



## Single Patient Classifier Assay, Microsatellite Instability, and Epstein-Barr Virus Status Predict Clinical Outcomes in Stage II/III Gastric Cancer: Results from CLASSIC Trial

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**Purpose:** Clinical implications of single patient classifier (SPC) and microsatellite instability (MSI) in stage II/III gastric cancer have been reported. We investigated SPC and the status of MSI and Epstein-Barr virus (EBV) as combinatory biomarkers to predict the prognosis and responsiveness of adjuvant chemotherapy for stage II/III gastric cancer.

**Materials and Methods:** Tumor specimens and clinical information were collected from patients enrolled in CLASSIC trial, a randomized controlled study of capecitabine plus oxaliplatin-based adjuvant chemotherapy. The results of nine-gene based SPC assay were classified as prognostication (SPC-prognosis) and prediction of chemotherapy benefit (SPC-prediction). Five quasi-monomorphic mononucleotide markers were used to assess tumor MSI status. EBV-encoded small RNA *in situ* hybridization was performed to define EBV status.

**Results:** There were positive associations among SPC, MSI, and EBV statuses among 586 patients. In multivariate analysis of disease-free survival, SPC-prognosis [hazard ratio (HR): 1.879 (1.101–3.205), 2.399 (1.415–4.067),  $p=0.003$ ] and MSI status (HR: 0.363, 95% confidence interval: 0.161–0.820,  $p=0.015$ ) were independent prognostic factors along with age, Lauren classification, TNM stage, and chemotherapy. Patient survival of SPC-prognosis was well stratified regardless of EBV status and in microsatellite stable (MSS) group, but not in MSI-high group. Significant survival benefit from adjuvant chemotherapy was observed by SPC-Prediction in MSS and EBV-negative gastric cancer.

**Conclusion:** SPC, MSI, and EBV statuses could be used in combination to predict the prognosis and responsiveness of adjuvant chemotherapy for stage II/III gastric cancer.

**Key Words:** Gastric cancer, microsatellite instability, single patient classifier, EBV

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•Jae-Ho Cheong, Yong-Min Huh, and Sung Hoon Noh are the founders and shareholders of Novomics Co., Ltd., and they have applied for a patent.

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## INTRODUCTION

Surgery and chemotherapy are the main treatment strategies against most solid cancers. Recently developed technologies and updates in the understanding of cancer biology have made it possible to classify cancers according to their molecular characteristics. These achievements have opened the era of precision medicine against cancer,<sup>1-3</sup> and various biomarkers and algorithms are being used in clinical practice to decide treatment strategies, as they can predict the prognosis and responsiveness to chemotherapy in various cancers.<sup>4-6</sup>

Gastric cancer is one of the most common and lethal malignancies in the world, especially in Korea. Various efforts have been made to understand gastric cancer at molecular level, hoping that the findings may have clinical utility.<sup>7-9</sup> For example, through integrated analyses using multi-omics platforms, the Cancer Genome Atlas study has identified four molecular subtypes of gastric cancer, including Epstein-Barr virus (EBV), microsatellite instability (MSI), chromosomal instability (CIN), and genomic stable (GS) types, with distinctive biological characteristics. Although a promising utility of MSI in making clinical decisions has been extensively reported,<sup>10-14</sup> concrete evidence is still needed to confirm clinical utility of other subtypes; currently, no assay is available for applying routine use to identify certain molecular subtypes such as CIN and GS, which prevents the accumulation of standardized evidence. In this regard, we have developed a predictive test on GMP-grade qRT-PCR assay platform for predicting the prognosis and response to adjuvant chemotherapy after D2 gastrectomy for stage II/III gastric cancer: single patient classifier (SPC).<sup>15,16</sup> Based on gene expression profiling, SPC translates clinically relevant molecular characteristics in gastric cancer (i.e., immune, stem-like, and epithelial type characteristics) into SPC-prognosis (low, intermediate, and high-risk groups) and SPC-prediction subtypes (responder and non-responder groups for adjuvant chemotherapy), serving as an accessible tool for the molecular subtyping of gastric cancer, along with EBV or MSI.

