Original Article

Single patient classifier as a prognostic biomarker in pT1N1 gastric cancer: Results from two large Korean cohorts

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Abstract

Objective: Benefits of adjuvant treatment in pT1N1 gastric cancer (GC) remain controversial. Additionally, an effective biomarker for early GC is the need of the hour. The prognostic and predictive roles of single patient classifier (SPC) were validated in stage II/III GC. In this study, we aimed to elucidate the role of SPC as a biomarker for pT1N1 GC.

Methods: The present retrospective biomarker study (NCT03485105) enrolled patients treated for pT1N1 GC between 1996 and 2012 from two large hospitals (the Y cohort and S cohort). For SPC, mRNA expression of four classifier genes (*GZMB*, *WARS*, *SFRP4* and *CDX1*) were evaluated by real-time reverse transcription-polymerase chain reaction assay. The SPC was revised targeting pT1 stages and the prognosis was stratified as high- and low-risk group by the expression of *SFRP4*, a representative epithelial-mesenchymal transition marker.

Results: SPC was evaluated in 875 patients (n=391 and 484 in the Y and S cohorts, respectively). Among 864 patients whose SPC result was available, 41 (4.7%) patients experience GC recurrence. According to revised SPC, 254 (29.4%) patients were classified as high risk [123 (31.5%) and 131 (27.1%) in the Y and S cohorts, respectively]. The high risk was related to frequent recurrence in both Y and S cohort (log-rank P=0.023, P<0.001, respectively), while there was no difference by *GZMB* and *WARS* expression. Multivariable analyses of the overall-cohort confirmed the high risk of revised SPC as a significant prognostic factor [hazard ratio (HR): 4.402 (2.293−8.449), P<0.001] of GC. A significant difference was not detected by SPC in the prognosis of patients in the presence and absence of adjuvant treatment (log-rank P=0.670).

Conclusions: The present study revealed the revised SPC as a prognostic biomarker of pT1N1 GC and suggested the use of the revised SPC for early-stage GC as like stage II/III.

Keywords: Gastric cancer; biomarker; prognosis; chemotherapy

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Introduction

Gastric cancer (GC) is one of the most frequent and lethal cancers worldwide (1,2). Surgical resection remains the fundamental treatment strategy for GC. The need for additional chemotherapy is determined by staging, which reflects the tumor burden in patients. While surgery is the sole therapy for regional cancer, systemic cancer is treated with surgery and chemotherapy (3,4). The pT1N1 stage of cancer, which is limited to the mucosal/submucosal layer of the stomach, with one or two metastatic lymph nodes (LNs), accounts for around 5% of GCs (5,6). This substage is regarded as regional cancer in the East, based on its classification as stage I (7,8), while it is considered as systemic cancer in the West, based on the involvement of metastatic lymph node(s) (9,10). Guidelines for therapy, therefore differ between the East and West, and the decision for including adjuvant therapy is left to the clinicians (7-10).

Advancing technology and several efforts have made it possible to understand GC at the molecular level (11-15). Few scientific results that provided promising evidences have been translated to real world applications (14,16-19). Single patient classifier (SPC, nProfiler® 1 Stomach Cancer Assay) is a readily applicable clinical *ex vivo* device that predicts prognosis and response to adjuvant chemotherapy in stage II/III GC (14,20,21). SPC is a four-gene-based real-time reverse transcription-polymerase chain reaction (RT-PCR) assay that classifies patients into low-, intermediate-, and high-risk groups based on prognosis. In addition, SPC is applied to classify patients into responsive and non-responsive groups to predict the extent of benefit that could be derived from chemotherapy. In situations wherein a similar biology of GC exists in stage I and II/III, SPC aids in the prediction of prognosis and response to chemotherapy in pT1N1, similar to stage II/III GC. Thus, in the present study, we enrolled two large patient cohorts from Korea targeting pT1N1 GC (22,23), to evaluate the applicability of SPC as a biomarker for identifying this specific substage of GC.

Materials and methods

Study cohorts and data collection

Clinical data and formalin-fixed paraffin-embedded (FFPE) tissues were obtained from patients diagnosed with GC between January 1990 and December 2012 at the Severance Hospital (Y cohort) and the Samsung Medical Center (S cohort). The inclusion criteria were as follows: 1) surgical R0 resection; 2) pathological stage T1 (mucosa/submucosa) and N1 (one or two metastatic LNs); 3) D1+ or higher LN dissection; 4) more than 15 retrieved LNs; and 5) availability of FFPE tissues. Patients who underwent neoadjuvant chemotherapy or radiotherapy prior to gastric surgery or who passed away within 30 d of surgery were excluded. The cohorts were previously a part of retrospective studies related to pT1N1 GC (22,23). Clinicopathological features, including sex, age, period of operation (1996–2006 and 2007–2012), histological type (differentiated, undifferentiated, and lymphoepithelioma-like cell), Lauren classification (intestinal, diffuse, and others), depth of tumor invasion (mucosa and submucosa), number of retrieved and metastatic LNs, tumor size (<2, 2–5, and >5 cm), location (upper/middle and lower body), adjuvant treatment (chemotherapy or chemoradiation therapy), microsatellite instability (MSI), and SPC status, were documented. In addition, patient survival and tumor recurrence were evaluated. This study was approved by the Institutional Review Boards of Severance Hospital (4-2017-0914) and Samsung Medical Center (2017-05-101), and the need for informed consent was waived. This retrospective biomarker study was registered at clinicaltrial.gov (NCT03485105).

