A Risk Signature with Immune and Inflammatory Cells Infiltration in Gastric Cancer Predicts Survival and Efficiency of Chemotherapy

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Research

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Abstract

Background In order to accurately predict outcomes of gastric cancer (GC), we developed a risk signature with tumor infiltration immune and inflammatory cells for prognosis.

Methods A risk signature model in combination with CD66b + neutrophils, CD3 + T, CD8 + T lymphocytes, and FOXP3 + regulatory T cells was developed in a training cohort of 327 GC patients undergoing surgical resection between 2011 and 2012, and validated in a validation cohort of 285 patients from 2012 to 2013.

Results High CD66b expression predicted poor disease-special survival (DSS) as well as inversely correlated with CD8 ($P < 0.05$) and FOXP3 expression ($P < 0.05$) in the training cohort, comparable disease-free survival (DFS) findings were observed in the validation cohort.Furthermore, a risk stratification was developed from integration of CD66b + neutrophils and T immune cells. In both DFS and DSS, the high-risk group all demonstrated worse prognosis than low-risk group in both the training cohort and the validation cohort (all $P < 0.05$). In addition, the high-risk group was associated with post-operative relapses. Furthermore, this risk signature model increase the predictive accuracy and efficiency for post-operative relapses. At last, the high-risk group identified a subgroup of GC patients who tend to not benefit from adjuvant chemotherapy.

Conclusions Incorporation of neutrophils into T lymphocytes could provide more accurate prognostic information in GC, and this risk stratification predicted survival benefit from post-operative adjuvant chemotherapy in GC.

Background

Gastric cancer (GC) is the fifth most common malignancy and the third leading cause

of cancer death worldwide[1]. The prognosis of GC is very poor, with a 5-year survival less than 30%[2]. Adjuvant treatment after surgery could reduce the rate of relapse for patients with advanced gastric cancer. For these patients, a 5-fluoropyrimidine-based chemotherapy is commonly used the first-line choice[3], and with the administration of a regimen of capecitabine plus platinum, patients with locally advanced gastric cancer can improve clinical prognosis with acceptable adverse effects[4]. Additionally, patients with tumor recurrence or metastasis might have a better outcomes when using chemotherapy combined with targeted treatments[3]. However, the prognosis of many patients were still low despite high initial response rates[5]. Therefore, there is a urgent to identify subgroup that can be used to better predict adjuvant chemotherapy and allow rational future therapies to be tailored for those patients.

Recent evidence has indicated that inflammatory reactions, always accompanied with immune response in tumor environment, play vital roles in tumor occurrence, development, and resistance to therapy[6]. Tumor-associated neutrophils are reported as pro-tumor or anti-tumor functions depending on the tumor microenvironment[7]. Thus, the relationships between neutrophils infiltration and prognostic outcomes

generated heterogeneous results[8, 9]. Typically neutrophils are strongly associated with poor clinical outcomes in many types of cancer, including GC[10]. Whereas some other study showed that neutrophils in GC predicted good prognosis[11]. These result revealed the multifaceted function roles of Neutrophils in different stages of some tumor. Indeed, there were phenotypically distinct sub-population of neutrophils with conflicting functions under different tumor niches[12]. Based on this plasticity, neutrophils showed different functions through polarization between an anti-tumoral (N1) and a pro-tumoral (N2) phenotype[13]. The nonspecific cross-reacting antigen-95 (NCA95/CD66b), a highly glycosylated CEA family protein encoded by the CGM6 gene that is an activation maker exclusively expressed in granulocytes and can be found on both neutrophils and eosinophils[14–16]. CD66b can be used to identify neutrophils on heath and inflammation using high-throughput screening flow cytometry with a purity of 99%[17], and has been used in many tumor to identify neutrophils, including GC[11].

The preliminary aim was to assess the association of neutrophils to patient clinical characteristics and outcome. Furthermore, Being an inflammation-associated tumor, GC is characterized with a variety of immune and inflammation cells, such as macrophages, granulocytes, T lymphocytes, NK, mast cells[18]. All these immune and inflammation cells contribute to tumor cell invasion and metastasis[19]. Some study have found that tumor associated immune and inflammation cells were correlated with many prognosis, including GC[18]. Hence the major cell types in T lymphocytes comprised of CD3, CD8, and FOXP3 Tregs were included in this study.

In order to accurately predict oncological outcomes of GC, we establish predictive biomarkers with integration of CD66b + neutrophils and T immune cells for prognosis and treatment response. Systematic analysis of CD66b + neutrophils and T immune cells in cancer tissue may add the prognostic value to further stratify and better manage patients with different prognosis. Although adjuvant treatment of GC relies on TNM staging system. However current prognostic model could not provide full prognostic information for not incorporating information from tumor environments. Thus combine TNM staging system with tumor environments information might improve the prognostic accuracy of current model.

In the present study, we combined CD66b + neutrophils and T immune cells in GC and investigated their relation with clinical outcomes, especially in patients receiving adjuvant chemotherapy. At last, a risk signature model has been developed and provides a possible predictive system to evaluate outcomes for patients received adjuvant chemotherapy.

Materials And Methods

Study population

This study included two independent cohorts of GC patients. Detailed CD66b, CD3, CD8, and FOXP3 expression data were study by IHC. The d training cohort enrolling 327 GC was obtained from The Affiliated Cancer Hospital of Zhengzhou University from 2011 and 2012. The validation cohort enrolling

285 GC patients was obtained from Harbin Medical University Cancer Hospital between 2012 and 2013. In this study, all patients were diagnosed with gastric adenocarcinoma and were not treated with neoadjuvant chemotherapy. Patients with infectious diseases, autoimmune disease or multi-primary cancers were excluded from the study.

Paraffin-embedded and formalin fixed tissues were obtained from 327 patients of GC in the training cohort and 285 patients of GC in the validation cohort. The medical data and follow-up information of GC patients were identified from our prospective database.10 fresh paired intratumoral and nontumoral (at least 5 cm distant from tumor site) samples were obtained for from patients with GC who underwent surgical resection.

The tumor size was defined according to the longest diameters of the samples.

The eighth edition of the AJCC TNM staging classification for carcinoma of the stomach was used for tumor staging. The Lauren classification was defined as intestinal type, diffuse type, and mixed type. The histological grade was classified as

G1, G2, G3 adenocarcinoma, signet ring cell carcinoma, and mucinous adenocarcinoma. Lymphovascular invasion and perineural invasion were diagnosed

by H&E-stained slides. Disease-free survival (DFS) was defined as the date of

surgery to the date of identification of disease recurrence, either radiological or histological. Disease-specific survival (DSS) was calculated from the date of surgery

to the date of death from gastric cancer. Patients who died of causes unrelated to the

disease were censored at the last follow-up. All patients have provided written informed consent. The research protocol was reviewed and approved by the institutional review board of the The Affiliated Cancer Hospital of Zhengzhou University.