Taken together, we believe that from a practical standpoint, it would be worthwhile to evaluate the combinatory effects of clinically relevant subtypes, such as SPC, MSI, and EBV, in order to make a more precise stratification of patients for their prognosis and predict their responsiveness to adjuvant chemotherapy.

## MATERIALS AND METHODS

### Study design and patients

From a total of 1035 patients enrolled in CLASSIC trial, which is a randomized controlled study of capecitabine plus oxaliplatin-based adjuvant chemotherapy for stage II/III gastric cancer,<sup>17,18</sup> we reviewed the medical records of 586 patients, for

whom it was possible to identify SPC, MSI and EBV statuses. Of these, 282 patients underwent D2 gastrectomy alone, while 304 patients underwent D2 gastrectomy plus adjuvant chemotherapy. This study was approved by the Institutional Review Board at Severance Hospital, Yonsei University Health System (IRB No: 4-2016-0068, 4-2016-0433).

### Definition of SPC status

We had previously analyzed the expression of four classifier genes (*GZMB* and *WARS* representing immune module, *SFRP4* representing stem-like module, and *CDX1* representing intestinal epithelial module) and five reference genes (*ACTB*, *ATP5E*, *HPRT1*, *GPX1*, and *UBB*) by performing a real-time RT-PCR assay (nProfiler 1 Stomach Cancer Assay, Novomics Co., Ltd., Seoul, Korea) using samples from CLASSIC trial cohort.<sup>15</sup> Prognostication (SPC-prognosis; low-risk, intermediate-risk or high-risk) and prediction of chemotherapy benefit (SPC-prediction; responder or non-responder) were defined according to the expression results of these nine genes from SPC assay.

### Definition of MSI status

Previously, we had also analyzed MSI status from CLASSIC trial cohort using a panel (MSI Analysis System, Version 1.2; Promega, Madison, WI, USA) of five quasi-monomorphic mononucleotide repeat markers (NR-21, BAT-25, MONO-27, NR-24, and BAT-26) that determined MSI status of the tumor with high accuracy.<sup>13</sup> MSI-H was defined as instability of two or more of the five markers, whereas MSI-L and microsatellite stable (MSS) were defined as instability of one marker and those without instability.<sup>19,20</sup>

### Epstein-Barr virus-encoded small RNA *in situ* hybridization

Two core tissue microarrays, with a diameter of 3 mm for each case, were constructed from formalin-fixed paraffin-embedded tumor blocks. Ventana Benchmark ISH system (Ventana ISH iView kit, Ventana Corporation, Tucson, AZ, USA) was used for Epstein-Barr virus-encoded small RNA *in situ* hybridization (EBER-ISH).<sup>21</sup> The presence of EBV in tumor cells in EBER-ISH analysis was considered as a positive result; the absence of EBV was considered an EBV-negative result.

### Statistical analysis

Chi-square test or Fisher's exact test were performed for categorical variables. Continuous variables were analyzed with independent t test. Disease-free survival (DFS) was defined as the time from surgery to recurrence or death, whichever occurred first. Survival data were censored at the last time of follow-up or at seven years for patients without these events. DFS curves were generated using Kaplan-Meier method and log-rank test. Cox proportional hazards regression models were used to obtain hazard ratios (HRs) with 95% confidence

interval (CI). The forward likelihood-ratio model was used for selecting inclusion variables in the final multivariable model, using an entry criterion between 0.15 and 0.25 as significance level. Two-sided *p* values less than 0.05 were considered statistically significant. Data were analyzed using IBM SPSS version 23.0 (IBM Corp., Armonk, NY, USA).

