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Original Article

Active Stat3 and Her-2 as combined survival predictors show superiority to TNM staging system for postoperative patients with gastric cancer

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Abstract: Objectives: TNM staging of gastric cancer (GC) is useful in predicting prognosis, but its definition is only possible after surgery. It is therefore desirable to develop a method that can predict prognosis and assist management options before surgery. Methods: This study investigated 110 GC patients after radical gastrectomy and followed-up for 136 months. Patients' complete clinicopathological data were collected and gastroscopically biopsied or surgically resected tissues were examined for the expression of Her-2, nm-23, CEA and phosphorylated Stat3 (p-Stat3) using immunohistochemistry (IHC). Univariate and multivariate ROC curves, Kaplan-Meier survival curves, and SPSS Version 22.0 and R (version 3.6.1) statistical software were used to analyze the data. Results: Three major findings were observed: (1) Tissue levels of p-Stat3, Her-2, CEA and nm-23 were correlated with GC patients' survival probability termed as survival prediction power (SPP). (2) Using 5-year survival as an end-point, the SPP of the p-Stat3+Her-2 combination was stronger (AUC=0.867) than that of TNM staging (AUC=0.755). (3) Using cut-off values derived from ROC curves, Kaplan-Meier analyses showed that the p-Stat3+Her-2 molecular combination could clearly predict overall survival rates between the predictive low-risk patients (69.2%) and the predictive high-risk patients (13.2%) with a discriminative difference as high as 56.0%. Conclusions: We conclude that area under the ROC curve (AUC) can be used to quantify SPP powers for biomarkers, making cross-comparisons possible among different survival predictors. This study has first established a multi-factor survival prediction model by which the p-Stat3+Her-2 combination has the best discriminative capability to differentiate low-risk patients from high-risk patients in terms of survival prognosis.

Keywords: Gastric cancer, survival prognosis, multivariate prediction for survival, ROC, survival prediction power, Her-2, Stat3

Introduction

Gastric cancer (GC) is common and poses a major threat to public health around the world. The Globocan 2018 has shown that GC is the fifth most prevalent cancer and the third leading cause of cancer-related death worldwide [1]. In China, the incidence and mortality rates of GC rank the second among all malignant tumors [2]. The 5-year survival rate of GC in China from 2010-2014 was 35.9%, far lower than that of South Korea and Japan [3].

In recent years, advanced screening methods in conjunction with improved public health awareness have improved early diagnosis of GC. For example, gastroscopy is the most effective screening tool for early detection of GC [4, 5] with a sensitivity of 60-80% [6]. However, late diagnosis of advanced GC is still common, which is the major cause leading to poor prognosis after radical gastrectomy [7]. Clinical TNM staging system is an established standard in predicting the overall survival (OS) for GC patients with GC [8]. The definition of TNM

staging, however, is only available after surgery, rendering the TNM staging little useful in pretreatment planning prior to surgery. On the other hand, biomarkers that can be detected before surgery and capable of predicting prognosis would be clinically useful in strategizing treatment options prior to surgery for patients with GC.

Signal transducer and activator of transcription 3 (Stat3) is a key molecule involved in Stat3 signaling pathway [9]. Phosphorylated Stat3 (p-Stat3) is the active form of Stat3 which has been found to be involved in the pathogenesis of colon cancer [10]. Human epidermal growth factor receptor 2 (Her-2), carcinoembryonic antigen (CEA), and non-metastasis-23 (nm-23) are involved in the pathogenesis of cancer and the overexpression of Her-2 [11] and CEA [12], and the low expression of nm-23 [13] are associated with poor prognosis of GC patients. It has been shown that GC patients with over-activated p-Stat3 have a shorter survival time [14], indicating its ability to predict prognosis of GC after gastrectomy.

Thus far, however, most studies have focused on using single biomarkers to predict prognosis of patients with GC in a postsurgical setting, which has little comprehension in terms of treatment precautions before surgery and therefore, provides little, if any, help to improve the prognosis. To establish precautions before surgery and improve the prognosis after surgery, this study aimed at two important aspects and obtained the following findings: (1) biomarkers that can be tested before surgery to predict prognosis are practical in reality, and (2) using combinations of more than one biomarker to increase sensitivity in predicting prognosis is superior to the TNM staging system. The findings described in this report have significant implications in clinical applications in terms of determining the potential outcome of GC patients before surgery.

Methods

Ethics statement

This study was approved by the Institutional Ethics Review Board (IERB No. 2017-070-01) of the First Affiliated Hospital, Shihezi University School of Medicine. Patients' informed consents were obtained orally by telephone during follow-up communications and standard university hospital guidelines in accordance with the Declaration of Helsinki including confidentiality and anonymity were followed in the handling patients' tissues and publication.

Patients and follow-ups

This study was a retrospective clinical analysis. GC patients (n=110) of ethnic Han nationality with histologically confirmed gastric cancer underwent surgery from 2007 to 2017 were selected from the First Affiliated Hospital of Shihezi University School of Medicine (see Supplementary Tables 1 and 2 for basic clinical information of GC patients studied). All of the 110 patients had complete clinicopathological data and follow-up information, and informed consent was obtained. Until October 2018, 110 patients with GC were followed up every year. Among these patients, the longest follow-up time was over 11 years. All patients did not receive chemotherapy and/or radiotherapy before surgery. After surgery, GC patients were followed up for 135 months with a median follow-up time of 25 months. Overall survival (OS) was defined from the date of surgery until the date of death or the date of the last follow-up. Patients who died within 30 days after surgery were defined as 0 month survival.

Measurements of p-Stat3, Her-2, nm-23 and CEA by immunohistochemistry

Tissue microarrays were made from paraffin-embedded tissue blocks from surgically dissected tissues of GC patients. The phosphorylated Stat3 (p-Stat3) is the active form of Stat3. Active levels of p-Stat3 and expressed levels of Her-2, nm-23 and CEA were detected on tissue microarray chips by immunohistochemical assay (IHC) [10] using specific antibodies against p-Stat3, Her-2, nm-23 and CEA, among which Her-2, nm-23 and CEA were routinely examined in clinical laboratories of our hospital.

Classifications of gastric cancer

Gastric adenocarcinoma was classified according to the histopathological classification criteria of the World Health Organization as follows: highly, moderately, and poorly differenti-

ated adenocarcinomas. TNM (tumor-node-metastasis) clinical staging (I-IV), depth of invasion (T_1-T_4), lymphatic and distant metastases were defined according to the American Joint Committee on Cancer [8].

Statistical analyses

Data analyses were performed using the statistical software package SPSS (version 22.0, InBM Corporation, Armonk, NY), R (version 3.6.1; http://www.R-project.org). Spearman ranking correlation method was employed to identify correlations between routine pathological data and four IHC detected tissue levels of p-Stat3, Her-2, nm-23 and CEA. Kaplan-Meier analysis was used to analyze survival curves against cell differentiation, TNM stages and the IHC-defined 4 molecule levels (see **Figures 2** and **4**). Kaplan-Meier survival analysis only classifies and compares one variable and cannot make comparisons among different variables to judge the abilities among variables as they may be all capable of predicting survival (**Figure 2**). Therefore, potential survival bio-predictors have to be quantified so they can be objectively cross-compared with each other. For this purpose, we introduced the ROC (receiver operating characteristic) curve and the area under the ROC curve (AUC) to quantify powers (or abilities) for survival predictors described. ROC is a graphical plot that illustrates the performance of a binary classifier system as its discrimination threshold is varied. On the other hand, AUC is a product of considering both sensitivity and specificity (**Figure 3**) which can be translated to discriminative power among different survival predictors. Therefore, the larger the AUC is, the more powerful the risk factor can be in predicting prognosis [15].

