

A prognostic risk model for patients with triple negative breast cancer based on stromal natural killer cells, tumor-associated macrophages and growth-arrest specific protein 6

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Key words

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The aim of this study was to establish a prognostic risk model for patients with triple negative breast cancer (TNBC). A total of 278 specimens of human TNBC tissues were investigated by immunohistochemistry for growth-arrest specific protein 6 expression, infiltrations of stromal natural killer cells and tumor-associated macrophages. According to their prognostic risk scores based on the model, patients were divided into three groups (score 0, 1–2, 3). Correlations of prognostic risk scores, clinicopathologic features and overall survival (OS) were analyzed. To study the clinical value of this stratification model in early disease recurrence or metastasis, 177 patients were screened out for further analysis. Based on disease free survival (DFS), 90 patients fell within the DFS ≤ 3 years group and 87 patients within the DFS ≥ 5 years group. We analyzed the differences in prognostic risk scores between the two groups. The prognostic risk scores were negatively related to tumor size, lymph node metastasis and P53 status ($P < 0.001$ for all). Patients with low prognostic risk scores had longer OS ($P = 0.001$). Using multivariate analysis, it was determined that TNM stage (HR = 0.432, 95% confidence interval [CI] = 0.281–0.665, $P = 0.003$), FOXP3 positive lymphocytes (HR = 1.712, 95% CI = 1.085–2.702, $P = 0.021$) and prognostic risk scores (HR = 1.340, 95% CI = 1.192–1.644, $P = 0.005$) were independent prognostic factors for OS. Compared with the DFS ≥ 5 years group, the DFS ≤ 3 years group patients had significantly higher prognostic risk scores ($P < 0.001$). In conclusion, the prognostic risk score of the model was a significant indicator of prognosis for patients with TNBC.

Triple-negative breast cancer (TNBC), immunohistochemically defined by lack of expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), is a highly aggressive subtype of breast cancer that accounts for approximately 15–20% of all breast cancers.^(1–3) The absence of ER, PR and HER2 protein receptors on TNBC precludes many therapeutic methods, such as endocrine therapy and anti-HER2 molecular targeted therapy, and, therefore, patients diagnosed with TNBC have a higher risk of disease relapse within 5 years and a higher death rate than patients with other types of breast cancers.^(4–6) To date, there are no effective therapeutic targets available against TNBC.^(7,8) Thus, identification and evaluation of new prognostic biomarkers and development of new therapeutic strategies are urgently needed to improve the treatment outcomes of TNBC patients.

It is well established that tumor microenvironment plays a crucial role in cancer development and metastasis.⁽⁹⁾ Natural killer (NK) cells, a type of cytotoxic lymphocyte, are a key component of the innate immune system. Compared with

circulating NK cells, the tissue-infiltrating NK cells in solid tumors appear to have a less robust response.^(10–12) In human solid tumors, NK cells were major components of immune cells in early tumor tissue; however, in advanced human neoplasms, NK cells were usually not found in large numbers.⁽¹³⁾ These findings indicate that NK cells play an important role in immune surveillance, but once tumor genesis occurs, the tumor microenvironment is suppressive for NK cells. Overcoming active immune suppression in the tumor microenvironment is an important consideration for tumor-infiltrating NK cells. However, few studies have focused on the role of NK cells in breast cancer, particularly in TNBC.

Growth arrest-specific protein 6 (Gas6), identified in 1995,^(14,15) acts as the ligand to the Axl/Tyro3/Mer family of tyrosine kinase receptors and exerts mitogenic activity when bound to these receptors.⁽¹⁶⁾ Gas6 binds to all three receptors, although Axl is the highest affinity receptor (Axl > Tyro3 > Mer); Gas6 has 3-fold to 10-fold higher affinity for Axl than Mer.^(17–21) Several studies have indicated that Axl plays a role in tumor invasion and metastasis in a number of

cancers, including breast cancer.⁽²²⁾ Interestingly, a separate study found that Gas6 correlated positively with a number of favorable prognostic variables.⁽²³⁾ However, this study enrolled all types of breast cancers and did not include further immunohistochemical characteristics. It has also been demonstrated that Axl is overexpressed in highly invasive breast cancer cell lines (such as MDA-MB-435s, MDA-MB-157, MDA-MB-436 and MDA-MB-231); in contrast, weakly invasive breast cancer cell lines do not or only weakly express Axl.⁽²⁴⁾ Is Gas6 also a good biomarker for prognosis of TNBC patients? That is a subject worthy of further study.