## RESULTS

### Baseline characteristics of study cohort

Table 1 shows clinical information of the study cohort (n=586) of the current study. Between the original CLASSIC cohort (n=1035) and this collected cohort, no significant differences were observed;<sup>13,15</sup> 70 (11.9%), 278 (47.4%), and 238 (40.6%) patients were classified into low-, intermediate-, and high-risk groups, respectively, by SPC-prognosis, and 318 (54.3%) and 268 (45.7%) of patients were classified as non-responders and

**Table 1.** Clinical Characteristics of Entire Cohort

	Entire cohort (n=586)
Age (yr)	56.1±11.6
Sex	
Male	410 (70.0)
Female	176 (30.0)
Lauren classification	
Intestinal	215 (36.7)
Non-intestinal	371 (63.3)
EBV status	
Negative	541 (93.0)
Positive	41 (7.0)
MSI status	
MSS/MSI-L	547 (93.3)
MSI-H	39 (6.7)
Treatment	
Surgery only	282 (48.1)
Surgery+CTx	304 (51.9)
pTNM stage*	
II	276 (47.1)
III	310 (52.9)
SPC-prognosis	
Low-risk	70 (11.9)
Intermediate-risk	278 (47.4)
High-risk	238 (40.6)
SPC-prediction	
Non-responder	318 (54.3)
Responder	268 (45.7)

EBV, Epstein-Barr virus; MSI, microsatellite instability; MSS, microsatellite stable; MSI-L, microsatellite instability-low; MSI-H, microsatellite instability-high; CTx, adjuvant chemotherapy (capecitabine plus oxaliplatin); SPC, single patient classifier.

Data are expressed as mean±standard deviation or as n (%).

\*According to American Joint Committee on Cancer 6th edition.

responders, respectively, by SPC-prediction. Furthermore, 39 (6.7%) patients showed MSI-H tumors and 41 patients (7.0%) showed EBV-positive tumors. Detailed baseline and clinical demographics by SPC and MSI have been reported in previous studies.<sup>13,15</sup> With regards to EBV status, no statistically significant distribution was observed between EBV-positive and -negative cases for clinical variables such as age, Lauren classification, pathologic stages, and treatment, but not gender; most cases of EBV-positive gastric cancer were observed in men (38 out of 41 patients, 92.7%, *p*=0.001; chi-square test) (Table 2).

### Associations of MSI and EBV statuses with respect to SPCs

With regards to SPC-prognosis, both MSI and EBV statuses had a positive association (Table 3). MSI-H and EBV-positive tumors were dominant in low-risk group compared to that in other groups (*p*<0.001 for both statuses; chi-square test). With regards to SPC-prediction, 27 (69.2%) of 39 MSI-H patients and 38 (92.7%) of 41 EBV-positive patients were enriched in non-responder group (*p*=0.066 and <0.001, respectively). Aside from SPC, MSI and EBV statuses were almost mutually exclusive; only two patients had both MSI-H and EBV-positive gastric cancer (*p*>0.999).

**Table 2.** Clinical Characteristics of Patients according to EBV Status

	EBV status		<i>p</i> value
	Negative (n=541)	Positive (n=41)	
Age (yr)	56.2±11.6	55.9±11.5	0.885
Sex			0.001
Male	371 (68.6)	38 (92.7)	
Female	170 (31.4)	3 (7.3)	
Lauren classification			0.486
Intestinal	201 (37.2)	13 (31.7)	
Non-intestinal	340 (62.8)	28 (68.3)	
pTNM stage*			0.274
II	259 (47.9)	16 (39.0)	
III	282 (52.1)	25 (61.0)	
pT classification*			0.408
pT1/T2	300 (55.5)	20 (48.8)	
pT3/T4	241 (44.5)	21 (51.2)	
pN classification*			0.379
pN0	48 (8.9)	2 (4.9)	
pN1/N2/N3	493 (91.1)	39 (95.1)	
Treatment			0.663
Surgery only	258 (47.7)	21 (51.2)	
Surgery+CTx	283 (52.3)	20 (48.8)	

EBV, Epstein-Barr Virus; CTx, adjuvant chemotherapy (capecitabine plus oxaliplatin).