SPC

SPC is an *ex vivo* device that validates biomarkers by evaluation of genomic applications in practice and prevention (EGAPP): 1) analytic validity; 2) clinical validity; and 3) clinical utility (24). RNA was extracted from tumor-enriched tissue cores measuring 3 mm obtained from two pieces of FFPE samples using the MasterPure complete DNA and RNA purification kit (Epicentre Technologies, Madison, WI, USA) as per the manufacturer's instructions. SPC is a commercial International Organization for Standardization (ISO)-13485 certified, Good Manufacturing Practice grade kit (nProfiler® 1 Stomach Cancer Assay, Novomics Co., Ltd., Seoul, Korea) based on four identified classifier genes (*GZMB*, *WARS*, *SFRP4*, and *CDX1*) and five reference genes (*ACTB*, *ATP5E*, *HPRT1*, *GPX1*, and *UBB*). For prognosis, the low-risk (highly immune) group is defined based on the positive expression of both *GZMB* and *WARS*. Among the others (not low-risk group), based on the expression of *SFRP4* [epithelial-mesenchymal transition (EMT)-related classifier gene], the patients were classified into

intermediate-risk (*SFRP4* negative) and high-risk (*SFRP4*-positive) groups. Responsive and non-responsive groups were defined based on the expression of *CDX1*; positivity for *CDX1* indicated response to chemotherapy. The low-risk group was classified as the non-responsive group, regardless of *CDX1* expression. The definition of positivity for each gene was based on the relative difference quantitation cycle (dCq) values compared to the five reference genes. The original cut-off dCq values for stage II/III GC are −5.18 for *GZMB*, −2.14 for *WARS*, −3.63 for *SFRP4*, and −2.69 for *CDX1* (14). Analytical validation was confirmed for stage II/III GC and the clinical utility was validated by multiple cohorts, including a randomized controlled trial cohort (14,20,21). Both retrospective and prospective validation multi-center studies for stage II/III GC are ongoing (NCT04600518, NCT04487717).

Revised SPC for pT1N1 GC

Because the distribution of expression in *SFRP4* in pT1N1 was different to stage II/III GC, and the expression of *GZMB* and *WARS* was not related to the prognosis in pT1N1 GC, we revised the SPC algorithm. The revised SPC classifies pT1N1 GC into two groups; *SFRP4*-positive (high-risk) and negative (low-risk), regardless of the *GZMB* and *WARS* expression levels with cut-off value of dCq $\geq$ −4.66. The details are in *Supplementary materials*.

MSI status

DNA was extracted from the 3 mm core of FFPE samples to evaluate the MSI status. Five quasimonomorphic mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24, and MONO-27) were evaluated using PCR. Instability in two or more markers was classified as MSI-high (MSI-H), while lack of instability was defined as microsatellite stable (MSS) (25,26).

Statistical analysis

Categorical data are described as numbers with percentages that were analyzed using the Chi-square or Fisher's exact test. Continuous variables are described as means with standard deviations analyzed using an independent *t*-test or the Mann-Whitney test, as appropriate. For survival analyses, GC-specific recurrence was analyzed. Presence of cancer in the remanent stomach was not considered as recurrence. Survival graphs were generated using the Kaplan-Meier method. Prognoses were compared using the log-rank test and Cox proportional hazards model and are described based on the hazard ratio (HR) and its 95% confidence interval (95% CI). Institution was used as an adjustment variable in univariate and multivariate analyses. For multivariable analyses, variables with a P-value less than 0.05 and possible clinical confounding factors related to the prognosis were selected. In addition, multicollinearity was considered. The revised cut-off value for *SFRP4* expression was determined using a step-Miner analysis that is used to identify the threshold to convert continuous gene expression value into Boolean values (High/Low) (27). The equivUMP package was used for an equivalence test with an equivalence limit of 0.5. Because the null hypothesis of this test is the values between two groups are different, statistical significance represents the values are equivalence. A P-value less than 0.05 was considered statistically significant. Statistical analyses were performed using R software (Version 3.6.1; R Foundation for Statistical Computing, Vienna, Austria), and "survival" package was mainly used for survival analysis.

Results

Populations

Table 1 and *Supplementary Table S1* summarize the baseline characteristics of the entire population and the cohorts. A total of 875 patients (391 and 484 in the Y and S cohort, respectively) were enrolled in this study. FFPE samples were not available for patients treated between the years 1990 and 1995. Comparison of demographics and clinicopathologic characteristics between the two cohorts showed significant differences, particularly related to adjuvant treatment: 11.5% (45/391) of patients were treated with chemotherapy following surgery in the Y cohort, while 56.0% (271/484) of patients received either adjuvant chemotherapy or chemoradiation therapy in the S cohort. Therefore, institution was used as an adjustment variable for survival (Cox) analyses. MSI and SPC statuses were available for 838 and 864 patients, respectively.

Characteristics and prognosis by revised SPC algorithm

A significant association was seen between the expression of revised SPC and clinicopathological characteristics: high risk frequently indicated a male patient (P=0.008), a smaller tumor size (P<0.001), lower body location (P=0.015), and submucosal invasion (P<0.001, *Table 2*). In contrast, there was no significant association between the revised SPC and histology or Lauren classification. The high risk of revised

Table 1 Baseline characteristics of enrolled patients

Characteristics	Total (N=875) [n (%)]
Age (year) ($\bar{x}\pm s$)	57.45±11.70
Sex	
Male	544 (62.2)
Female	331 (37.8)
Period	
1996–2006	420 (48.0)
2007–2012	455 (52.0)
Size (cm) (N=874)	
<2	139 (15.9)
2–5	542 (62.0)
>5	193 (22.1)
Location	
MB/UB	323 (36.9)
LB	552 (63.1)
Histology	
Differentiated	404 (46.2)
Undifferentiated	462 (52.8)
LELC	9 (1.0)
Lauren classification	
Intestinal	463 (52.9)
Diffuse	293 (33.5)
Others	119 (13.6)
Depth of invasion	
Mucosa	90 (10.3)
Submucosa	785 (89.7)
No. of metastatic LNs	
1	592 (67.7)
2	283 (32.3)
No. of retrieved LNs ($\bar{x}\pm s$)	37.48±12.55
Adjuvant treatment	
None	559 (63.9)
CTx	203 (23.2)
CTx+RTx	113 (12.9)

MB, mid-body; UB, upper-body; LB, lower-body; LELC, lymphoepithelioma-like cell; LN, lymph node; CTx, chemotherapy; RTx, radiation therapy.