Immunohistochemistry (ihc) Staining

Immunohistochemical staining was performed using the avidin-biotin-peroxidase complex method. Appropriate primary antibodies (anti-CD66b antibody, BD, anti-CD3, CD8, FOXP3 antibody, Biosciences) and Envision-plus detection system (anti-mouse polymer or anti-robbat polymer) were applied for detection of immune and inflammatory cells. The immunostaining was evaluated by two pathologists blinded to the clinical information. Discordances were resolved by re-review to consensus or the third reviewer. The results were averaged. The median value derived from the training cohort was defined as cutoff for high or low cells infiltration, and applied to the training and validation cohort.

Prognostic Prediction

The risk signature model was generated by combining expression pattern of CD66b, CD3, CD8, and FOXP3 by using Nearest Template Prediction (NTP) algorithm, in which a model of risk stratification is made as implemented in the NTP module of the GenePattern analysis toolkit (http://software.broadinstitute. org/cancer/software/genepattern/). Neutrophil infiltration was regarded as cells down regulation host immune response to cancer, while CD3, CD8, and FOXP3 T cells as cells immunoactivities. Hence, the predictive score was summed by following scores: (1) high CD66b defined as 1 point; (2) low CD66b defined as 0 point; (3) high CD3, CD8, and FOXP3 as 0 point, and (4) low CD3, CD8, and FOXP3 as 1 point. The final cutoff value was defined as two points in total.

Statistical analysis

SPSS 19.0 software (Version 19.0, Chicago, IL, USA) and Graphpad prism 5.0 were used for all statistical analyses. Results were expressed as mean ± S.D. Analyses of variance and Pearson chi-square tests were utilized for exploratory comparisons between variables. Clinical outcomes were calculated with the Kaplan-Meier method and the log-rank (Mantel-Cox) test. Stepwise multivariate Cox proportion analysis was performed. The level of significance permitting multivariate analysis inclusion and the statistical significance for all other tests used was set at $P < 0.05$. The R software version 3.4.3 and the “rms” package (R Foundation for Statistical Compution) were applied to perform the nomogram analysis and calibration plot.

Results

Association of CD66b + neutrophils with survival

In total, 612 patients were enrolled in the study. In the training cohort by IHC, based on the cutoff value of 66 in neutrophils (Fig. 1A and 1B), Then patients with high and low neutrophils infiltration were identified for further analysis (< 66 as low neutrophils infiltration group and > 66 as high infiltration group). Patients with high number of CD66b + neutrophils had a significantly worse DSS than those with low number of CD66b + neutrophils ($P < 0.05$, Fig. 1D). A tendency of worse DFS was found to be associated with high number of CD66b + neutrophils,in spite of no statistical significance ($P = 0.68$, Fig. 1C). Not only CD66b + cells, but also CD3+, CD8+, and FOXP3 + cells were observed in gastric cancer tissues. The ability of neutrophils expressing CD66b to regulate the immune-cell infiltration may account for this survival profit. To investigate the relationship of neutrophils and immune and inflammatory cells, the relationships of CD66b with CD3, CD8, and FOXP3 expression were assessed. The pattern of CD66b expression was negatively correlated with CD8 ($r^2 = 0.386$, $P < 0.001$, Fig. 2A), and negatively correlated with FOXP3 ($r^2 = 0.367$, $P < 0.001$, Fig. 2B). No significant correlation of CD66b with CD3 ($P > 0.05$) was seen.

In the validation cohort, based on the cutoff value of 66 in neutrophils, Then patients with high and low neutrophils infiltration were identified for further analysis (< 66 as low neutrophils infiltration group and > 66 as high neutrophils infiltration group). Kaplan-Meier analysis also showed that high neutrophils were associated with worse DFS of GC patients ($P = 0.020$, **Figure E**). A tendency of worse DSS was found to

be associated with high number of CD66b + neutrophils,in spite of no statistical significance ($P = 0.74$, Fig. 1F). To validate the relationship of neutrophils and immune and inflammatory cells, the distributions of positively labeled cells with CD66b, CD3, CD8, and FOXP3 were assessed. The number of neutrophils expressing CD66b was negatively correlated with CD8 cells ($r^2 = 0.383$, $P < 0.001$, Supplementary Fig. 1A), and negatively correlated with FOXP3 ($r^2 = 0.569$, $P < 0.001$, Supplementary Fig. 1B). No significant correlation of CD66b with CD3 ($P > 0.05$) was seen.

To validate the result that neutrophils was negatively linked with CD8 + cells and Treg cells in cancer tissue, multi-color immunofluorescence was performed using antibodies recognizing CD66b, CD8, FOXP3 and DAPI. The number of CD8 + cells was significantly higher in tumors, when CD66b expression decreased (Fig. 2C). When CD66b expression increased, the number of CD8 cells was significantly lower (Fig. 2D). In addition, the number of FOXP3 + cells was significantly higher in tumors, when CD66b expression decreased (Fig. 2E). When CD66b expression increased, the number of FOXP3 + cells was significantly lower (Fig. 2F).

Risk Stratification Based On Cd66b + neutrophils And T Immune Cells

To illustrate the correlation of tumor-infiltration immune and inflammatory cells with clinicopathological factors and prognosis, we developed a risk signature model prediction survival based on neutrophils and immune cells. We divided the patients into high-risk and low-risk groups based on the four immune cells with an NTP algorithm to assess the prognostic impact of immune profile in GC patients. What is more, we transposed the risk signature into a predictive score to make the model easier to use in further study. The high-score group determined by predictive score > 2 had been identified as the high-risk group, and the low-risk group with the score ≤ 2 (Table 1and Supplementary Tables 1).

In the training cohort, patients in the high-risk group had worse survival of DFS and DSS than those in the low-risk group ($P < 0.001$ and $P < 0.001$, Fig. 3A **and** Fig. 3B). In addition, Kaplan-Meier analysis also showed that the high-risk group was associated with worse DFS and DSS of GC patients ($P < 0.001$ and $P < 0.001$, Fig. 3C and 3D) in the validation cohort.