Data are expressed as mean±standard deviation or as n (%), unless otherwise specified.

\*According to American Joint Committee on Cancer 6th edition.

**Prognosis according to markers**

In the study cohort, both SPC and MSI statuses were prognostic factors, but EBV was not ( $p=0.001$  in SPC-prognosis,  $p=0.003$  in MSI-H vs. MSS/MSI-L,  $p=0.547$  in EBV positive vs. EBV negative; log-rank test).

We first examined the combinatory effects of SPC and MSI statuses in prognostication. In Fig. 1A, different prognoses of

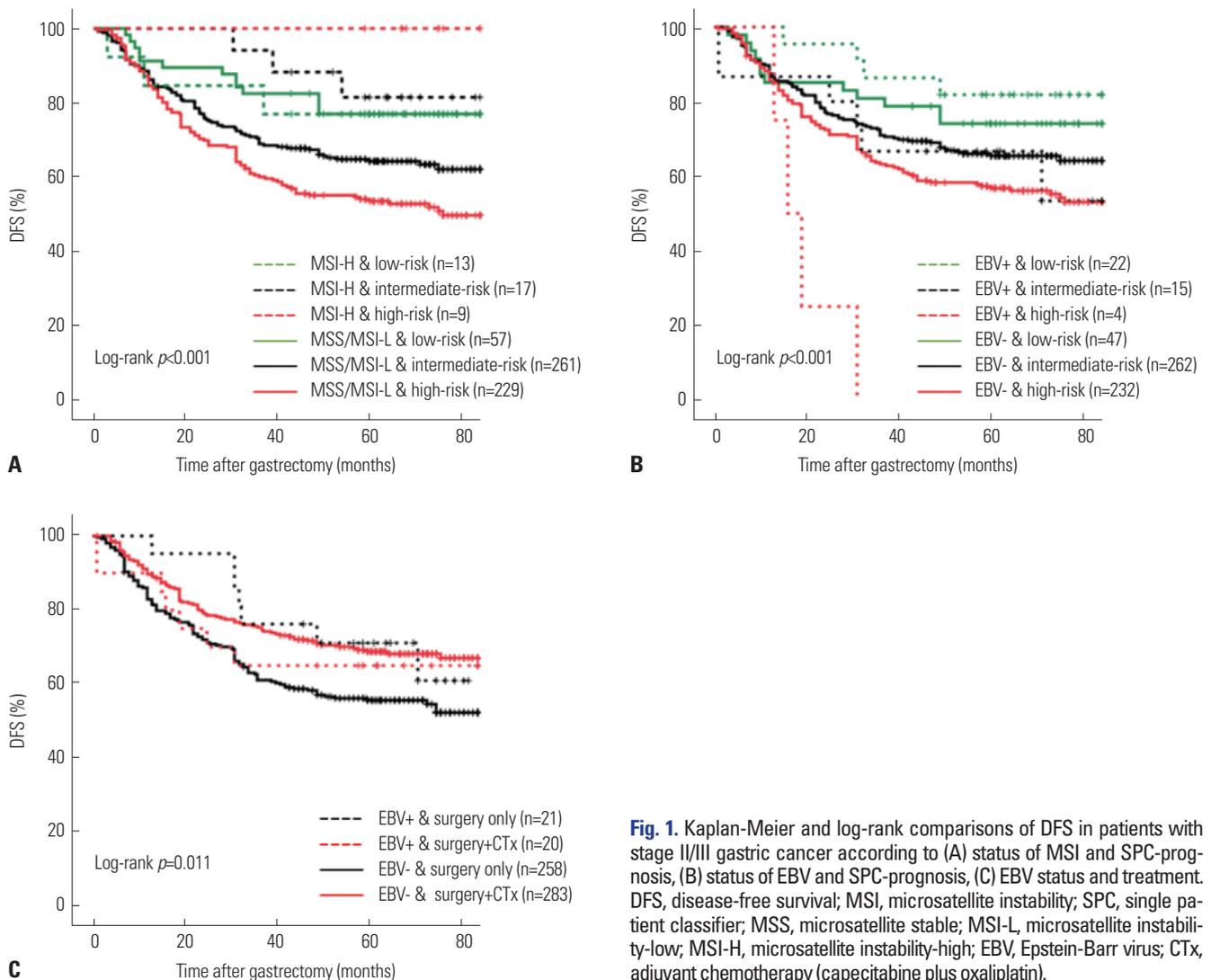
low-, intermediate-, and high-risk groups obtained from SPC-prognosis were well-preserved in MSS/MSI-L patients [HR: 1.787 (1.000–3.191) in intermediate-risk, 2.510 (1.411–4.463) in high-risk group, log-rank  $p=0.001$ ], but not in MSI-H patients (log-rank  $p=0.316$ ), indicating that SPC and MSI statuses were independent of one another in prognostication. A multivariate analysis using Cox proportional hazards regres-