Table 2 Association between expression of revised SPC and clinical features

Variables	Revised SPC [n (%)]		P
	Low risk (N=610)	High risk (N=254)	
Age (year) ($\bar{x}\pm s$)	57.08±12.10	58.41±10.61	0.107
Sex			0.008
Male	360 (59.0)	175 (68.9)	
Female	250 (41.0)	79 (31.1)	
Period			0.905
1996–2006	295 (48.4)	121 (47.6)	
2007–2012	315 (51.6)	133 (52.4)	
Size (cm)*			<0.001
<2	89 (14.6)	50 (19.7)	
2–5	364 (59.7)	172 (67.7)	
>5	156 (25.7)	32 (12.6)	
Location			0.015
MB/UB	240 (39.3)	77 (30.3)	
LB	370 (60.7)	177 (69.7)	
Histology			0.066
Differentiated	268 (43.9)	132 (52.0)	
Undifferentiated	334 (54.8)	121 (47.6)	
LELC	8 (1.3)	1 (0.4)	
Lauren classification			0.151
Intestinal	215 (35.2)	75 (29.5)	
Diffuse	311 (51.0)	148 (58.3)	
Mixed/undetermined	84 (13.8)	31 (12.2)	
Depth of invasion			<0.001
Mucosa	79 (13.0)	10 (3.9)	
Submucosa	531 (87.0)	244 (96.1)	
No. of metastatic LNs			0.749
1	412 (67.5)	175 (68.9)	
2	198 (32.5)	79 (31.1)	
No. of retrieved LNs ($\bar{x}\pm s$)	37.57±12.85	37.25±11.90	0.727
Adjuvant treatment			0.069
No	379 (62.1)	175 (68.9)	
Yes	231 (37.9)	79 (31.1)	

SPC, single patient classifier; MB, mid-body; UB, upper-body; LB, lower-body; LELC, lymphoepithelioma-like cell; LN, lymph node. *, No. of low-risk revised SPC was 609.

SPC was associated with significant *GZMB* and *WARS* positivity (P<0.001 and P=0.004, respectively) and reduced *CDX1* positivity (P<0.001), while MSI status was not (P=0.813, *Supplementary Table S2*).

The prognosis of pT1N1 GC that was stratified by the revised SPC was significantly different in the Y cohort: 5-year GC-specific recurrence-free probability of the low- and high-risk groups were 96.5% (95% CI: 94.3–98.8) and 90.0% (95% CI: 84.8–95.5) in the Y cohort, 98.9% (95% CI: 97.7–100) and 90.6% (95% CI: 85.3–96.1) in the S cohort, and 97.8% (95% CI: 96.6–99.0) and 90.3% (95%

CI: 86.6–94.2) in the overall cohort, respectively (P=0.023, <0.001, and <0.001, respectively, *Figure 1*). The institution-adjusted HR of the high-risk compared to low-risk group was 4.274 (95% CI: 3.950–8.701, P<0.001). The influence of multiple factors associated with prognosis and the revised SPC was adjusted in pT1N1 GC by selecting significant variables in univariate Cox analyses (*Supplementary Table S3*) and factors that were related to prognosis, such as sex, depth of invasion, number of metastatic LNs, and adjuvant treatment. Considering multicollinearity, the Lauren classification was selected, as its P-value was lower than that of histology. In multivariable analyses, the revised SPC was an independent prognostic factor, with an HR of 4.402 (95% CI: 2.293–8.449, *Table 3*). The most frequent recurrent pattern of pT1N1 GC was hematogenous recurrence (46.3%, 19/41, *Table 4*), unlike peritoneal recurrence in advanced GC (3). The type of recurrence did not differ according to the revised SPC.

Benefit from adjuvant treatment by SPC

Presence or absence of adjuvant therapy did not influence the prognosis of pT1N1 GC, which was in accordance with the previously published data (adjusted HR: 1.626, 95% CI: 0.782–3.378, *Supplementary Table S3*) (17,22). This result indicates an absence of beneficial effect of adjuvant treatment in pT1N1 GC. Fifty-four percent of patients (465 out of 864) were *CDX1*-positive, and 23 of the *CDX1*-positive patients were *GZMB* and *WARS*-positive; therefore, 442 patients were classified into the responsive group (chemotherapy sensitive), and the remaining 422 patients were classified into the non-responsive group (chemotherapy resistance) by SPC prediction. The prognosis was similar among the responsive and non-responsive groups in the presence and absence of adjuvant treatment (P=0.670, *Figure 2A*), probably due to the lack of any beneficial effect of adjuvant treatment in this cohort. Taken together, our results indicate that SPC was not effective in predicting the response to chemotherapy in