Risk Stratification with CD66b + TANs and T immune cells is an independent

Predictor Of Dfs And Dss

Univariate and multivariate analyses for DFS and DSS were carried out (Tables 2 and Tables 3). Univariate analysis of the training cohort for DFS revealed that age, tumor location, tumor size, TNM stage, Lauren classification, lymphovascular invasion, risk signature and postoperative adjuvant chemotherapy were significantly associated with poor DFS (all $P < 0.05$) (Tables 2). Multivariate analysis of the training cohort balancing those factors showed that TNM stage, postoperative adjuvant

chemotherapy and high-risk signature (HR 2.777; 95% CI 1.945–3.966; $p < 0.001$) were independent predictors of poor DFS (Tables 2). Regarding disease-special survival, univariate analysis of the training cohort found that age, tumor location, tumor size, TNM stage, Lauren classification, tumor differentiation, lymphovascular invasion, perineural invasion, risk signature and postoperative adjuvant chemotherapy were significantly associated with poor DSS (Tables 3). Tumor size, TNM stage, postoperative adjuvant chemotherapy and high-risk signature (HR3.233; 95% CI 2.247–4.651; $p < 0.001$) were independent predictors of poor DFS in multivariate analysis (Tables 3).

Table 1
The clinicopathologic and treatment-related characteristics of the patients

Variables	Inflammation risk signature		*p* value
	Low risk (n = 213)	High risk (n = 114)	
Gender			0.450
Male	156	79	
Female	57	35	
Age			0.916
≤70	184	98	
>70	29	16	
ASA score			0.945
I	49	28	
II	148	78	
III	16	8	
BMI(kg/m2)	21.433 ± 3.437	21.796 ± 3.263	0.301
Tumor location			0.814
Upper third	65	31	
Middle third	70	40	
Lower third	78	43	
Tumor size			0.662
≤ 5.0	136	70	
> 5	77	44	
TNM stage			0.031
I	32	24	
II	52	35	
III	97	49	
	32	6	
Lauren classification			0.081

ECOG: Eastern Cooperative Group Performance Status; ASA: The American Society of Anesthesiologists; BMI: body mass index

Variables	Inflammation risk signature		*p* value
Intestinal	154	81	
Diffuse	32	10	
Mixed	27	23	
Tumor differentiation			0.113
G1/ G2	78	52	
G3/signet ring cell/mucinous	135	62	
Lymphovascular invasion			0.958
No	147	66	
Yes	79	35	
Perineural invasion			0.951
No	102	55	
Yes	111	59	
Fu[a]			0.002
Yes	99	90	
No	72	29	

ECOG: Eastern Cooperative Group Performance Status; ASA: The American Society of Anesthesiologists; BMI: body mass index

Table 2
Univariate and multivariate analysis for DFS.

	HR (95% CI)	P value	HR (95% CI)	P value
Gender		0.555		
Male	1			
Female	0.911(0.669–1.241)			
Age		0.003		0.193
≤70	1		1	
>70	1.688(1.185–2.403)		1.295(0.878–1.911)	
ASA score		0.284		
I	1			
II	1.301(0.920–1.841)			
III	1.407(0.799–2.478)			
Tumor location		< 0.001		0.183
Upper third	1		1	
Middle third	0.916(0.661–1.269)		1.340(0.931–1.927)	
Lower third	0.544(0.384–0.771)		0.995(0.669–1.481)	
Tumor size		< 0.001		0.129
≤ 5.0	1		1	
> 5	2.246(1.702–2.963)		1.287(0.929–1.783)	
TNM stage		< 0.001		< 0.001
I	1		1	
II	1.965(1.104–3.498)		0.977(0.483–1.976)	

ECOG: Eastern Cooperative Group Performance Status; ASA: The American Society of Anesthesiologists; BMI: body mass index

	HR (95% CI)	P value	HR (95% CI)	P value
III	4.503(2.662–7.619)		2.625(1.334–5.165)	
□	8.372(4.591–15.267)		4.632(2.157–9.947)	
Lauren classification		0.015		0.178
Intestinal	1		1	
Diffuse	1.703(1.158–2.503)		1.334(0.892–1.996)	
Mixed	1.325(0.910–1.928)		1.352(0.912–2.004)	
Tumor differentiation		0.308		
G1/ G2	1			
G3/signet ring cell/mucinous	1.159(0.873–1.538)			
Lymphovascular invasion		0.031		0.684
No	1		1	
Yes	1.374(1.029–1.835)		1.076(0.755–1.534)	
Perineural invasion		0.060		
No	1			
Yes	1.301(0.988–1.714)			
Risk signature		< 0.001		< 0.001
Low	1		1	
High	2.681(1.930–3.723)		2.777(1.945–3.966)	
Fu[a]		< 0.001		0.001
Yes	1		1	
No	1.695(1.281–2.242)		1.713(1.256–2.338)	
ECOG: Eastern Cooperative Group Performance Status; ASA: The American Society of Anesthesiologists; BMI: body mass index				

Table 3
Univariate and multivariate analysis for DSS.

	HR (95% CI)	P value	HR (95% CI)	P value
Gender		0.342		
Male	1			
Female	0.849(0.606–1.190)			
Age		0.001		0.105
≤70	1		1	
>70	1.861(1.289–2.686)		1.388(0.934–2.064)	
ASA score		0.196		
I	1			
II	1.304(0.911–1.868)			
III	1.614(0.910–2.862)			
Tumor location		0.003		0.174
Upper third	1		1	
Middle third	0.930(0.666-1.300)		1.425(0.983–2.066)	
Lower third	0.567(0.397–0.811)		1.209(0.805–1.816)	
Tumor size		< 0.001		0.013
≤ 5.0	1		1	
> 5	2.532(1.906–3.364)		1.519 (1.092–2.113)	
TNM stage		< 0.001		< 0.001
I	1		1	
II	2.285(1.221–4.276)		1.128(0.536–2.371)	
III	5.223(2.938–9.321)		3.020(1.480–6.164)	
	10.219(5.366–19.462)		5.668(2.556–12.570)	
Lauren classification		0.012		0.075

ECOG: Eastern Cooperative Group Performance Status; ASA: The American Society of Anesthesiologists; BMI: body mass index

	HR (95% CI)	P value	HR (95% CI)	P value
Intestinal	1		1	
Diffuse	1.698(1.147–2.514)		1.367(0.908–2.057)	
Mixed	1.417(0.966–2.078)		1.439(0.964–2.146)	
Tumor differentiation		0.159		
G1/ G2	1			
G3/signet ring cell/mucinous	1.232(0.921–1.649)			
Lymphovascular invasion		0.008		0.481
No	1		1	
Yes	1.487(1.108-1. 995)		1.133(0.800-1.604)	
Perineural invasion		0.009		0.282
No	1		1	
Yes	1.454(1.095–1.930)		1.155(0.836–1.596)	
Risk signature		< 0.001		< 0.001
Low	1		1	
High	2.862(2.040–4.015)		3.233(2.247–4.651)	
Fu[a]		< 0.001		< 0.001
Yes	1		1	
No	1.818(1.367–2.412)		1.844(1.342–2.534)	
ECOG: Eastern Cooperative Group Performance Status; ASA: The American Society of Anesthesiologists; BMI: body mass index				