**Table 3.** Associations between SPC, MSI, and EBV Status

	SPC-prognosis				SPC-prediction			EBV		
	Low-risk	Intermediate-risk	High-risk	<i>p</i> value	Non-responder	Responder	<i>p</i> value	Negative	Positive	<i>p</i> value
MSI status				<0.001			0.066			>0.999
MSS/MSI-L	57 (81.4)	261 (93.9)	229 (96.2)		291 (91.5)	256 (95.5)		504 (93.2)	39 (95.1)	
MSI-H	13 (18.6)	17 (6.1)	9 (3.8)		27 (8.5)	12 (4.5)		37 (6.8)	2 (4.9)	
EBV				<0.001			<0.001			
Negative	47 (68.1)	262 (94.6)	232 (98.3)		276 (87.9)	265 (98.9)				
Positive	22 (31.9)	15 (5.4)	4 (1.7)		38 (12.1)	3 (7.3)				

SPC, single patient classifier; MSI, microsatellite instability; EBV, Epstein-Barr virus; MSS, microsatellite stable; MSI-L, microsatellite instability-low; MSI-H, microsatellite instability-high.

Data are expressed as n (%).



**Fig. 1.** Kaplan-Meier and log-rank comparisons of DFS in patients with stage II/III gastric cancer according to (A) status of MSI and SPC-prognosis, (B) status of EBV and SPC-prognosis, (C) EBV status and treatment. DFS, disease-free survival; MSI, microsatellite instability; SPC, single patient classifier; MSS, microsatellite stable; MSI-L, microsatellite instability-low; MSI-H, microsatellite instability-high; EBV, Epstein-Barr virus; CTx, adjuvant chemotherapy (capecitabine plus oxaliplatin).

sion models showed that both MSI status (HR: 0.363, 95% CI: 0.161–0.820,  $p=0.015$ ) and SPC-prognosis [HR: 1.879 (1.101–3.205) in intermediate-risk, 2.399 (1.415–4.067) in high-risk group,  $p=0.003$ ] were independently significant prognostic factors for DFS, in addition to age, chemotherapy, pTNM stage, and Lauren classification (Table 4).

In contrast, a combination of SPC and EBV statuses revealed a distinct trend whereby the survival by SPC-prognosis was well-stratified regardless of EBV status (Fig. 1B). Intriguingly, in EBV-positive patients, the difference in DFS between SPC-prognosis groups was highly significant [HR: 2.608 (0.735–9.254) in intermediate-risk, 17.656 (3.839–81.200) in high-risk group, log-rank  $p<0.001$ ], necessitating further investigation. In a multivariate analysis, EBV status was not found to be a significant prognostic factor for DFS [HR: 0.735 (0.425–1.268),  $p=0.268$ ], in addition to age, sex, chemotherapy, and pTNM stage.