The predictive probabilities of multiple bio-indicator combinations were obtained using logistic regression analysis [16] and ROC curves were obtained using a 5-year survival as an end-point (outcome), which gave an optimal cut-off value of 0.576 (**Figure 4**; **Table 5**). GC patients with a cut-off value of ≥0.576 were defined as being predictive high risk (PHR) while GC patients with a cut-off value of <0.576 were defined as being predictive low risk (PLR). GC patients categorized as PLR or PHR were then subjected to Kaplan-Meir survival analysis (**Figure 4**). Spearman ranking correlation method was employed to identify correlations among preoperative and/or postoperative variables. In this study, we introduced a concept termed as survival prediction power (SPP) which was quantifiable by AUC (area under the ROC curve) (**Tables 2** and **5**).

Results

IHC staining of p-Stat3, Her-2, CEA and nm-23 in gastric cancer tissues

Figure 1 shows negative and positive IHC staining of p-Stat3, Her-2, CEA and nm-23 in gastric cancer tissues. Negative IHC staining was shown as light blue while positive IHC staining was shown as brown (light brown to dark brown). The active p-Stat3 was located in the nucleus while CEA and nm-23 were stained in the cytoplasm. Her-2 is a receptor and thus the positive staining was shown on the cell membrane. The basic clinical information of 110 GC patients and their IHC results were summarized in Supplementary Tables 1 and 2.

Correlations among various indicators affecting survival in postoperative GC patients

We first examined correlations among IHC-defined bio-indicators (p-Stat3, Her-2, CEA, nm-23) and clinical indicators. As shown in **Table 1**, positive correlations were present between cell differentiation and T, N, M, and TNM staging, respectively ($P<0.01$). Further analyses showed that the preoperative survival predictors of p-Stat3, Her-2, and CEA were positively correlated with T, N, M, as well as TNM stages ($P<0.05$), respectively, while nm-23 was negatively correlated with T, N, M, and TNM stages ($P<0.05$), respectively. The negative correlation of nm-23 with TNM stages was further confirmed by Kaplan-Meir survival analysis (see **Figure 2**).

Tissue levels of p-Stat3, Her-2, CEA and nm-23 impact on survival of GC patients after surgery

Patient follow-ups ranged from 0 (<1 month) to 135 months after surgery until October 10, 2018 and the median follow-up time was 25 months. The longest survival time of GC patients after surgery reached 11 years. By October 1, 2018 (135 months), 30 (27.3%) GC

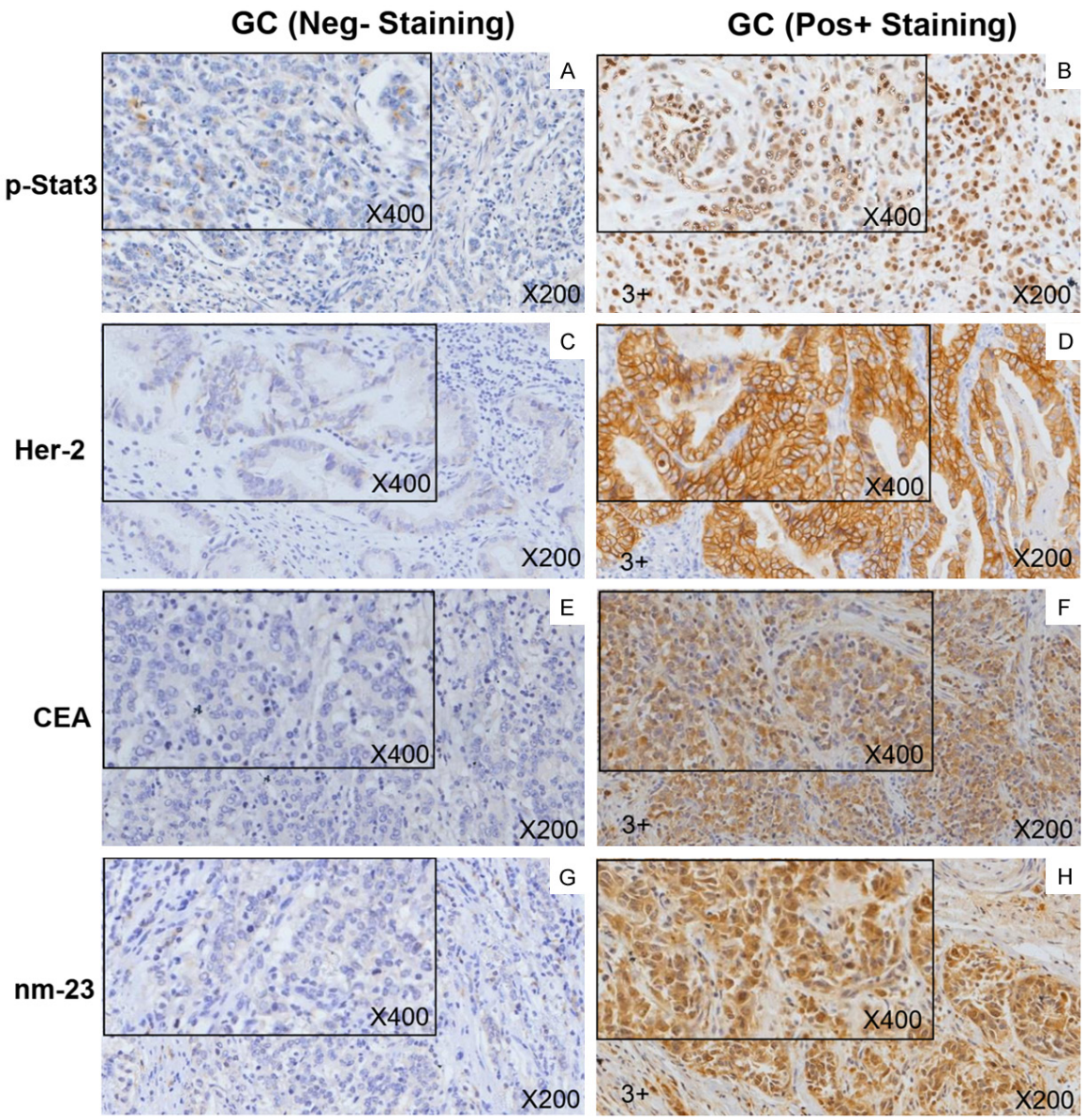

Figure 1. Immunohistochemical (IHC) staining of specific antibodies against p-Stat3, Her-2, CEA and nm-23 in tissues of gastric cancer patients. As shown are eight representative panels of GC tissues using IHC staining. Antibody-staining against p-Stat3 is mainly localized in the nucleus (B, positive staining of yellow-brown) and Her-2 staining is mainly localized on the membrane (D, positive staining of yellow-brown), while antibody-staining against CEA and nm-23 are primarily in the cytoplasm (F and H, positive staining of yellow-brown). It can be seen that p-Stat3 is strongly stained in GC tissue (B) (scored as 3+) as compared with GC tissue (A) (scored as negative or 0). Similarly, for Her-2, CEA and nm-23, tissue (D, F, H) show strong staining (scored as 3+) but tissue (C, E, G) show little or no staining (scored as negative or 0). Microscopic magnification was ×200 with inserts of ×400.