Univariate analysis of the validation cohort for DFS revealed that tumor location, tumor size, TNM stage, Lauren classification, lymphovascular invasion, perineural invasion, risk signature and postoperative adjuvant chemotherapy were significantly associated with poor DFS (Supplementary Tables 2). Multivariate analysis of the validation cohort balancing those factors showed that tumor size, TNM stage, postoperative adjuvant chemotherapy and high-risk signature (HR2.126; 95% CI 1.463–3.090; $p < 0.001$) were independent predictors of poor DFS (Supplementary Tables 2). Regarding disease-special survival, univariate analysis of the validation cohort found that tumor location, tumor size, TNM stage, Lauren classification, lymphovascular invasion, perineural invasion, risk signature and postoperative

adjuvant chemotherapy were significantly associated with poor DSS (Supplementary Tables 3). Tumor size, TNM stage, postoperative adjuvant chemotherapy and high-risk signature (HR1.986; 95% CI 1.372–2.874; $p < 0.001$) were independent predictors of poor DSS in multivariate analysis (Supplementary Tables 3).

Risk Stratification of CD66b + TAN and T immune cells is correlated with distant metastases

To address why a high-risk signature had a correlation with poor prognosis, relapse patterns were deeply determined. All patients with relapse could be properly assessed for this analysis. Relapse involving anastomosis and pelvic lymph nodes were defined as local. Relapse involving organs such as the liver, lungs, peritoneum, and retroperitoneal nodes were defined as distant. Relapse involving serum tumor markers (including CEA and CA199) also indicate recurrence. In the training cohort, the high-risk signature was found to correlate with local and distant recurrence significantly (Fig. 4C). CEA levels and CA199, two of the most widely used tumor markers for detection recurrence in GC, were included in this comparative analysis. A significantly greater increase in CEA and CA199 was observed in the high-risk signature group than that in the low-risk signature group (Fig. 4A and 4B). In the validation cohort, the high-risk signature was found to correlate with local and distant recurrence significantly (Fig. 4F). A significantly greater increase in CEA and CA199 was also observed in the high-risk signature group than that in the low-risk signature group (Fig. 4D and 4E).

Extension of the distant metastases prognostic model with risk signature

To improve the prognostic accuracy for current TNM staging system, we developed a predictive model for GC patients by combining TNM stage and this risk signature with neutrophils plus T immune cells. In the training cohort, the area under the curve (AUC) as prediction based on the TNM staging (0.703) was comparable with that for the risk signature with neutrophils plus T immune cells (0.657), and the combination of both factors achieved the highest AUC value (0.782) (Fig. 4G**)**. In the validation cohort, the area under the curve (AUC) as prediction based on the T staging (0.727) was comparable with that for the risk signature with neutrophils plus T immune cells (0.614), and the combination of both factors achieved the highest AUC value (0.768) (Fig. 4H**)**.

Predictive Value Of Risk Signature For Adjuvant Chemotherapy Benefit

Increased levels of immune and inflammation cells have been reported to promote tumor invasion and reduce chemotherapy efficacy and immunologic death. Thus, we evaluated the benefit of fluorouracil-based adjuvant chemotherapy according to the risk signature model of neutrophils plus T immune cells. In the training cohort, patients who received postoperative chemotherapy had better DFS and DSS (Supplementary Fig. 2A and 2B, $p < 0.001$ and $p < 0.001$, respectively). Similar results were obtained from the training cohort (Supplementary Fig. 2C and 2D, $p < 0.001$ and $p < 0.001$, respectively).

In addition, high risk signature patients was not associated with a better DFS and DSS in patients with postoperative adjuvant chemotherapy (Fig. 5A and 5B, HR 1.248; 95% CI 0.909–1.715; p = 0.171, HR 1.348; 95% CI 0.976–1.863; p = 0.069, respectively). The result of validation cohort further conformed that patients with high risk signature patients did not have improved DFS and DSS with postoperative adjuvant chemotherapy (Fig. 5C **and D**, HR 1.273; 95% CI 0.876–1.848; p = 0.204, HR 1.353; 95% CI 0.932–1.964; p = 0.111, respectively). In contrast, low risk signature patients was associated with reduced risk of DFS and DSS in patients who received postoperative adjuvant chemotherapy (Fig. 5E and 5F, p = 0.004 and p = 0.002, respectively). Low risk signature patients was also associated with reduced risk of DFS and DSS in the validation cohort (Fig. 5G and 5H, p = 0.003 and p = 0.005, respectively).

Discussion

Tumor-infiltrating immune and inflammatory cells was frequently observed in GC, and several previous studies have found that the infiltration of both inflammatory cells and T lymphocytes were linked to different prognosis, including GC[18]. In this study, we investigated the survival impact of CD66b + neutrophils in GC. We found that high number of CD66b + neutrophils were negatively correlated with patient survival. In addition, we developed a risk signature model predicting prognosis independent of TNM staging based on tumor-infiltration immune and inflammatory cells. Further more, it was demonstrated that the high-risk signature could also predict the efficiency of chemotherapy.

Cancer-related inflammation is recognized as the seventh hallmark of cancer[20].

Besides tumor cells, a variety of immune stromal cells are the main components of the GC environment. Mounting evidence has emerged suggesting that systemic immune and inflammatory cells such as neutrophils may contribute to tumor cell invasion and metastasis[2]. It is reported that elevated peripheral blood neutrophil and neutrophil/lymphocyte ratio predicts poor outcomes in many types of cancer, including GC[10, 21]. So as to neutrophils infiltrated in tumors[10]. On the contrary, some other study showed that presence of neutrophils predicted good prognosis in GC [8]. This inconsistency may result from the methods used in assessing neutrophil infiltration. Hematoxylin-eosin staining alone or CD15 + immunohistochemical staining for neutrophils were utilized in variety of studies[11]. Apart from this, CD15 has been reported on other tumor cells, we decided to use CD66b to identify neutrophils[11].