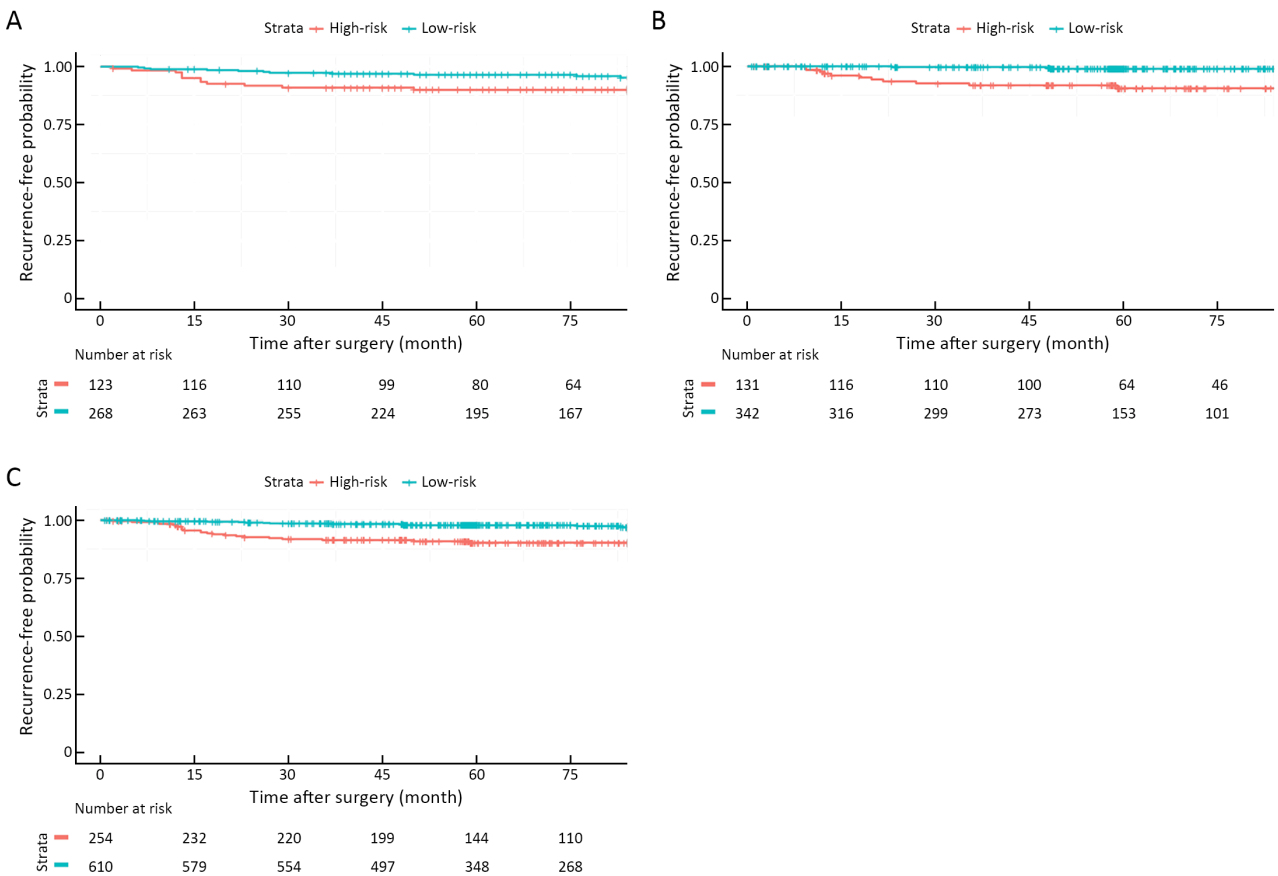

Figure 1 Kaplan-Meier curves of pT1N1 gastric cancer recurrence according to revised expression of *SFRP4* in (A) Y cohort (training cohort) (P=0.023); (B) S cohort (validation cohort) (P<0.001); and (C) overall cohort (P<0.001).

Table 3 Multivariable Cox proportional hazard model for recurrence of pT1N1 gastric cancer

Variables	Adjusted HR (95% CI)	P
Age	1.040 (1.010–1.077)	0.019
Sex		0.505
Male	1	
Female	0.800 (0.430–1.163)	
Period		0.005
1996–2006	1	
2007–2012	0.362 (0.178–0.736)	
Lauren classification		
Intestinal	1	
Diffuse	0.328 (0.123–0.879)	0.027
Mixed/undetermined	0.602 (0.223–1.624)	0.316
Depth of invasion		0.557
Mucosa	1	
Submucosa	0.692 (0.203–2.365)	
No. of metastatic LNs		0.876
1	1	
2	1.055 (0.541–2.056)	
Revised SPC		<0.001
Low risk	1	
High risk	4.402 (2.293–8.449)	
Adjuvant treatment		0.021
No	1	
Yes	2.600 (1.154–5.861)	

LN, lymph node; SPC, single patient classifier; HR, hazard ratio; 95% CI, 95% confidence interval.

pT1N1 GC. In multivariable analyses, adjuvant treatment was associated with poor prognosis compared to surgery alone, and the difference was statistically significant [HR: 2.600 (95% CI: 1.154–5.861), P=0.021, *Table 3*]. The benefits of adjuvant treatment did not differ with the revised SPC (P<0.001; *Figure 2B*).

Discussion

Based on the current scientific knowledge related to the prognosis of resectable GC (11-14,17,20), tumors of the highly immune subtypes, including MSI-H, Epstein-Barr virus (EBV)-positive, and low risk of SPC types, have better prognosis compared to those of the EMT-related subtypes that are associated with poor prognosis. In the present study, the highly immune, which is *GZMB*- and *WARS*-positive subtypes of SPC did not differentiate between the prognoses of pT1N1 GC, similar to the MSI in other cohorts of early GC (28). Prognosis of stage I GC is already promising (<5% of tumor recurrence), therefore the space for better survival will be very small. Conversely, enhanced expression of *SFRP4* (high risk of the revised SPC), an EMT marker, is associated with poor prognosis of pT1N1, similar to stage II/III GC. This suggests that EMT mainly explains tumor recurrence in overall resectable GC, regardless of early and advanced stages.