patients were still alive, whereas 80 (73.0%) died. First, we analyzed the impacts of the well-established TNM staging system on the survival of GC patients by Kaplan-Meir method, whose results validated this GC patient cohort (**Figure 2**). Having validated the patient cohort for survival analysis, we then analyzed the impacts of p-Stat3, Her-2, CEA and nm-23 on the survival curves of the GC patients. As shown in **Figure 2**, the tissue levels of p-Stat3, Her-2, and CEA showed correlations with patient survival, i.e., the higher the tissue levels were, the poorer the survival was for those patients. On the other hand, however, lower levels of nm-23

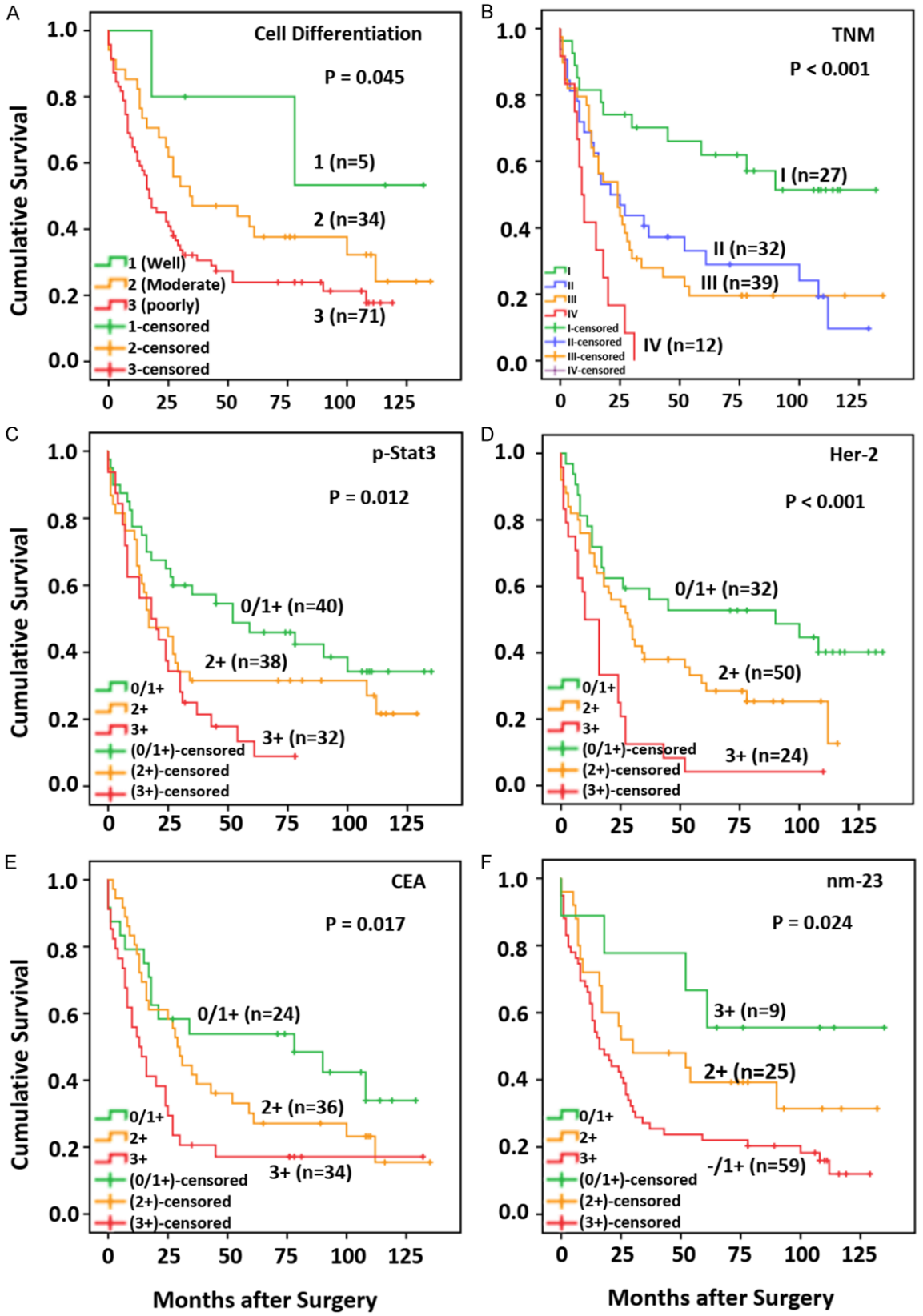

Figure 2. Kaplan-Meier analyses reveal that the tissue levels of p-Stat3, Her-2, CEA or nm-23 correlate with the survivals of GC patients after surgery. This study analyzed 110 total GC cases for survival, of which 110 cases were available for p-Stat3, 106 cases for Her-2 and 94 cases for CEA and nm-23, respectively. To validate this GC cohort

in terms of possible sampling bias that could affect survival analysis, overall survival (OS) is first analyzed against well-established risk factors affecting survival in GC patients using Kaplan-Meier method. As can be seen in cell differentiation in (A), patients with well differentiated GC (1) have the best OS while patients with poor differentiated GC (3) have the worst OS, and patients with moderately differentiated GC (2) show OS in between. Furthermore, in TNM staging as shown in (B), a typical OS hierarchy is shown that patients with the earliest stage I present the best OS while patients with the latest stage IV show the poorest OS. Having validated this patient cohort using well-established risk factors, it is immediately clear that the tissue levels of p-Stat3, Her-2, CEA, and nm-23 are correlated with GC patients' survival. (C-F) The tissue levels of p-Stat3, Her-2, and CEA showed correlations with patient survival, i.e., the higher the tissue levels were, the poorer the survival was for those patients. However, lower levels of nm-23 correlated with a poorer survival of GC patients.