### Responsiveness to adjuvant chemotherapy according to markers

Statistically significant survival benefit from adjuvant chemotherapy was observed in responder group for SPC-prediction, MSS/MSI-L groups, and EBV-negative groups ( $p=0.001$ , 0.002, and 0.001, respectively; log-rank test), but not in non-responder group for SPC-prediction, MSI-H groups, and EBV-positive groups ( $p=0.243$ , 0.931, and 0.687, respectively) (Fig. 1C).

To explore the survival benefit from adjuvant chemotherapy by joint markers, we categorized patients into the follow-

**Table 4.** Disease-Free Survival Analyzed by a Multivariate Cox Proportional-Hazards Model

	HR (95% CI)	p value
Age	1.015 (1.003–1.027)	0.013
Treatment		<0.001
Surgery only	Reference	
Surgery+CTx	0.621 (0.475–0.811)	
pTNM stage*		<0.001
II	Reference	
III	2.160 (1.621–2.879)	
Lauren classification		0.024
Intestinal	Reference	
Non-intestinal	1.421 (1.048–1.927)	
MSI status		0.015
MSS/MSI-L	Reference	
MSI-H	0.363 (0.161–0.820)	
SPC prognosis		0.003
Low-risk	Reference	
Intermediate-risk	1.879 (1.101–3.205)	0.021
High-risk	2.399 (1.415–4.067)	0.001

HR, hazard ratio; CI, confidence interval; CTx, adjuvant chemotherapy (capecitabine plus oxaliplatin); SPC, single patient classifier; MSI, microsatellite instability; MSS, microsatellite stable; MSI-L, microsatellite instability-low; MSI-H, microsatellite instability-high.

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ing: 1) responder/MSI-H, non-responder/MSI-H, responder/MSS/MSI-L, and non-responder/MSS/MSI-L for joint analysis of SPC and MSI and 2) responder/EBV-positive, non-responder/EBV-positive, responder/EBV-negative, and non-responder/EBV-negative for joint analysis of SPC and EBV.