Tumor-associated macrophages (TAM), which are often abundantly present in malignant tumors, share many common features with alternatively activated anti-inflammatory macrophages (M2).⁽²⁵⁾ CD163 is a scavenger receptor upregulated by macrophages in an anti-inflammatory environment,⁽²⁶⁾ and regarded as a highly specific monocyte/macrophage marker for M2 macrophages.^(27–29) In addition, previous studies revealed that TAM were the main source of Gas6 within the tumor microenvironment, and Gas6 expression was upregulated by IL-10 and macrophage colony stimulating factor (M-CSF).⁽³⁰⁾ In Chitu *et al.*,⁽³¹⁾ M-CSF was the primary cytokine that induced M2 polarization of macrophages. Gas6 can suppress the proliferation and IFN- γ production of NKG2D-activated NK cells.⁽³²⁾ A previous study showed that the infiltration of CD163 macrophages into tumor stroma, but not tumor nest is of clinical relevance for breast cancer patients.⁽³³⁾ Stromal lymphocytic infiltration constitutes a robust prognostic factor in TNBC.^(34,35) In another study, comparing Gas6 expression in tumor-infiltrating macrophages and resident tissue macrophages, the researchers found a strong upregulation of Gas6 in tumor stromal macrophages.⁽³⁰⁾ This means that Gas6 is prominently expressed by leukocytes, in particular by macrophages in tumor stroma. We think stromal immune microenvironment is strongly correlated with prognosis for TNBC subtype. Therefore, we proposed that there exists a TAM–Gas6–NK cell axis in the breast cancer microenvironment. Stromal TAM would negatively influence the infiltration of NK cells by producing Gas6 protein. The aim of this study was to investigate the prognostic values of stromal NK cells and Gas6 in TNBC, and to eventually establish a prognostic risk model for patients with TNBC.

Materials and Methods

Patients and clinical samples. This study used archival materials from the Department of Pathology at the Cancer Hospital of Harbin Medical University (CHHMU, Harbin, China), including breast cancer tissue samples from 278 patients with histologically proven TNBC (ER negative, PR negative and HER-2 negative). The specimens of breast cancer tissues were collected and snap-frozen from breast cancer patients who had surgery between 2006 and 2008 at the CHHMU. All breast cancers were invasive ductal carcinomas, neoadjuvant-free and collected before systemic treatments. Patients were treated according to standard practice guidelines. Clinicopathological information and follow-up results were obtained for all breast cancer patients along with informed consent. Four-micron tissue sections were prepared from a formalin-fixed and paraffin-embedded sample. For P53, positive staining of more than 10% of the tumor cells was defined as positive tumor expression and staining of 10% or fewer of the cells as negative tumor expression.⁽³⁶⁾ All protocols were reviewed and approved by the Ethical Committee of Harbin Medical

University in Harbin, China. Use of human breast tumors in immunohistochemistry experiments was approved by the Institutional Review Board at CHHMU, and was performed in accordance with the policies of CHHMU.

Follow up. The clinical and pathological records of all patients in the study were reviewed periodically. Examinations were performed every 3–6 months for the first 5 years and every 12 months thereafter during the follow-up period. Patients were followed regularly for a minimum of 5 years of follow up at CHHMU. The clinical records were obtained from the departments providing follow-up care. Survival was calculated in months from the date of diagnosis to whichever of the following occurred first: the date of death, the date last known to be alive, or 11 August 2015, which was the follow-up cut-off date used in our analysis. The median follow-up time was 76 months (range, 4–116 months).

Immunohistochemistry and pathologic assessment. Detection of CD8 positive lymphocytes, forkhead box protein 3 (FOXP3) positive lymphocytes and Gas6 was performed with anti-CD8 polyclonal antibody (1:100, ab4055; Abcam, Cambridge, MA, USA), anti-FOXP3 monoclonal antibody (1:75, ab20034; Abcam) and polyclonal antibodies directed against Gas6 (1:100, ab136249; Abcam), respectively. Detection of NK cells and M2 TAM was performed with polyclonal antibodies directed against NKp46 (1:100, ab199128; Abcam) and monoclonal anti-CD163 antibody (1:200, ab156769; Abcam). The specificities of these antibodies have been previously demonstrated.^(27–29,37)