To prove the role of biomarkers in predicting drug response, the interaction between drug treatment and the biomarker should be statistically significant (29). We speculated that SPC might play an important role in predicting the beneficial effects of chemotherapy in pT1N1 as like stage II/III GC, at least in the high-risk group; however, such a role was not observed in this study. In addition, there was no benefit of adjuvant treatment; instead, this treatment seemed to be harmful in the present cohort. Therefore, evaluating biomarkers for predicting the chemotherapy response might be insignificant in a cohort. The role of adjuvant treatment in stage I GC remains uncertain. Hence, predictive biomarker studies in this subgroup might not be promising.

EMT is a representative molecular characteristic related to cancer metastasis, poor prognosis, and refractory behavior to conventional chemotherapy in both gastric and colorectal cancers (11,12,14,30,31). In GC, the subtype

Table 4 Patterns of recurrence of pT1N1 gastric cancer expressing revised SPC

Patterns	Revised SPC [n (%)]			P
	Low risk (N=15)	High risk (N=26)	Total	
Recurrence				0.549
Hematogenous	7 (46.7)	12 (46.2)	19 (46.3)	
LN	5 (33.3)	5 (19.2)	10 (24.4)	
Peritoneum	1 (6.7)	6 (23.1)	7 (17.1)	
Any combination	2 (13.3)	3 (11.5)	5 (12.2)	

SPC, single patient classifier; LN, lymph node.

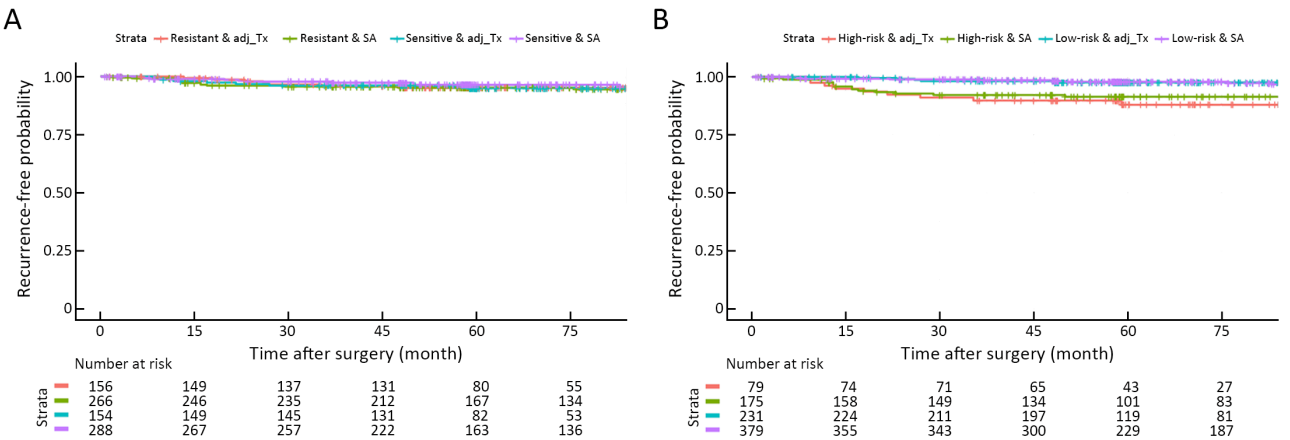

Figure 2 Kaplan-Meier curves of pT1N1 gastric cancer recurrence according to adjuvant treatment. (A) SPC prediction for chemotherapy (P=0.670); (B) revised SPC in overall cohort (P<0.001). Resistant, resistant to chemotherapy; Sensitive, sensitive to chemotherapy; adj Tx, adjuvant treatment; SA, surgery alone; SPC, single patient classifier.

with EMT is genomically stable, with fewer somatic mutations, and is resistant to the beneficial effects of targeted therapy or immune checkpoint inhibitors and is thus clinically challenging to treat (11-14). Development of novel therapies targeting the EMT subtype of GC is the need of the hour. Few preclinical studies have shown the possibility of cancer metabolism-related therapy targeting this refractory subtype of GC (32,33). These EMT-targeted treatment strategies might prove to be helpful for *SFRP4*-positive GC, although the beneficial effects need to be validated at the clinical level.

These results could be translated for clinic use: 1) for low-risk (*SFRP4*-negative) pT1N1 with a very low likelihood of tumor recurrence (<2.5%) and a possible stage modification from the current IB–IA; 2) for high-risk (*SFRP4*-positive) pT1N1 with a 10 % tumor recurrence risk and lack of beneficial effects from adjuvant treatment, accordingly, the follow-up schedule (short-term as stage II) could be suitably revised. In addition, this study showed the possibility of using the revised SPC even for early-stage GC with a relatively good prognosis; therefore, expanding this study to other substages of stage I GCs (pT1N0, pT2N0) is worthy of investigation. We are evaluating the revised SPC by focusing on patients diagnosed with stage I GC (except pT1N1) associated with tumor recurrence. The results that will be obtained from the ongoing study would indicate whether the revised SPC could be applicable in patients with overall resectable GC, including stage I GC.

The role of revised SPC as a prognostic marker for pT1N1 GC was evaluated and validated in two independent cohorts with a large number of patients, considering the rarity of this specific substage of GC. As there has been no randomized controlled trial covering this specific stage of GC, the results of the present study provide are novel; however, they need to be validated in other cohorts, especially in different countries, covering various ethnicities. Since the number of events and tumor recurrence is small, type II errors need to be considered, especially for subgroup analysis. Despite the pre-planned biomarker study design, the retrospective nature of the cohort and various adjuvant treatment strategies in the cohort are other possible limitations of this study.

Conclusions

The present results indicated the low risk of revised SPC, low *SFRP4* expression, as a better prognostic biomarker for pT1N1 GC. Accordingly, it can be said that the revised SPC is effective for the stratification of prognoses of patients with pT1N1 GC and for precision medicine strategies.