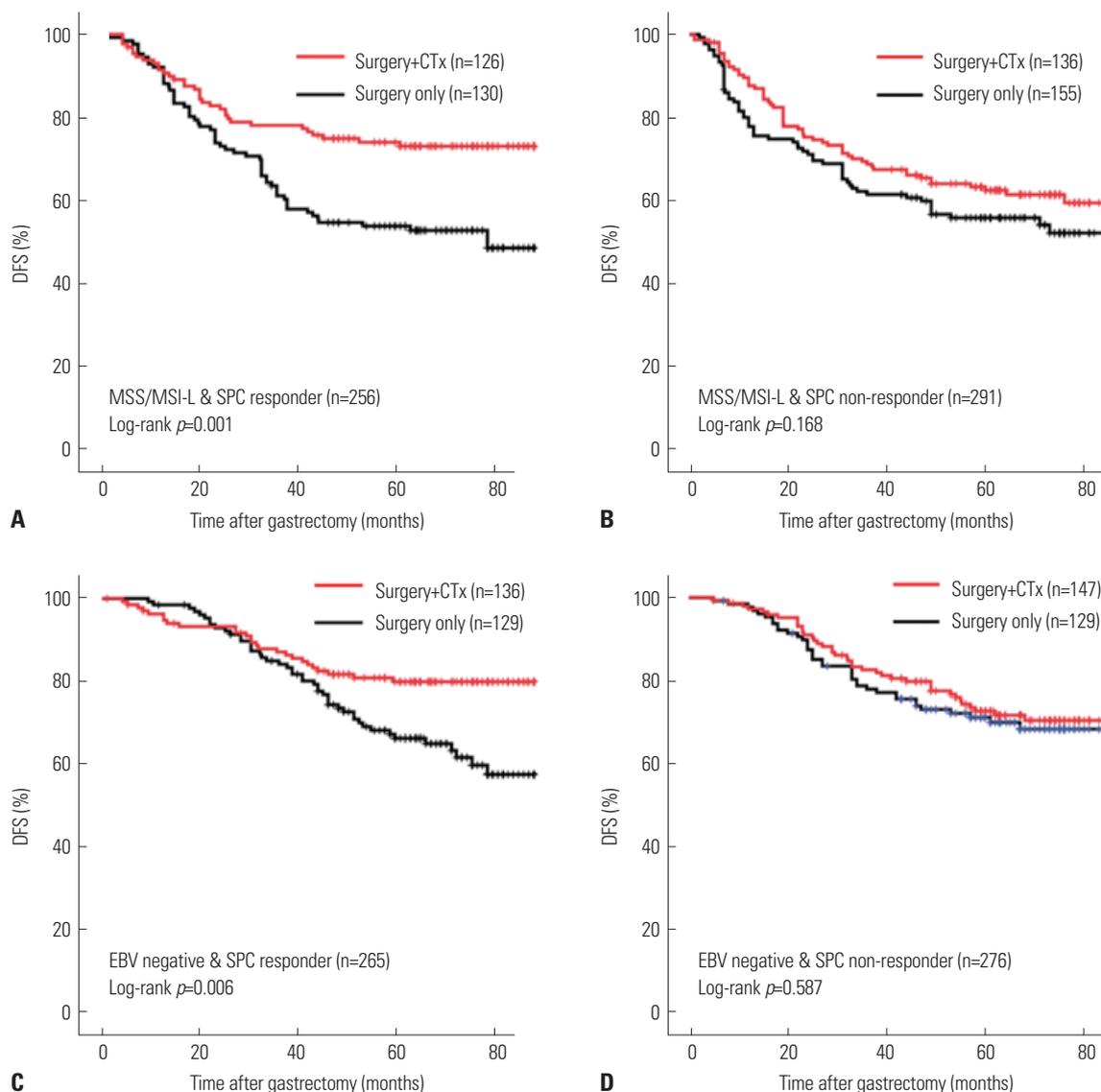
In both non-responder/MSI-H and responder/MSI-H populations, no prognostic difference was observed between surgery alone and surgery plus chemotherapy ( $p=0.630$  and 0.317, respectively). In contrast, in MSS/MSI-L patients, a significant survival benefit from adjuvant chemotherapy was seen in responder group of SPC-prediction ( $p=0.001$ ) (Fig. 2A), but not in non-responder group ( $p=0.168$ ) (Fig. 2B).

In case of joint SPC/EBV, for responder/EBV-positive patients, survival benefit from adjuvant chemotherapy was not analyzed since all of the three patients underwent surgery alone. In the remaining population, the trend of survival benefit in joint SPC/EBV was similar to that for joint SPC/MSI. In non-responder/EBV-positive patients, no survival benefit from adjuvant chemotherapy was observed ( $p=0.491$ ;  $n=18$  and 20 for surgery alone and adjuvant chemotherapy group, respectively). For EBV-negative patients, responder group of SPC-prediction gained a significant survival benefit from adjuvant chemotherapy ( $p=0.006$ ) (Fig. 2C), whereas non-responder group did not ( $p=0.587$ ) (Fig. 2D).

## DISCUSSION

We examined the combinatory effect of clinically relevant subtypes, SPC, MSI, and EBV, in the prediction of prognosis and chemotherapy response after surgery using a cohort of 586 patients enrolled in CLASSIC trial, a randomized controlled study of capecitabine plus oxaliplatin-based adjuvant chemotherapy for stage II/III gastric cancer. The three markers used in this study, SPC, MSI, and EBV, were based on different biological, pathological, or etiological grounds. SPC classified patients by immune, stem-like, and epithelial characteristics from the gene expression profile of tumor tissues, while MSI-H and EBV-positive tumors were associated with genetic hypermutability due to impaired DNA mismatch repair and exogenous viral infection, respectively. Thus, we expected that our joint marker analysis would discover new findings clinically meaningful to patient selection. It was also considered that the use of these markers or some of their combinations would be comparatively feasible to be introduced into practice.