Immunohistochemical staining was performed on 4- μ m thick formalin-fixed paraffin sections after heating overnight at 55°C followed by subsequent deparaffinization in xylene and rehydration through graded alcohols. After deparaffinization and hydration, sections were washed in PBS (3 min \times 3) and incubated in 0.01-M citrate buffer (pH = 6.0) for 10 min in a microwave for antigen retrieval. After washing in PBS (5 min \times 3), sections were treated with 3% H₂O₂ in the dark for 10–20 min. Each section was incubated with antibodies recognizing of CD8, FOXP3, CD163, NKp46 or Gas6, at 4°C overnight. After washing in PBS (5 min \times 3), each section was incubated with secondary antibody at room temperature for 30 min. After washing in PBS (5 min \times 3), each section was treated with diaminobenzidine (DAB) working solution at room temperature for 3–10 min and counterstained with hematoxylin. As negative controls, the primary antibody was substituted by PBS. Positive controls included lung cancer tissues with positive expression of stromal CD8 positive lymphocytes, FOXP3 positive lymphocytes, NK cells, TAM and Gas6. Details of scoring systems and cut-off points for high or low infiltrations of immune cells and Gas6 expressions are described in previous studies (Figs 1–5).^(33,38–40) The immunostaining and histopathologic results were evaluated by two pathologists who had no access to clinical data.

Prognostic risk model for patients with triple-negative breast cancer. We developed a prognostic risk model based on our hypothesis of a TAM–Gas6–NK cell axis. The risk factors were low infiltration of stromal NK cells, high infiltration of stromal TAM and Gas6 expression positive, respectively (Table 1). According to the immunohistochemistry results, patients were graded using this model. The prognostic risk scores ranged from 0 to 3. We found that there were no significant differences in overall survival (OS) between score 1 and 2 ($P = 0.245$). Therefore, we divided patients into three groups based on their prognostic scores in our model (score = 0, 1–2 or 3).

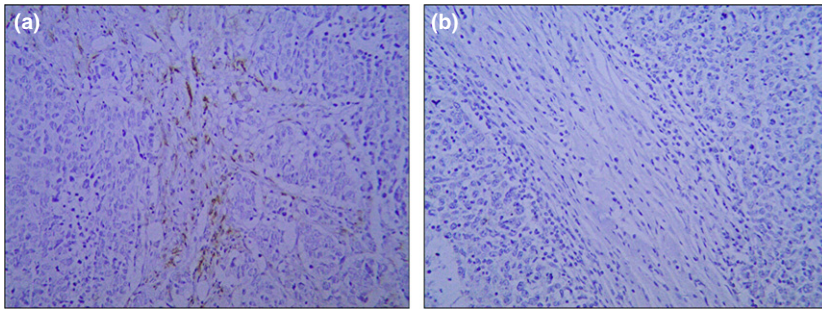


Fig. 1. Immunohistochemical staining of stromal natural killer (NK) cells in triple negative breast cancer (TNBC) tissues (200 \times). (a) High infiltration level of stromal NK cell. (b) Stromal NK cell negative staining.

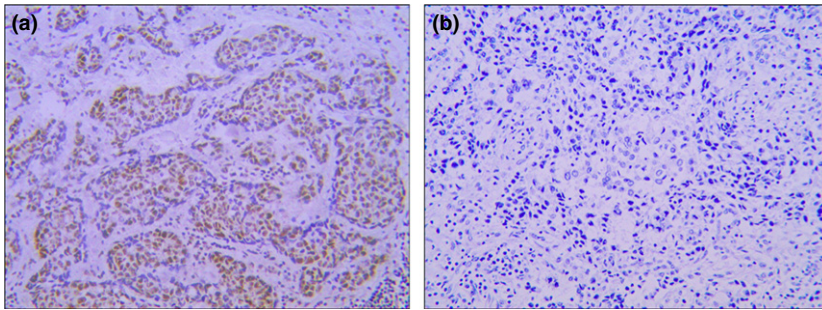


Fig. 2. Immunohistochemical staining of growth-arrest specific gene 6 (Gas6) protein in triple negative breast cancer (TNBC) tissues (200 \times). (a) Gas6 positive staining. (b) Gas6 negative staining.

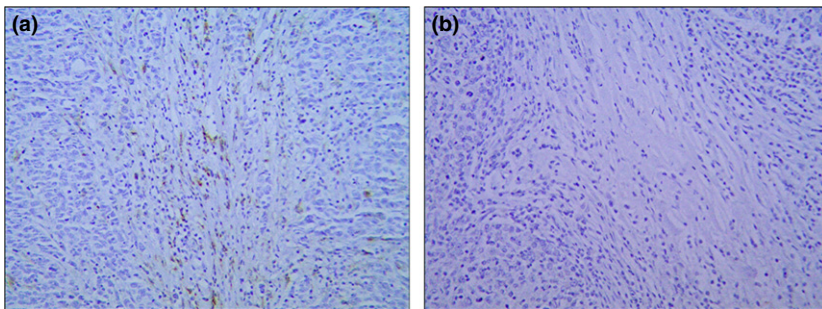


Fig. 3. Immunohistochemical staining of stromal tumor-associated macrophages (TAM) in triple negative breast cancer (TNBC) tissues (200 \times). (a) High infiltration level of stromal TAM. (b) Stromal TAM negative staining.