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Footnote

Conflicts of Interest: Jae-Ho Cheong, Yong-Min Huh, and

Sung Hoon Noh are the founders and shareholders of Novomics Co., Ltd. All the remaining authors have no conflicts of interest to declare.

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Supplementary materials

Revision of single patient classifier (SPC) algorithm for pT1N1 gastric cancer (GC)

The SPC algorithm was developed and validated primarily in advanced GC (stage II/III), wherein the tumor usually invades over the proper muscle layer, and the tumor-enriched core originates from the outer layer of the gastric wall. Conversely, in pT1N1 GC, the tumor core originates from the inner layer of the gastric wall, in the mucosal/submucosal layer. Thus, the expression pattern of the four classifier genes of SPC (*GZMB*, *WARS*, *SFRP4*, and *CDX1*) might be different in pT1N1 compared to stage II/III GC. Therefore, we compared the expression levels of each gene between the present cohort (pT1N1, n=864) and previous cohorts covering stage II/III (n=1,586) (1-3). As shown in *Supplementary Figure S1*, the expression of *SFRP4* was not equivalent between pT1N1 and stage II/III GC (P>0.990), but the expression of the other genes (*GZMB*, *WARS*, and *CDX1*) was similar between these cohorts (P<0.001). These results implied the necessity to revise the cut-off for *SFRP4* expression in early GC (cancer invading the mucosa/submucosa).

Based on the pre-planned original SPC algorithm, the pT1N1 tumors were classified as low-, intermediate-, and high-risk in 7.6% (n=66), 78.9% (n=682), and 13.4% (n=116) of patients, respectively. Survival analyses based on GC-specific recurrence indicated statistically significant distinct prognosis, as determined by SPC (log-rank P=0.021, *Supplementary Figure S2A*). Although high risk was related to a higher likelihood of tumor recurrence, low risk was not associated with better prognosis. Similar to the MSI status in early GC (4), low-risk SPC, which represents a high immune signature, was not helpful in stratifying the prognosis of pT1N1 GC. In addition, *SFRP4*-positive patients had poorer prognosis in the *GZMB*-positive and MSI-H subgroups (*Supplementary Figure S2B–D*). Therefore, we decided to revise the SPC algorithm targeting pT1N1.

Because of the differential distribution of *SFRP4* expression between pT1N1 and stage II/III GC (early and advanced GC), a new cut-off value [difference quantitation cycle (dCq) ≥−4.66] for *SFRP4* was decided based on the step-Miner analysis (5) in the Y cohort and was applied to the S and overall cohorts (*Supplementary Figure S3*). In addition, immune-related markers, *GZMB* and *WARS*, were not helpful in stratifying the prognoses of pT1N1 GC. The prognosis group patients were roughly classified as *SFRP4* positive (high-risk) and negative (low-risk), regardless of the *GZMB* and *WARS* expression levels (*Supplementary Figure S4*).

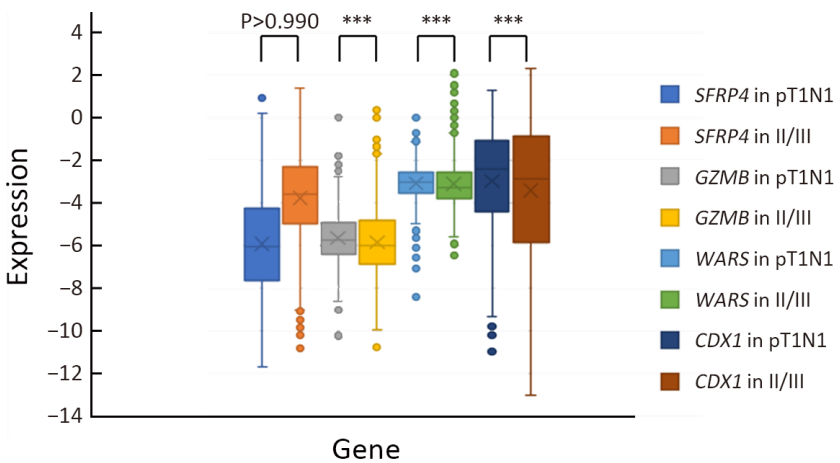

Figure S1 Comparison of expression of each single patient classifier gene (*GZMB*, *WARS*, *SFRP4*, and *CDX1*) between pT1N1 (n=864) and stage II/III (n=1,586) gastric cancer. The statistical comparison was conducted by equivUMP and statistical significance (P<0.05) represents that the expression was equivalent, otherwise the expression was different. ***, P<0.001.