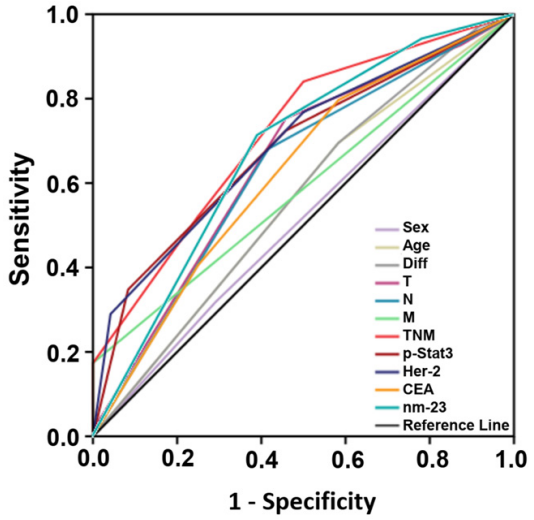

Figure 3. ROC curves and AUCs reveal performance abilities or power of risk factors affecting patients' survival. The TNM staging is an established risk factor capable of predicting survival prognosis for GC patients. In ROC analysis, the larger the AUC (area under the curve) of a factor has, the higher the ability or power of the factor in predicting survival. In other words, the AUC is quantified performance that reflects the reliability and accuracy of a risk factor in terms of predicting patient's survival. As shown, TNM staging has the largest AUC (AUC=0.717) followed by Her-2 (AUC=0.691), nm-23 (AUC=0.675), and CEA (AUC=0.626) (see **Table 2** for statistical comparisons). It is interesting to note that Her-2 has an AUC slightly less than TNM staging.

correlated with a poorer survival of GC patients (**Figure 2**), in keeping with the previous observations and, importantly, further confirming that this GC patient cohort was reliable in terms of testing new bio-indicators which were potential risk factors for survival among postoperative GC patients.

Survival prediction power (SPP) of different bio-indicators in postoperative GC patients

As shown in **Table 2**, the numerical value of an AUC represents the power or weight of a risk factor that impacts on survival prognosis, which we have previously termed as impact weight (IW) [15]. The higher the AUC/IW of a factor is, the more powerful that factor impacts on survival prognosis and, therefore, a concept of survival prediction power (SPP) has been derived. As SPP is AUC-quantified probability of survival for a particular bio-indicator, the clinical usefulness of that bio-indicator becomes mathematical. Like other clinical tests, the final destiny of survival prediction should be quantifiable or semi-quantifiable. A hierarchy of AUC was obtained as follows: Her-2 (AUC=0.691, P<0.001), p-Stat3 (AUC=0.683, P=0.008), nm-23 (AUC=0.675, P=0.012), and CEA (AUC=0.626, P=0.068). All these 4 molecules could predict the survival of postoperative GC patients, of which Her-2 had the greatest ability to predict the survival time, which was slightly lower than the TNM staging (AUC=0.717, P=0.002) but higher than all other indicators, suggesting a high SPP for Her-2 on survival prediction.

Risk factors affecting the prognosis of GC patients

As shown in **Table 3**, univariate analyses demonstrated that, in GC patients, survival prognosis was associated with cell differentiation (P=0.025), invasion depth (P=0.006), lymph node metastasis (P=0.002), distant metastasis (P=0.001), TNM staging (P=0.004), and tissue levels of p-Stat3 (P=0.014), Her-2 (P=0.006) and nm-23 (P=0.013). Her-2 and p-Stat3 were independent factors affecting the survival of GC patients. Furthermore, multivariate analyses showed only Her-2 (HR=2.273, P=0.010) and cell differentiation (HR=2.057, P=0.020) to be independent factors in predicting survival for GC patients (see **Table 4**). These results suggested that clinicopathological factors and bio-indicators may work in concert, with a few being the major players, to impact on patients' survival, which was in keeping with the paradigm that GC is a polygenic disease with multistaged progressions.

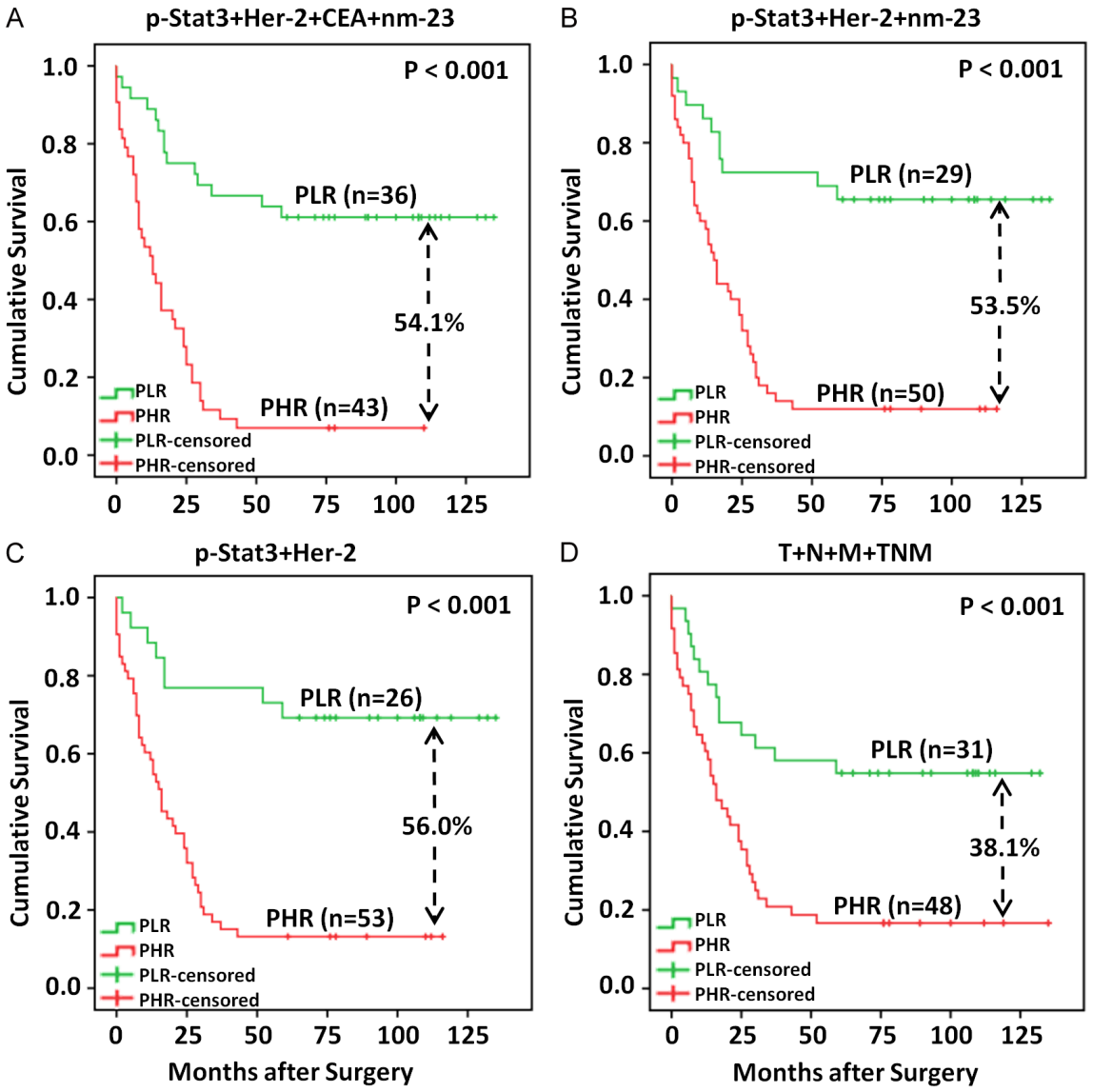

Figure 4. Combinations of IHC-defined bio-indicators show optimal survival predictions among postoperative GC patients with defined predictive risks. Based on IHC scores of bio-indicators and TNM stages, postoperative GC patients were grouped into PLR (predictive low risk) or PHR (predictive high risk) patients by logistic regression and ROC analyses using 5-year survival as an end point (see Methods and **Figure 2**). A-D. Represent Kaplan-Meir survival analyses for particular combinations of bio-indicators, respectively. Between the upper survival curve of PLR patients and the lower survival curve of PHR patients is a wide interval termed as survival discriminative interval (SDI) expressed as percent difference in cumulative survival rates (%) between PLR and PHR curves. SDI is a function of discriminating power in terms of survival prediction. The wider the SDI is, the higher the discriminative power has for the bio-indicator combination. These results have three important indications: (1) any combinations of two or more bio-indicators show better discriminative power than individual bio-indicators shown in **Figure 2**; (2) all bio-indicator combinations have higher SDI (>53.0%) than that of TNM staging (SDI=38.1%) which is a gold standard in survival prediction; and (3) the best bio-indicator combination is the p-Stat3+Her-2 combination which gives the highest SDI (56.0%) and the least bio-indicators needed.

