with the alternative promoters also do not show obvious immune or tumor microenvironment associations, consistent with AP usage likely being tumor-specific rather than occurring in the tumor microenvironment.

We found that nearly a third of gastric tumors express high levels of these alternate promoter isoforms. Interestingly, alternate promoter utilization does not associate with common clinicopathological prognostic factors such as age, gender or histological subtype, nor with other immune-related markers such as PD-L1 IHC scores or mutational load. The AP_{high} group had only one MSI tumor and no EBV tumors, suggesting that this mechanism of resistance appears to be largely restricted to the CIN/GS subgroups. Analysis of the CIN/GS AP_{high} group's PFS survival curves suggest a clear lack of benefit from pembrolizumab, with most patients progressing within 3 months of treatment. These findings form the basis of considering alternate promoter utilization as a predictive biomarker for selecting patients for ICI.

Despite initial enthusiasm for ICIs in gastric cancer following the ATTRACTION-2 [5] and KEYNOTE-059 [6] studies, newer randomized phase III studies have yielded disappointing results. Pembrolizumab failed to demonstrate benefit compared with the second-line paclitaxel chemotherapy [16], while avelumab (an anti-PDL1 antibody) failed when compared with chemotherapy in the third line [17]. However, from the large number of patients treated on these studies—one common pattern appears to emerge: there are a small group of patients who derive great benefit from exposure to immunotherapy and appear to have prolonged, durable responses, of several months to years. This phenomenon, unique to immunotherapy, has led to rapidly increasing demands by patients and utilization by clinicians, however low the chances of success. Cost of immunotherapy is a major prohibitive factor, leading to socio-economic imbalances. In this treatment landscape, negative predictive biomarkers (that identify subjects with clear lack of benefit from specific therapy) are of particular interest. An example of commonly used negative predictive biomarkers in clinical practice in gastrointestinal cancers are RAS mutations to identify lack of benefit from anti-EGFR therapy in metastatic colorectal cancer [18]. MSI is one of the few established biomarkers to identify gastric cancer patients who benefit from ICI, and this remains a primary-site agnostic indication for this therapy [19]. Biomarkers such as PD-L1 expression using immunohistochemistry and tumor mutational burden have been explored, but conflicting data exist and there is currently a lack of consensus on the utility of these biomarkers with varying results according to different ICIs (i.e. pembrolizumab vs nivolumab) [20]. Further validation of the findings in our study is required in larger clinical data sets. However, the availability of fresh tissue biopsies to carry out RNAseq is limited and platforms such as Nanostring may need to be optimized to analyze transcriptomic data from archival tissue. Optimization of the bioinformatic analysis pipeline would also be required to allow this to be brought into routine clinical practice. Finally, biomarker-driven randomized studies would need to be conducted to fully establish the utility of this biomarker. In conclusion, alternate promoter utilization may potentially be a powerful negative predictive biomarker for selecting patients for clinical trials involving immune checkpoint inhibitors in addition to existing positive biomarkers such as MSI and EBV in gastric cancer.

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Disclosure

The subject matter in this article has been submitted as a Technology Disclosure to the institutional Technology Transfer Office, for potential intellectual property protection. The authors have declared no conflicts of interest.

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