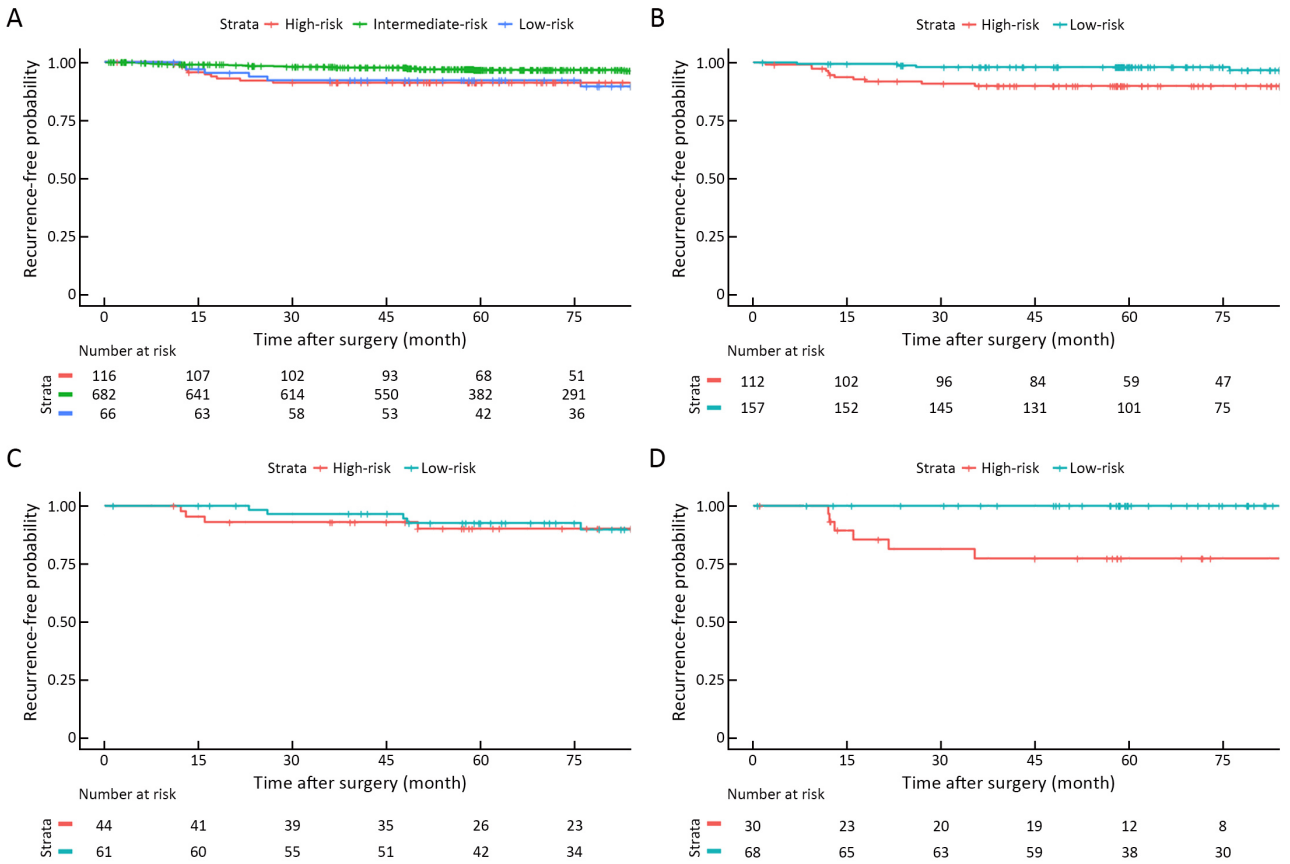

Figure S2 Kaplan-Meier curves indicating recurrence of pT1N1 gastric cancer (A) by original SPC in overall cohort (P=0.021), and by revised SPC (revised expression of *SFRP4*) in (B) *GZMB*-positive group (P=0.005), (C) *WARS*-positive group (P=0.830), and (D) MSI-high group (P<0.001). SPC, single patient classifier; MSI, microsatellite instability.

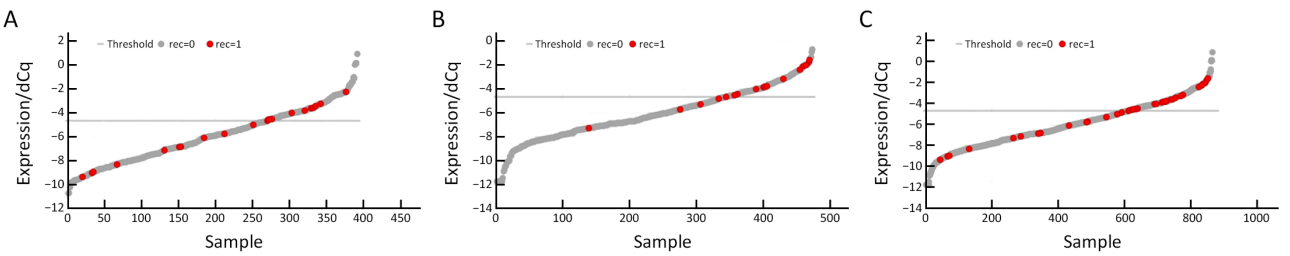

Figure S3 Distribution of *SFRP4* expression in (A) Y cohort, (B) S cohort, and (C) overall cohort. The linear line on the y-axis represents the revised cut-off point for *SFRP4* positivity determined by the step-miner analysis that identify the threshold to convert continuous gene expression values into Boolean values (High/Low) in the Y cohort. Based on this new cut-off of *SFRP4* expression, the revsied single patient classifier was defined high risk for *SFRP4*-positive and low risk for *SFRP4* negative. Each circle represents a sample, and red color represents recurrence.

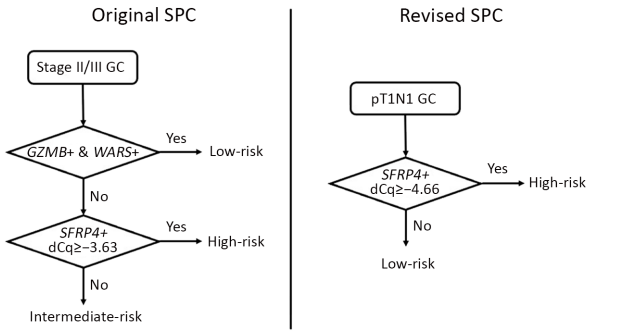

Figure S4 Diagram defining original SPC algorithm and revised version in pT1N1 GC. SPC, single patient classifier; GC, gastric cancer; dCq, difference quantitation cycle. The original SPC algorithm classifies stage II/III GC into 3 groups for the prognosis: *GZMB*- & *WARS*-positive classified into low-risk group with favorable prognosis, and others were classified into intermediate- and high-risk based on the expression of *SFRP4* (left). For the revised SPC in this study, because *GZMB* and *WARS* expression was not related to the prognosis in pT1N1 GC, we neglected the expression of *GZMB* and *WARS*. The prognosis of pT1N1 GC can be stratified by the expression of *SFRP4*, and the revised SPC classifies pT1N1 GC into two groups, high- and low-risk (right).