Multiple survival predictors in combination are superior to single survival predictors as defined by ROC curve (AUC) analyses

Gastric cancer is polygenic in terms of pathogenesis and disease progression, and therefore, the power of a single risk factor/bio-indicator as a predictor for GC patients' survivals is limited. Different from our previous studies [15], this study took a new approach aimed at enhancing the discriminative power in predicting survival using combinations of multiple IHC-

Table 1. Cross-correlation analyses between various clinicopathological factors and IHC-detected biomarkers in GC patients

Variables	Clinicopathologic factors							IHC-detected molecules			
	Gender	Age	Cell diff.	Depth of invasion	Lymph node metastasis	Distant metastasis	TNM staging	p-Stat3	Her-2	CEA	nm-23
Gender	1										
Age	-0.123	1									
Cell diff.	0.18	-0.163	1								
Depth of invasion	-0.037	0.137	0.141	1							
Lymph node metastasis	-0.037	0.077	0.129	0.423**	1						
Distant metastasis	0.105	-0.129	0.257**	0.164	0.280**	1					
TNM staging	0.015	0.019	0.262**	0.701**	0.686**	0.565**	1				
p-Stat3	0.036	-0.093	0.260**	0.236*	0.117	0.210*	0.285**	1			
Her-2	-0.044	0.289**	-0.047	0.317**	0.371**	0.159	0.372**	-0.004	1		
CEA	-0.005	0.154	0.055	0.377**	0.417**	0.157	0.394**	0.155	0.370**	1	
nm-23	-0.075	0.199	-0.093	-0.311**	-0.262*	0.058	-0.207*	-0.125	-0.131	-0.203	1

Note: Values in the main table are correlation coefficients or r values. Cell diff.=cell differentiation; T=invasion depth; N=lymph node metastasis; M=distant metastasis; TNM=TNM staging. *=P<0.05, **=P<0.01.

Table 2. Areas under ROC curves (AUC) and other characteristics of survival predictors

Indicators	AUC	95% CI	*P* value	Sensitivity	Specificity	Youden's Index	Cut-off value
Reference curve	0.500	N/A	N/A	N/A	N/A	N/A	N/A
Sex	0.514	(0.380, 0.648)	0.843	31.9%	70.8%	0.03	N/A
Age	0.556	(0.420, 0.692)	0.414	65.2%	54.2%	0.19	61.5
Cell differentiation	0.563	(0.425, 0.700)	0.361	69.6%	41.7%	0.11	2.5
Invasion depth	0.647	(0.516, 0.778)	0.033	75.4%	54.2%	0.30	2.5
Lymph node metastasis	0.632	(0.501, 0.764)	0.055	68.1%	58.3%	0.26	0.5
Distant metastasis	0.587	(0.464, 0.710)	0.206	17.4%	100.0%	0.17	0.5
TNM staging	0.717	(0.601, 0.834)	0.002	84.1%	50.0%	0.34	1.5
p-Stat3	0.683	(0.565, 0.800)	0.008	72.5%	54.2%	0.27	1.5+
Her-2	0.691	(0.574, 0.807)	0.006	76.8%	50.0%	0.27	1.5+
CEA	0.626	(0.493, 0.759)	0.068	79.7%	41.7%	0.21	1.5+
nm-23	0.675	(0.542, 0.809)	0.012	70.0%	57.9%	0.28	1.5+

Note: The optimal cut-off point was defined as the closest point on the ROC curve to the point (0, 1) where false positive rate of zero and sensitivity of 100% (also see **Figure 3** and **Table 5**). *P* values were obtained by comparing various indicators with the reference curve in ROC analyses. Statistical significance was defined as P<0.05 for all analyses.

Table 3. Analyses using Cox single factor regression model on clinical and IHC variables in GC patients

Variables	Univariate Analysis	
	HR (95% CI)	*P* value
Sex (Female vs. Male)	1.393 (0.862, 2.251)	0.176
Age (≥60 vs. <60)	1.270 (0.790, 2.042)	0.323
Cell diff. (Poor vs. Moderate+Well)	1.727 (1.072, 2.783)	0.025
Invasion depth (T_3+T_4 vs. T_1+T_2)	2.075 (1.233, 3.493)	0.006
Lymph node metastasis (Yes vs. No)	2.158 (1.337, 3.485)	0.002
Distant metastases (M1 vs. M0)	2.954 (1.563, 5.581)	0.001
TNM stages (III+IV vs. I+II)	1.946 (1.242, 3.050)	0.004
p-Stat3 levels (2+/3+ vs. -/1+)	1.824 (1.127, 2.953)	0.014
Her-2 levels (2+/3+ vs. -/1+)	2.137 (1.247, 3.662)	0.006
CEA levels (2+/3+ vs. -/1+)	1.791 (0.993, 3.231)	0.053
nm-23 levels (2+/3+ vs. -/1+)	0.516 (0.306, 0.869)	0.013

Note: Cell diff.=cell differentiation; HR=hazard ratio; CI=confidence interval.

defined bio-indicators in the analyses. As mentioned above, ROC curves and AUC are means to quantify powers or abilities of survival predictors. As shown in **Table 5**, the predictive power for the combination of p-Stat3+Her-2 was the strongest because the combination produced an AUC of 0.867 (95% CI, 0.784-0.950), similar to the full four molecular combination of p-Stat3+Her-2+CEA+nm-23, which had an AUC of 0.891 (95% CI, 0.820-0.963). Surprisingly, the AUC of the p-Stat3+Her-2 combination was much higher than that of TNM staging, which is the established standard, that only produced an AUC of 0.755 (95% CI, 0.645-0.865) (P< 0.001 for all above comparisons).