Neutrophils infiltration could be recognized as an independent prognostic factor in this study. Whether, incorporation of neutrophils into T immune cells could provide more accurate prognostic information for the risk stratification of GC. So we developed a risk signature model with four types of cells (Neutrophils, CD3+, CD8 + T cells, and FOXP3 + cells), and this model showed correlation with DFS and DSS ($p < 0.001$ and $p < 0.001$, respectively) in the training cohort, comparable DFS and DSS findings were observed in the validation cohort. This risk signature model including neutrophils and T lymphocytes showed better correlation with survival prognosis than neutrophil infiltration alone. The number of neutrophils was significantly correlated with that of CD8 + T cells, suggesting that those cells cooperate to induce inflammation affecting cancer invasion, although there were not apparent associations in neutrophils

with CD3 + cells. So, we believed that neutrophil cells somehow moderated immune cell status relative to tumor progression.

Besides the clinical relevance of the risk-signature to outcomes, the risk signature was also associated with postoperative relapse. The mechanisms behind the clinical relevance of the risk-signature to recurrence pattern may mainly lie in

that tumor-induced alteration of neutrophils and T lymphocytes acted to produce premetastatic niches then promote distant metastasis[6, 22, 23]. Exactly, neutrophil cells in cancer niches were able to inhibit anti-tumor T-cells such as CD8 cells. As expected, this high-risk signature represented a more aggressive phenotype in the cohort, accompanied advanced TNM staging. Furthermore, study showed that increased levels of immune and inflammation cells have been reported to promote tumor invasion and reduce chemotherapy efficacy and immunologic death. Thus, we evaluated the relation between this risk signature and fluorouracil-based adjuvant chemotherapy. The result showed that patients with low risk signature tended to have longer DFS and DSS. No survival benefit was founded in patients with high risk signature. These indicated that the risk signature could be an important factor for predicting the efficiency of chemotherapy. Present studies also showed that inflammatory and immune cells were responsible in patients most likely to benefit from chemotherapy. In HCC, neutrophils could recruited macrophages and Treg cells promoting neovascularization and resistance to antiangiogenesis therapy by expressing cytokines[24]. Additionally, interferon derived from CD8 + T cell reversed stroma-mediated chemoresistance in the tumor niches[25]. The increased proinflammatory cytokines, including IL-6 and IL-8 levels, were a part of explanations for multidrug and apoptosis resistance in cancers[26, 27]. Therefore, the roles of Neutrophils and T immune cells tend to be more clear, and indicated that this risk signature based on these cells might be utilized to stratify patients by tumor niche status and immune cell profile.

There were several limitations in the present study. Firstly, the study is a retrospective study in nature, possible selection bias, detection bias, and performance of analysis bias might be confounded. Secondly, we developed a risk signature model of neutrophils and T immune cells involved in tumor progression, the underlying mechanisms through which those major immune cells crosstalk with tumor cells remains unrevealed. Thirdly, prognostic circulating marker for risk signature will be easier to use, which are our next concern and under investigation.

Conclusions

In summary, CD66b + neutrophils could be used to predict survival in GC. Incorporation of neutrophils into T lymphocytes could provide more accurate prognostic information for the risk stratification. So, this risk signature model combined with neutrophils and T lymphocytes cells has been developed and predict survival benefit from post-operative adjuvant chemotherapy.

Abbreviations

GC
Gastric cancer; DSS:Disease-special survival; DFS:Disease-free survival; NCA95/CD66b:Nonspecific cross-reacting antigen-95; IHC:Immunohistochemistry; NTP:Nearest Template Prediction; AUC:Area under the curve.

Declarations

Ethical approval

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions.

Consent for publication

The author(s) declared consent for publication.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Declaration of conflicting interests

The author(s) declared no potential conflicts of interest.

Author contributions

Sen Li and Wenpeng Wang: Conception, design, data analysis, and writing-original draft; Sen Li, Wenpeng Wang, Pengfei Ma, Junli Zhang, Yanghui Cao, Chenyu Liu, Xijie Zhang, Li Chen, and Shubing Song: Provision of study materials or patients, reagents and analysis tool; Sen Li, Wenpeng Wang, and Pengfei Ma analyzed the data. Zhiguo Li and Yuzhou Zhao: Financial support, technical help and fruitful discussion.

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28. Legend.
29. Sup. **Figure**-1 **The association between CD66b + neutrophils and CD8 + T and Treg cells in GC**. The pattern of CD66b expression was negatively correlated with CD8 (A), and negatively correlated with FOXP3 (B).
30. Sup. **Figure**-2 **The correlation between risk signature model and postoperative adjuvant chemotherapy in training cohort and validation cohort.** Kaplan-Meier analysis of DFS and DSS for postoperative adjuvant chemotherapy in training cohort (A,B) and validation cohort (C, D).