In the analyses evaluating the combinatory prognostic effects of SPC, MSI, and EBV, joint SPC/MSI and SPC/EBV showed their respective clinical implications. 1) SPC and MSI were independent prognostic factors. Although i) MSI-H tumors have been known for their activated immune response and ii) SPC and MSI have a positive association, the proportion of MSI-H was slightly higher in low-risk group than in



**Fig. 2** Kaplan-Meier and log-rank comparisons of DFS in patients with stage II/III gastric cancer following treatment in subgroups of (A) MSS/MSI-L and SPC responder group, (B) MSS/MSI-L and SPC non-responder group, (C) EBV-negative and SPC responder group, and (D) EBV-negative and SPC non-responder group. DFS, disease-free survival; CTx, adjuvant chemotherapy (capecitabine plus oxaliplatin); MSS, microsatellite stable; MSI-L, microsatellite instability-low; SPC, single patient classifier; EBV, Epstein-Barr virus.

other groups ( $p < 0.001$ ; chi square test) and low-risk/MSS/MSI-L patients had as good a prognosis as MSI-H patients ( $p = 0.001$ ; log-rank test), indicating that aside from MSI status, SPC identifies patients whose long-term survival is anticipated. 2) EBV was not a significant prognostic factor in this cohort. SPC/EBV, however, showed the most dramatic stratification between low-risk and high-risk patients in EBV-positive group. As low-risk and high-risk groups in SPC scheme were associated with immune and stem-like characteristics, respectively, the result implies that not all EBV-positive patients might have the same immune response, supporting the existence of molecular heterogeneity in EBV-associated gastric cancers.<sup>21,22</sup>

SPC, MSI, and EBV statuses identified two groups of patients in the present CLASSIC cohort: those who gained sur-

vival benefit from adjuvant chemotherapy and those who did not. The combination of SPC/MSI or SPC/EBV led to a more elaborate selection of these patient groups. 1) It has generally been accepted that MSI-H patients do not benefit from chemotherapy, whereas MSS/MSI-L patients do.<sup>5,12,13</sup> The combinatory SPC and MSI statuses further classified patients in MSS/MSI-L population who did not benefit from chemotherapy. 2) SPC/EBV worked in a manner similar to that of SPC/MSI. EBV-positive patients did not gain chemotherapy benefits, while EBV-negative patients did. Joint SPC/EBV further selected patients from EBV-negative population who did not gain chemotherapy benefits. The combinatory use of SPC, MSI, and EBV might guide clinical decisions with regards to chemotherapeutic treatment.

Currently, accumulating studies have reported that patients

with MSI-H and EBV-positive gastric cancer dramatically responded to immune checkpoint inhibitors.<sup>23</sup> As defined by the immune-related characteristics, low-risk/non-responder SPC subtype could be another companion diagnostic candidate for immune therapy. Otherwise, the combinatory use of SPC, MSI, and EBV may yield meaningful knowledge on the immunological landscape of gastric cancer, leading to a better selection of patients for immune therapy and improvement in response rates.

This study had some limitations. First, although the distribution of clinical variables of the study cohort (n=586) was similar to that of the original CLASSIC trial cohort (n=1035), the possibility of selection bias cannot be overlooked. Second, due to a limited number of cases and events, type II error needs to be considered, especially in subgroup analysis. Although the present results were from a randomized controlled trial (RCT), additional validation with qualified specimens should be conducted to reach a substantial consensus of the clinical role of these markers in patients with gastric cancer. Using specimens from other RCTs, such as MAGIC,<sup>12,24</sup> ARTIST,<sup>25</sup> or FLOT<sup>26</sup> trials, would be the most reliable and practical option.

In conclusion, SPC, MSI, and EBV positivity could be used in a combinatory manner to predict the prognosis and responsiveness of adjuvant chemotherapy for stage II/III gastric cancer.

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