These results indicated that the combination of p-Stat3 plus Her-2 would be the best combination in terms of the least bio-indicators needed and the reasonable AUC value presented, to predict possible outcomes of GC patients five years after surgery. One could then make the following individualized judgments based on the results in **Table 5**. For example, one could input the scores of p-Stat3 and Her-2, respectively into the logistic equation and then obtain

Table 4. Analyses using Cox multiple factor regression on clinical and IHC variables in GC patients

Variables	Multivariate Analysis	
	HR (95% CI)	*P* value
Cell diff. (poor vs. well+moderate)	2.057 (1.120, 3.777)	0.020
Invasion depth (T_3/T_4 vs. T_1/T_2)	1.155 (0.571, 2.336)	0.689
Lymph node metastasis (Yes vs. No)	1.654 (0.840, 3.259)	0.146
Distant metastases (M1 vs. M0)	1.575 (0.761, 3.260)	0.221
TNM stages (III/IV vs. I/II)	0.654 (0.310, 1.383)	0.267
p-Stat3 levels (2+/3+ vs. -/1+)	1.299 (0.681, 2.479)	0.428
Her-2 levels (2+/3+ vs. -/1+)	2.273 (1.219, 4.239)	0.010
nm-23 levels (2+/3+ vs. -/1+)	0.700 (0.389, 1.262)	0.236

Note: Cell diff.=cell differentiation; HR=hazard ratio; CI=confidence interval.

Table 5. ROC analyses provide cross-comparisons among different bio-indicator combinations using multiple parameters

Combinations of indicators	AUC	95% CI	*P* value	Sensitivity	Specificity	Accuracy	Youden's Index	Cut-off value
Reference curve	0.500	N/A	N/A	N/A	N/A	N/A	N/A	N/A
p-Stat3+Her-2+CEA+nm-23	0.891	(0.820, 0.963)	<0.001	74.1%	88.0%	78.5%	0.621	0.728
p-Stat3+Her-2+CEA	0.872	(0.791, 0.953)	<0.001	90.7%	72.0%	84.8%	0.627	0.436
p-Stat3+Her-2+nm-23	0.883	(0.810, 0.956)	<0.001	81.5%	76.0%	79.7%	0.575	0.709
p-Stat3+CEA+nm-23	0.832	(0.734, 0.930)	<0.001	74.1%	80.0%	75.9%	0.541	0.705
p-Stat3+Her-2	0.867	(0.784, 0.950)	<0.001	85.2%	72.0%	81.0%	0.572	0.576
p-Stat3+CEA	0.804	(0.698, 0.911)	<0.001	61.1%	88.0%	69.6%	0.491	0.759
p-Stat3+nm-23	0.794	(0.689, 0.900)	<0.001	70.4%	72.0%	70.9%	0.424	0.707
Her-2+CEA	0.766	(0.659, 0.872)	<0.001	53.7%	88.0%	64.6%	0.417	0.764
Her-2+nm-23	0.785	(0.678, 0.892)	<0.001	66.7%	80.0%	70.9%	0.467	0.739
CEA+nm-23	0.771	(0.663, 0.880)	<0.001	81.5%	64.0%	75.9%	0.455	0.618
T+N+M+TNM	0.755	(0.645, 0.865)	<0.001	74.1%	68.0%	72.2%	0.421	0.644

Note: Larger AUC suggests higher SPP power in predicting survival. *P* values were obtained in comparisons with the reference curve (AUC=0.500) by ROC analyses. Statistical significance was defined as P<0.05 for all analyses.

a joint prediction probability value of p-Stat3 and Her-2 for possible death events (**Figure 5**). If the joint score is greater than the best critical probability value of 0.576, one could predict and evaluate the death of GC patients five years after surgery with an accuracy of 81.0% (**Table 5**).

Combinations of multiple survival predictors are superior to individual predictors by Kaplan-Meier survival analyses among GC patients

Area under the ROC curve (AUC) is a measure that quantifies the probability of survival prediction for an indicator, which we have defined as survival prediction power (SPP). Having analyzed the SPPs for various combinations of bio-indicators (**Table 5**), we then transformed the SPP results to survival curves using Kaplan-Meir method. As mentioned in the Methods, GC patients were categorized into predictive low risk (PLR) and predictive high risk (PHR), respectively, according to 5-year survivals as an end point. As shown in **Figure 4**, it was immediately clear that combinations of two or more bio-indicators showed a wide discriminative distance between PLR patients' survival curves and PHR patients' survival curves, indicating a superior power when compared with those using only individual bio-indicators (**Figures 2** vs. **4**). Interestingly, among four bio-indicators, the combination of p-Stat3 plus Her-2 (p-Stat3+Her-2) showed the very similar discriminative power compared with the combinations with three bio-indicators (p-Stat3+Her-2+CEA) or even four bio-indicators (p-Stat3+Her-2+CEA+nm-23) (**Table 5**). Surprisingly, on the other hand, all three combinations of IHC-

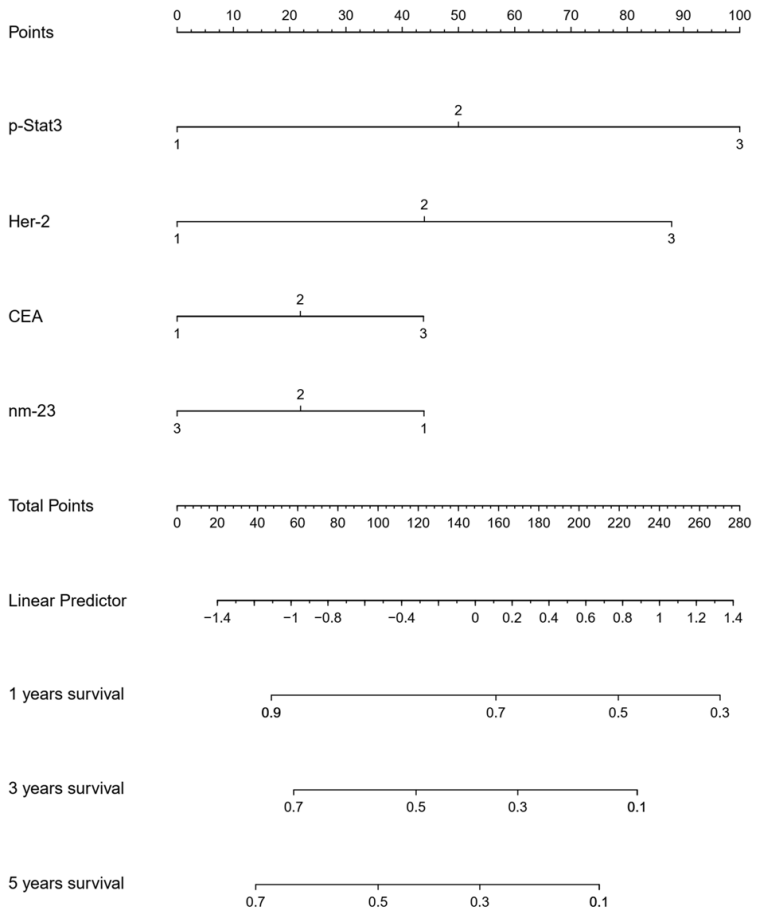

Figure 5. Nomogram in predicting individual patients' overall survival of 1, 3 and 5 years based on preoperative clinical indices. The value of an individual patient is located on each variable axis, and the predictor points ("points" scale at top) correspond to each variable. The sum of all five variables is located on the total point axis.

defined bio-indicators, from two to four bio-indicators, exhibited superior discriminative power to TNM staging in terms of AUC and other comparing parameters (**Table 5**) and also survival curves between PLR and PHR patients (**Figure 4**), verifying the reliability and rationality of p-Stat3 plus Her-2 (p-Stat3+Her-2) as the optimal combination (**Table 5**; **Figure 4**). It was interesting to note that the results shown in **Table 5** and **Figure 4** were supporting each other.