Figures

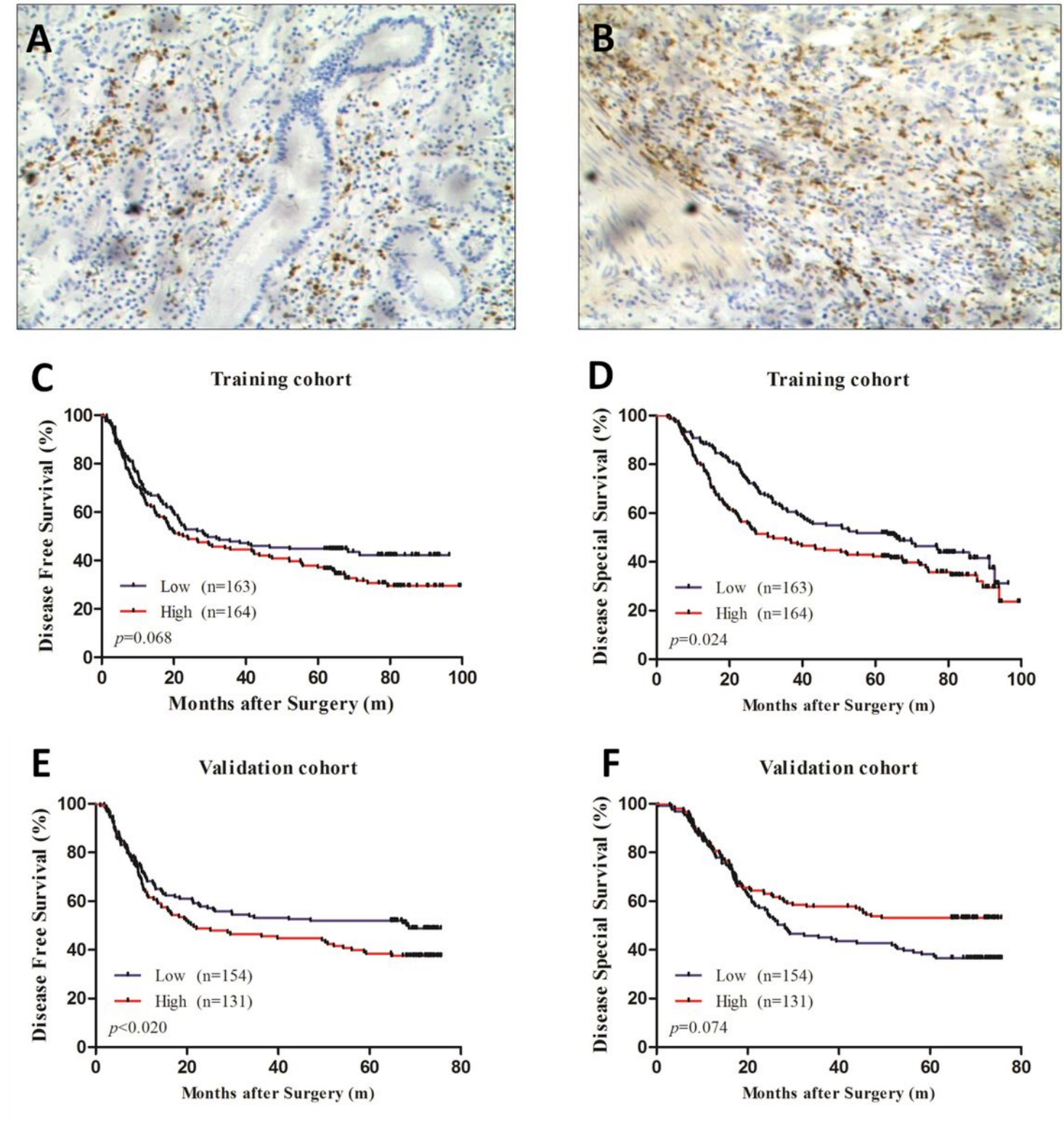

Figure 1

CD66b+neutrophils in GC from training cohort and validation cohort. Representative image of immunohistochemical staining of CD66b+ neutrophils as low (A), or high (B). Kaplan-Meier analysis showed that CD66b+neutrophils was not identified as an independent prognostic factor for DFS (C), while

high CD66b+neutrophils was significantly associated with worse DSS (D) in training cohort. In validation cohort, Kaplan-Meier analysis showed high CD66b+neutrophils was significantly associated with worse DFS (E), while CD66b+neutrophils was not identified as an independent prognostic factor for DSS (F).

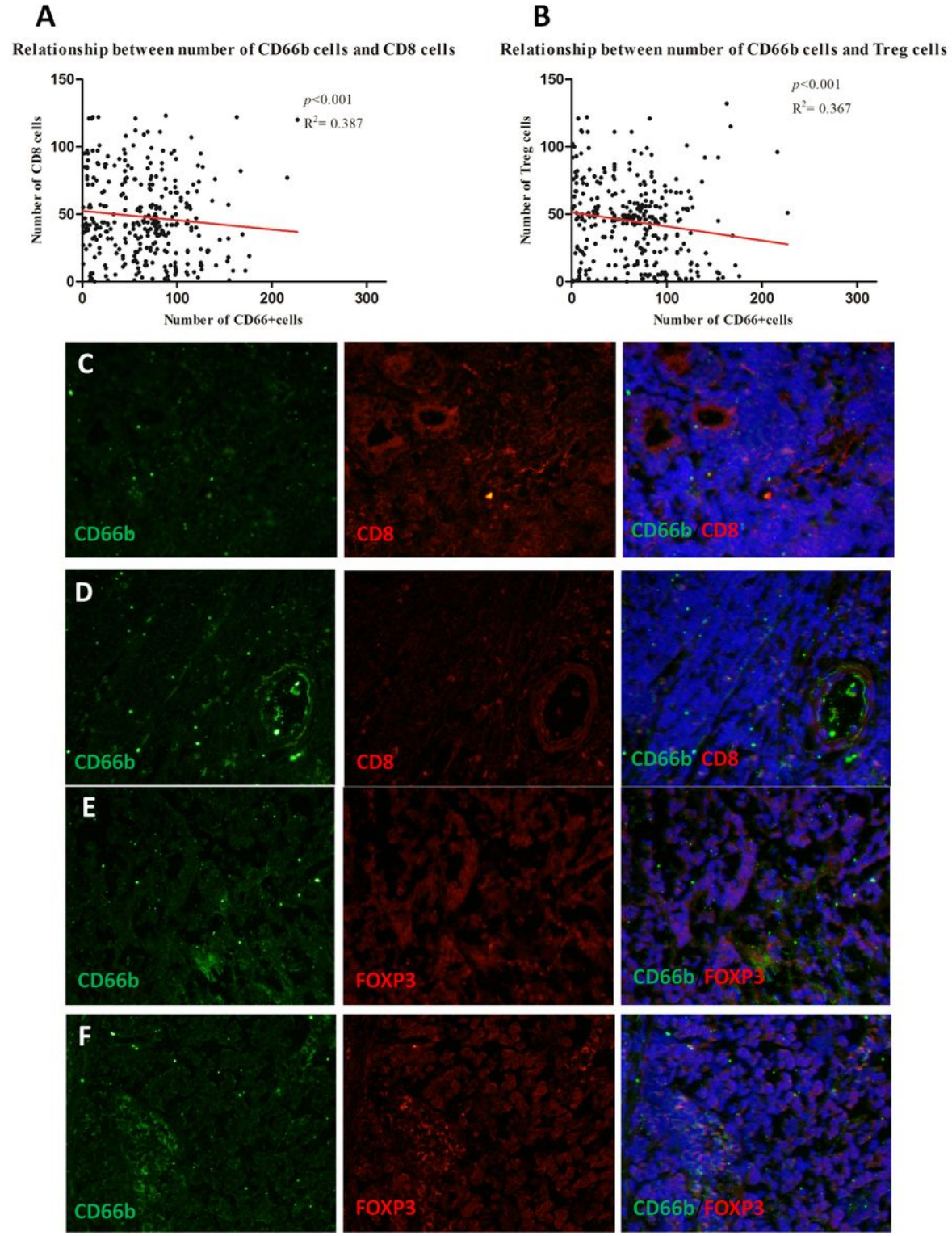

Figure 2

The association between CD66b+ neutrophils and CD8+ T and Treg cells in GC. The pattern of CD66b expression was negatively correlated with CD8 (A), and negatively correlated with FOXP3 (B).