Table S1 Baseline characteristics of population by cohorts

Variables	n (%)		P
	Y cohort (N=391)	S cohort (N=484)	
Age (year) ($\bar{x}\pm s$)	58.10±12.10	56.90±11.40	0.142
Sex			0.934
Male	242 (61.9)	302 (62.4)	
Female	149 (38.1)	182 (37.6)	
Period			0.128
1996–2006	176 (45.0)	244 (50.4)	
2007–2012	215 (55.0)	240 (49.6)	
Size (cm) (N=874)			<0.001
<2	94 (24.1)	45 (9.3)	
2–5	247 (63.3)	295 (61.0)	
>5	49 (12.6)	144 (29.7)	
Location			0.467
MB/UB	150 (38.4)	173 (35.7)	
LB	241 (61.6)	311 (64.3)	
Histology			0.278
Differentiated	186 (47.6)	218 (45.0)	
Undifferentiated	199 (50.9)	263 (54.3)	
LELC	6 (1.5)	3 (0.7)	
Lauren classification			<0.001
Intestinal	221 (56.5)	242 (50.0)	
Diffuse	99 (25.3)	194 (40.1)	
Others	71 (18.2)	48 (9.9)	
Depth of invasion			0.006
Mucosa	53 (13.6)	37 (7.6)	
Submucosa	338 (86.4)	447 (92.4)	
No. of metastatic LNs			0.889
1	266 (68.0)	326 (67.4)	
2	125 (32.0)	158 (32.6)	
No. of retrieved LNs ($\bar{x}\pm s$)	36.48±12.46	38.29±12.58	0.034
Adjuvant treatment			<0.001
None	346 (88.5)	213 (44.0)	
CTx	45 (11.5)	158 (32.6)	
CTx+RTx	0 (0)	113 (23.4)	
MSI status (N=827)			<0.001
MSS	330 (92.7)	408 (84.6)	
MSI-high	26 (7.3)	74 (15.4)	
SFRP4 (N=864)*			0.202
Negative	325 (83.1)	409 (86.5)	
Positive	66 (16.9)	64 (13.5)	
GZMB (N=864)			0.025
Negative	285 (72.9)	310 (65.5)	
Positive	106 (27.1)	163 (34.5)	
WARS (N=864)			<0.001
Negative	323 (82.6)	436 (92.2)	
Positive	68 (17.4)	37 (7.8)	
CDX1 (N=864)			>0.999
Negative	181 (46.3)	218 (46.1)	
Positive	210 (53.7)	255 (53.9)	

*, original cut-off. MB, mid-body; UB, upper-body; LB, lower-body; LELC, lymphoepithelioma-like cell; LN, lymph node; CTx, chemotherapy; RTx, radiation therapy; MSI, microsatellite instability; MSS, microsatellite stable.

Table S2 Association between expression of revised SPC and MSI status, expression of *GZMB*, *WARS*, and *CDX1*

Variables	n (%)		P
	Low risk (N=610)	High risk (N=254)	
MSI status (N=827)			0.813
MSS	516 (88.4)	213 (87.7)	
MSI-high	68 (11.6)	30 (12.3)	
GZMB			<0.001
Negative	453 (74.3)	142 (55.9)	
Positive	157 (25.7)	112 (44.1)	
WARS			0.004
Negative	549 (90.0)	210 (82.7)	
Positive	61 (10.0)	44 (17.3)	
CDX1			<0.001
Negative	259 (42.5)	140 (55.1)	
Positive	351 (57.5)	114 (44.9)	

SPC, single patient classifier; MSI, microsatellite instability; MSS, microsatellite stable.

Table S3 Univariable Cox proportional hazard model for recurrence of pT1N1 gastric cancer

Variables	Adjusted HR* (95% CI)	P
Age	1.040 (1.011–1.069)	0.007
Sex		0.094
Male	1	
Female	0.555 (0.279–1.105)	
Period		0.019
1996–2006	1	
2007–2012	0.441 (0.223–0.874)	
Size (cm)		0.700
<2	1	
2–5	1.542 (0.591–4.025)	
>5	1.508 (0.491–4.633)	
Location		0.190
MB/UB	1	
LB	1.565 (0.801–3.061)	
Histology		0.050
Differentiated	1	
Undifferentiated	0.431 (0.226–0.822)	
LELC	1.393 (0.188–10.309)	
Lauren classification		0.010
Intestinal	1	
Diffuse	0.293 (0.122–0.704)	0.006
Mixed/undeterminated	0.550 (0.213–1.416)	0.217
Depth of invasion		0.379
Mucosa	1	
Submucosa	1.698 (0.522–5.522)	
No. of metastatic LNs		0.700
1	1	
2	1.040 (0.548–1.976)	
No. of retrieved LNs	0.993 (0.969–1.018)	0.600
Adjuvant treatment		0.193
No	1	
Yes	1.626 (0.782–3.378)	
MSI status		0.205
MSS	1	
MSI-high	1.702 (0.748–3.872)	
Revised *SFRP4* (revised SPC)		<0.001
Negative (low risk)	1	
Positive (high risk)	4.274 (3.950–4.598)	

Table S3 (*continued*)

Table S3 (*continued*)

Variables	Adjusted HR* (95% CI)	P
GZMB		0.256
Negative	1	
Positive	1.441 (0.767–2.708)	
WARS		0.105
Negative	1	
Positive	1.861 (0.878–3.942)	

*, adjusted by institution. MB, mid-body; UB, upper-body; LB, lower-body; LELC, lymphoepithelioma-like cell; LN, lymph node; MSI, microsatellite instability; MSS, microsatellite stable; SPC, single patient classifier; HR, hazard ratio; 95% CI, 95% confidence interval.

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