Discussion

Gastric cancer (GC) is one of the most life-threatening diseases [2]. The high mortality of GC is largely due to a late-stage diagnosis during the disease course and early diagnosis is often difficult due to a lack of massive screening, which, at the present, is a mission impossible in developing countries and low-resource communities in more developed countries. Treatment of late-stage GC is ineffective resulting in a low five-year survival rate in China and other developing countries [2]. While early diagnosis is critical, GC diagnosed at a late stage may also face challenges in terms of treatment options. Surgery, such as gastrectomy, is an option for many late-stage GC patients, however, not all late-stage GC patients are beneficial from gastrectomy [17, 18] in terms of survival and quality of life. We have previously proposed the option to test some preoperative bio-indicators such as BMI (body mass index) and blood metabolites that can predict survival of GC patients prior to surgery [15]. Based on this concept, this study takes a novel approach to incorporate multiple bio-indicators that: (1) can be tested preoperatively; and (2) can increase the sensitivity or power in predicting survival before surgery.

Using IHC method, bio-indicators or biomarkers, namely p-Stat3, Her-2, CEA and nm-23 were detected (**Table 1**), respectively, among which p-Stat3 is an activated form of the Stat3 signaling pathway [15]. The correlation analysis has indicated a significant correlation of high levels of p-Stat3 with cell differentiation, depth of invasion, TMN stage, and lymph node metastasis in GC patients, in keeping with previous studies of ours and others [19, 20]. Importantly, p-Stat3 is also involved in cell differentiation, cell proliferation, apoptosis, invasion, angiogenesis, and poor prognosis in other cancers including breast cancer, pancreatic cancer, and lung cancer [21-23]. Increased expression of Her-2 and CEA has been shown to correlate with many features of cancer such as differentiation, proliferation, invasion, and metastasis [24, 25]. Here we show that tissue

levels of Her-2 and CEA are correlated with patient's age, T, N, M, or TNM stages (**Table 1**), in agreement with previous findings [26-28]. On the contrary, nm-23 is a tumor metastasis inhibitors and the deceased expression of nm-23 promotes tumor invasion and vascular dispersal in cancer tissues [29]. Studies have found that the expression of nm-23 is decreased in gastric cancer tissues with lymph node metastasis [30]. In this study, the expression of nm-23 is negatively correlated with T, N and TNM staging (**Table 1**), consistent with previous observations [31, 32].

Having tested the correlations among various clinical and bio-indicators, we hypothesize that, given the correlations of those bio-indicators with TNM staging system as shown in **Table 1**, they should have, similarly to TNM staging, impacts on the survival of GC patients. As shown in **Figure 2**, well-established cell differentiations and TNM stages have significantly impact on the survival of GC patients, validating the usefulness of this GC patient cohort for newly tested bio-indicators. Indeed, Kaplan-Meir analyses have indicated that tissue levels of p-Stat3, Her-2, CEA, and nm-23 have impact on the survival prognosis of this GC patients cohort, of which nm-23 negatively impacts the survival (**Figure 2**), in keeping with the correlation analyses in **Table 1**.

Kaplan-Meier analysis compares different groupings within one variable (risk factor) for its ability to discriminate each other in survival [15]. For example, as shown in **Figure 2**, differences in survival are discriminated by patient groupings as defined within cell differentiation variable or within TNM variable. However, this analysis cannot cross-compare the discriminative ability of cell differentiation variable (risk factor) from TNM variable (risk factor) (**Figure 2**) as these abilities are not quantified. To compare discriminative abilities between or among different risk factors so as to understand their abilities in predicting survival prognosis, we have used ROC curve and AUC to quantify the ability or power of a survival predictor in gastric cancer, by which the potentials of different survival predictors were cross-compared (**Figure 3** and **Table 2**). As shown in **Table 2**, bio-indicators with higher AUCs suggest higher discriminative powers in predicting prognosis, and therefore, the clinical usefulness of these bio-indicators in predicting survival among GC patients (**Figure 3** and **Table 2**). Based on these findings, here we introduce a new concept of survival prediction power (SPP) in terms of ROC/AUC-quantifying and cross-comparing risk factors that impact survival. SPP is defined as a power or ability that can discriminate the groupings of GC patients based on one risk factor or a collection of risk factors.

Gastric cancer, like many other cancers, is polygenic in terms of pathogenesis and multi-staged in terms of disease progression. Therefore, the power of a single risk factor, which may represent a single gene, as a survival predictor for GC patients is limited. We favor the hypothesis that there should be multiple risk factors affecting the prognosis of GC patients and incorporating multiple risk factors (bio-indicators) in the survival analysis would be able to reveal a more powerful survival prediction for postoperative GC patients [33]. To test this hypothesis, we have taken a new approach aimed at enhancing the SPP power in predicting survival using combinations of multiple IHC-defined bio-indicators in the analyses. As shown in **Table 5**, the SPP for the combination of p-Stat3+Her-2 is the strongest because the combination produces an AUC of 0.867, close to the four molecular combination of p-Stat3+Her-2+CEA+nm-23 (AUC=0.891). To our surprise, the AUC of the p-Stat3+Her-2 combination is much higher than that of TNM staging with an AUC of 0.755 (**Table 5**).

Next, we used Kaplan-Meir method to translate the AUC results (**Table 5**) to survival curves using GC patients categorized into predictive low risk (PLR) and predictive high risk (PHR), respectively. As shown in **Figure 4**, it is immediately demonstrated that combinations of two or more bio-indicators show wide discriminative distances between the survival curves of PLR patients and PHR patients for all four panels. Clearly, SPP powers with multiple bio-indicators are superior to those with single bio-indicators (**Figures 2** vs. **4**). There are two interesting observations in **Figure 4**: (1) the twofactor combination of p-Stat3+Her-2 has the same or very similar SPP power (56.0%) to those of the three-factor combination of p-Stat3+Her-2+nm-23 (53.5%) and even the

four-factor combination of p-Stat3+Her-2+CEA+nm-23 (54.1%); (2) surprisingly, all three combinations of the above bio-indicators exhibit superior SPP powers to that of TNM staging (38.1%) in terms of discriminating PLR patients from PHR patients. The echoing observations in **Table 5** and **Figure 4** have demonstrated that ROC/AUC quantification results can be reproduced by Kaplan-Meir survival curves, indicating the reliability of the new approach.

The combination of p-Stat3 plus Her-2 with the best SPP power is important because the combination needs the least biomarkers making its clinical use practical and economic. Furthermore, as all four bio-indicators described can be tested on biopsies obtained from screening gastroscopy and many clinicopathological characteristics can also be obtained preoperatively, this new approach may be indeed useful and practical in routine clinic.