Representative image of GC tissue in (C) high CD8+ T cells (red) with low density of CD66b+ (green), compared with the low CD8+ T cells (red) with high density of CD66b+ (green) (D). Representative image of GC tissue in (E) high Treg cells (red) with low density of CD66b+ (green), compared with the low Treg cells (red) with high density of CD66b+ (green) (F).

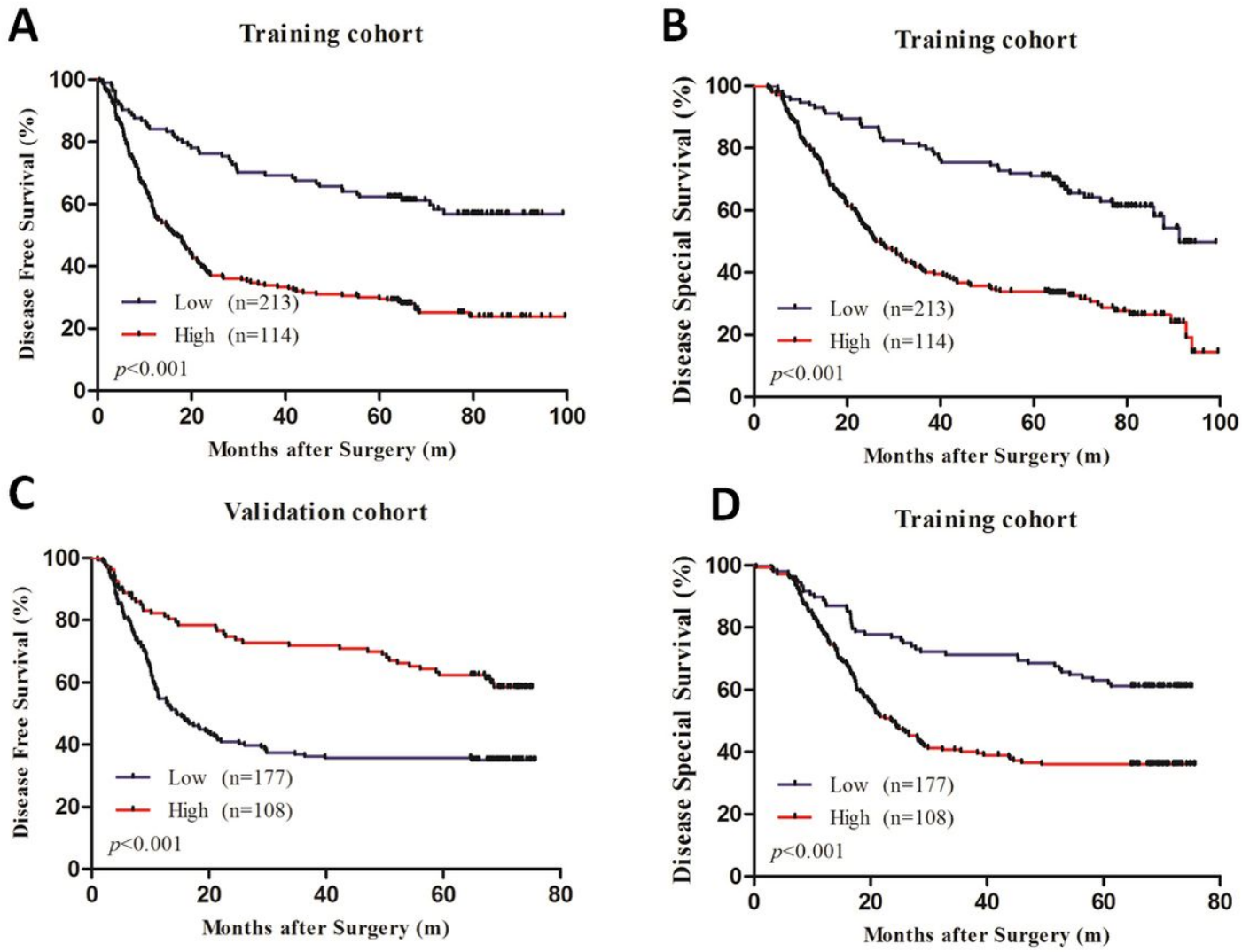

Figure 3

The prognostic significance of Risk signature model of tumor-infiltrating CD66b+ neutrophils plus immune cells. Kaplan-Meier analysis showed high-risk signature was significantly associated with worse DFS (A) and DSS (B) in training cohort. Kaplan-Meier analysis showed high-risk signature was significantly associated with worse DFS (C) and DSS (D) in validation cohort.