In summary, using a cohort of 110 GC patients with more than 10 years follow-up, we have established a multi-factor survival prediction model for gastric cancer patients after surgery. This study has observed three major findings: (1) IHC-tested tissue levels of p-Stat3, Her-2, CEA, and nm-23 are survival predictors among which p-Stat3 and Her-2 can jointly serve as a survival prediction combination for GC patients after surgery. (2) ROC curve analysis and AUC areas can be used to quantify survival prediction powers (SPP) of biomarkers involved in survival prediction which makes cross-comparisons possible for individual survival predictors for GC patients. (3) We have for the first time demonstrated that multiple bio-indicators have higher SPP than those of single bio-indicators as well as those of TNM staging system in survival prediction in postoperative GC patients. As p-Stat3 and Her-2 can be tested on biopsies obtained from gastroscopy, the combinational enhancement in survival prediction may be indeed useful to serve as “pre-warning indicators” in management decisions before surgery. In this context, these “pre-warning indicators” may assist in personalized treatment for GC patients such as pre-surgical planning, optimal radio-chemotherapy and appropriate follow-up intervals after surgery.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. The correlation between the levels of p-Stat3 or Her-2 and the clinicopathological characters in GC patients

	n	p-Stat3			*P* value	n	Her-2			*P* value
	110	-/1+ (%)	2+ (%)	3+ (%)		106	-/1+ (%)	2+ (%)	3+ (%)	
Sex										
Male	79	31 (39.2)	24 (30.4)	24 (30.4)	0.708	75	23 (30.7)	33 (44.0)	19 (25.3)	0.652
Female	31	9 (29.0)	14 (45.2)	8 (25.8)		31	9 (29.0)	17 (54.8)	5 (16.1)	
Age										
<60	36	9 (25.0)	17 (47.2)	10 (27.8)	0.333	34	15 (44.1)	17 (50.0)	2 (5.9)	0.003
≥60	74	31 (41.9)	21 (28.4)	22 (29.7)		72	17 (23.6)	33 (45.8)	22 (30.6)	
Cell differentiation										
Well	5	4 (80.0)	1 (20.0)	0 (0)	0.015	4	1 (25.0)	3 (75.0)	0 (0)	0.701
Moderate	34	17 (50.0)	9 (26.5)	8 (23.5)		33	7 (21.2)	19 (57.6)	7 (21.2)	
Poorly	71	19 (26.8)	28 (39.4)	24 (33.8)		69	24 (34.8)	28 (40.6)	17 (24.6)	
Invasion depth										
T_1	9	5 (55.6)	3 (33.3)	1 (11.1)	0.040	9	5 (55.6)	4 (44.4)	0 (0)	0.011
T_2	26	15 (57.7)	5 (19.2)	6 (23.1)		25	11 (44.0)	12 (48.0)	2 (8.0)	
T_3	73	19 (26.0)	29 (39.7)	25 (34.2)		70	16 (22.9)	32 (45.7)	22 (31.4)	
T_4	2	1 (50.0)	1 (50.0)	0 (0)		2	0 (0)	2 (100)	0 (0)	
Lymph node metastasis										
No	43	20 (46.5)	11 (25.6)	12 (27.9)	0.221	42	21 (50.0)	17 (40.5)	4 (9.5)	<0.001
Yes	67	20 (29.9)	27 (40.3)	20 (29.9)		64	11 (17.2)	33 (51.6)	20 (31.3)	
Distant metastasis										
No	98	38 (38.8)	35 (35.7)	25 (25.5)	0.029	94	30 (31.9)	45 (47.9)	19 (20.2)	0.104
Yes	12	2 (16.7)	3 (25.0)	7 (58.3)		12	2 (16.7)	5 (41.7)	5 (41.7)	
TNM staging										
I	27	17 (63.0)	5 (18.5)	5 (18.5)	0.014	26	15 (57.7)	11 (42.3)	0 (0)	0.001
II	32	11 (34.4)	11 (34.4)	10 (31.3)		32	9 (28.1)	14 (43.8)	9 (28.1)	
III	39	10 (25.6)	19 (48.7)	10 (25.6)		36	6 (16.7)	20 (55.6)	10 (27.8)	
IV	12	2 (16.7)	3 (25.0)	7 (58.3)		12	2 (16.7)	5 (41.7)	5 (41.7)	

Note: Kaplan-Meier survival analysis was used in the statistical analyses.

Supplementary Table 2. The correlation between the expression of CEA or nm-23 and the clinico-pathological characters in GC patients

	n	CEA			*P* value	n	nm-23			*P value*
	94	-/1+ (%)	2+ (%)	3+ (%)		93	-/1+ (%)	2+ (%)	3+ (%)	
Sex										
Male	65	17 (26.2)	24 (36.9)	24 (36.9)	0.965	67	41 (61.2)	19 (28.4)	7 (10.4)	0.469
Female	29	7 (24.1)	12 (41.4)	10 (34.5)		26	18 (69.2)	6 (23.1)	2 (7.7)	
Age										
<60	32	11 (34.4)	12 (37.5)	9 (28.1)	0.137	31	24 (77.4)	5 (16.1)	2 (6.5)	0.057
≥60	62	13 (21.0)	24 (38.7)	25 (40.3)		62	35 (56.5)	20 (32.3)	7 (11.3)	
Cell differentiation										
Well	4	2 (50.0)	1 (25.0)	1 (25.0)	0.658	3	2 (66.7)	1 (33.3)	0 (0)	0.565
Moderate	28	6 (21.4)	13 (46.4)	9 (32.1)		32	18 (56.3)	10 (31.3)	4 (12.5)	
Poorly	62	16 (25.8)	22 (35.5)	24 (38.7)		58	39 (67.2)	14 (24.1)	5 (8.6)	
Invasion depth										
T_1	8	8 (100)	0 (0)	0 (0)	<0.001	8	3 (37.5)	3 (37.5)	2 (25.0)	0.030
T_2	22	7 (31.8)	8 (36.4)	7 (31.8)		20	8 (40.0)	11 (55.0)	1 (5.0)	
T_3	63	9 (14.3)	28 (44.4)	26 (41.3)		63	46 (73.0)	11 (17.5)	6 (9.5)	
T_4	1	0 (0)	0 (0)	1 (100)		2	2 (100)	0 (0)	0 (0)	
Lymph node metastasis										
No	36	16 (44.4)	15 (41.7)	5 (13.9)	<0.001	36	17 (47.2)	14 (38.9)	5 (13.9)	0.012
Yes	58	8 (13.8)	21 (36.2)	29 (50.0)		57	42 (73.7)	11 (19.3)	4 (7.0)	
Distant metastasis										
No	82	22 (26.8)	33 (40.2)	27 (32.9)	0.131	82	53 (64.6)	21 (25.6)	8 (9.8)	0.576
Yes	12	2 (16.7)	3 (25.0)	7 (58.3)		11	6 (54.5)	4 (36.4)	1 (9.1)	
TNM staging										
I	23	14 (60.9)	6 (26.1)	3 (13.0)	0.001	21	8 (38.1)	10 (47.6)	3 (14.3)	0.029
II	28	5 (17.9)	13 (46.4)	10 (35.7)		27	18 (66.7)	6 (22.2)	3 (11.1)	
III	31	3 (9.7)	14 (45.2)	14 (45.2)		34	27 (79.4)	20 (14.7)	2 (5.9)	
IV	12	2 (16.7)	3 (25.0)	7 (58.3)		11	6 (54.5)	4 (36.4)	1 (9.1)	

Note: Kaplan-Meier survival analysis was used in the statistical analyses.