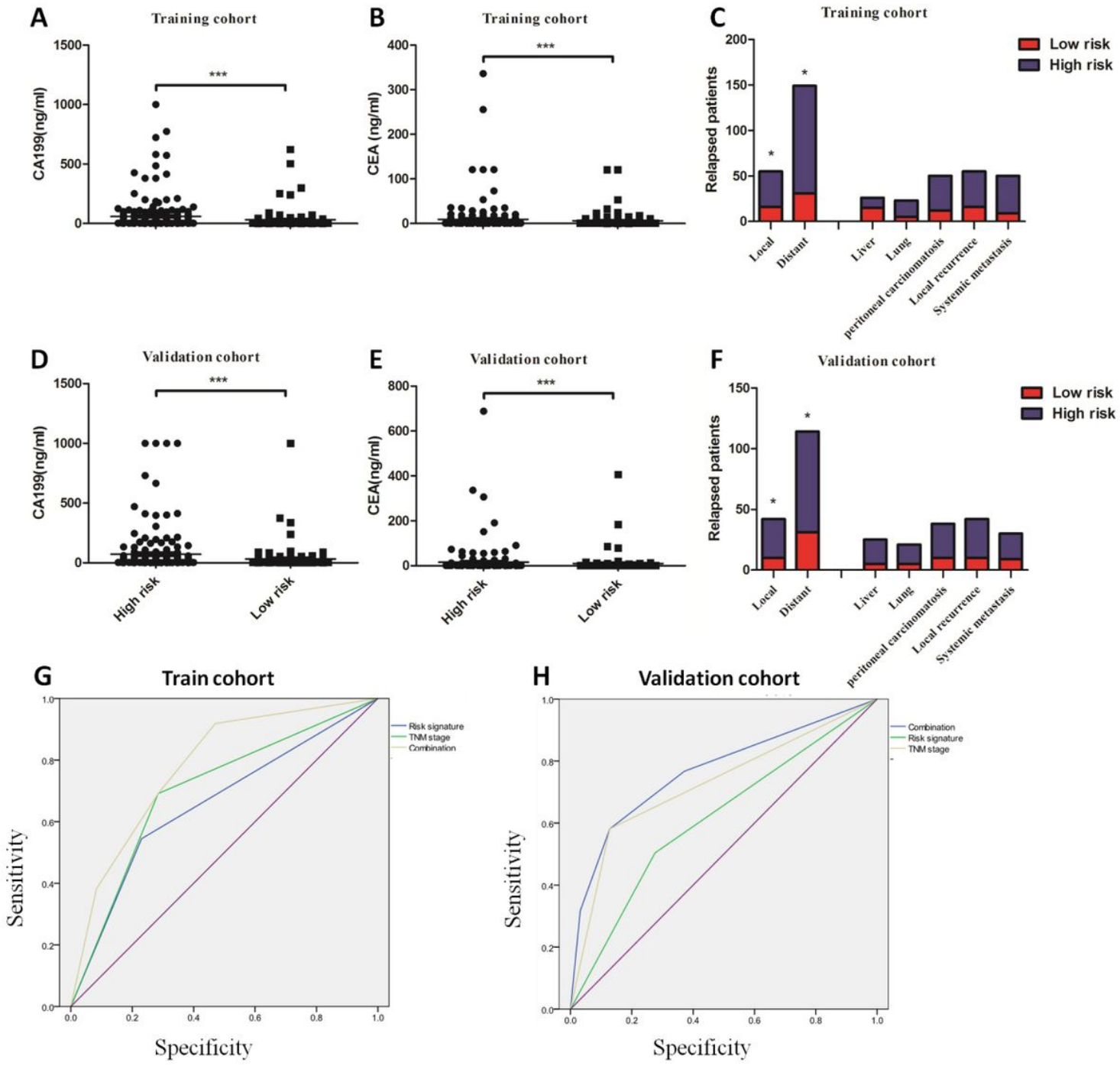

Figure 4

Risk signature model of tumor-infiltrating CD66b+ neutrophils plus immune cells. A significantly greater increase in CEA and CA199 was observed in the high-risk signature group than the low-risk group (A, B). The high-risk signature was correlated with distant recurrence significantly (C). The receiver operating characteristic (ROC) curves for predicting patient distant metastases using the risk signature, TMN stage or a combination of the two factors (D).

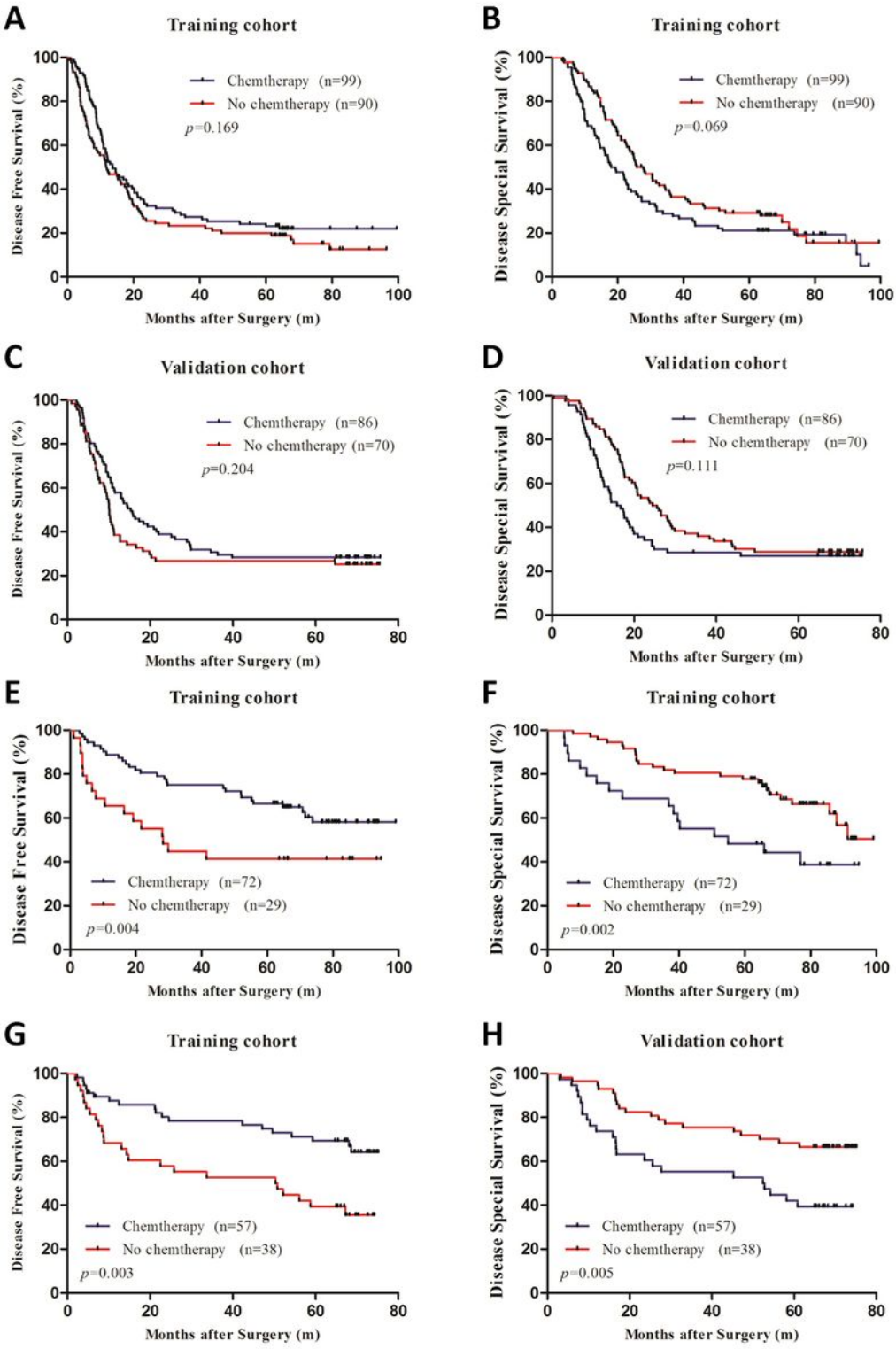

Figure 5

The correlation between risk signature model and postoperative adjuvant chemotherapy in training cohort and validation cohort. Kaplan-Meier analysis of DFS and DSS for postoperative adjuvant chemotherapy in high risk cohort (A, B) and validation cohort (C, D). Kaplan-Meier analysis of DFS and DSS for postoperative adjuvant chemotherapy in low risk cohort in training cohort (E, F) and validation cohort (G, H).

Supplementary Files

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