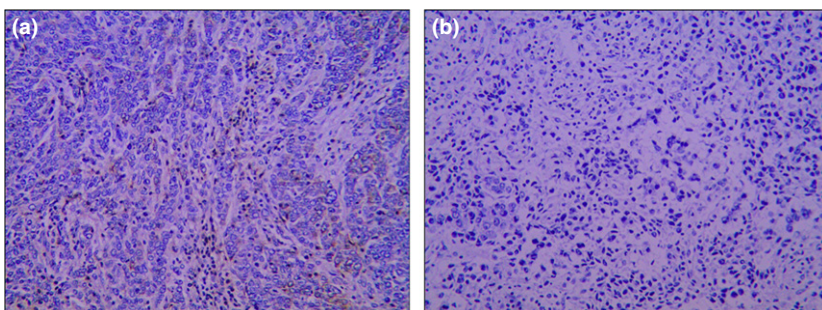


Fig. 4. Immunohistochemical staining of CD8 positive lymphocytes in triple negative breast cancer (TNBC) tissues (200 \times). (a) High infiltration level of stromal CD8 positive lymphocytes. (b) Low infiltration level of stromal CD8 positive lymphocytes.

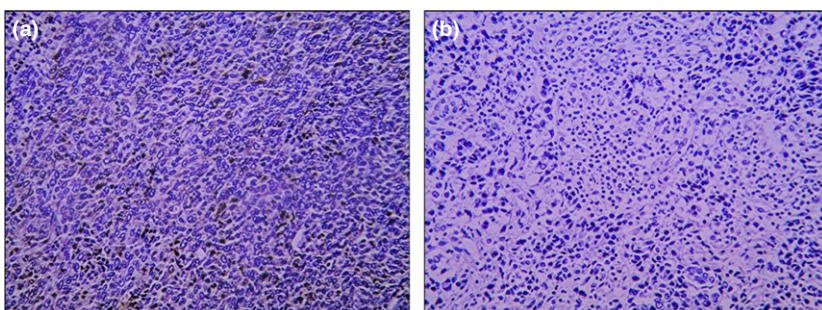


Fig. 5. Immunohistochemical staining of forkhead box protein 3 (FOXP3) positive lymphocytes in triple negative breast cancer (TNBC) tissues (200 \times). (a) High infiltration level of stromal FOXP3 positive lymphocytes. (b) Low infiltration level of stromal FOXP3 positive lymphocytes.

Table 1. Prognostic risk model

Risk factors	Score
Low stromal NK cell infiltration	1
Stromal TAM positive	1
Gas6 expression positive	1

Gas6, growth-arrest specific gene 6; NK, natural killer; TAM, tumor-associated macrophage.

Statistical analysis. Statistical analysis was performed using SPSS v 21 (Chicago, IL, USA). Associations of stromal NK cell infiltrations, Gas6 expression and patients' clinicopathological features were assessed using the Pearson chi-square test. This study employs the method of Spearman correlation analysis to investigate the relationships of stromal NK cells, Gas6 expression and stromal TAM. The Kaplan–Meier method was used to estimate OS. The influence of different variables on survival was assessed using Cox multivariate regression analyses. Hazard ratios and their 95% confidence intervals (CI) were recorded for each marker. For continuous variables, Student's *t*-test was performed. *P*-values of less than 0.05 were considered significant.

Results

Patients' characteristics. Analyses for the infiltration of NK cells, TAM and Gas6 expression were performed using

specimens from 278 untreated female patients with invasive TNBC. The median age was 50 years (range, 28–75). Of all the patients, 115 patients (52.2%) had tumors sized ≤ 2 cm and 163 patients (47.8%) had tumors sized > 2 cm. Lymph node metastasis (LNM) was present in 145 patients (52.2%), and absent in 133 patients (47.8%). A total of 231 patients (83.1%) were classified at TNM stage I or II, and 46 (16.9%) were TNM stage III. A total of 196 patients (70.5%) were classified as histological grade I or II, and 72 patients were grade III (29.5%). P53-negative tumors were observed in 103 patients (37.1%), and 175 patients (62.9%) were P53 positive. Epidermal growth factor receptor (EGFR)-positive tumors were present in 194 patients (70.0%), and absent in 84 patients (30.0%). A total of 135 (48.6%) patients were classified as having low infiltration of CD8 positive stromal lymphocytes, and 143 (51.4%) patients scored as high infiltration. A total of 194 patients (69.8%) were classified as low infiltration of FOXP3 positive stromal lymphocytes, and 84 patients (30.2%) were high infiltration.

Correlations of stromal natural killer cells, Gas6 expression and clinicopathological features. We analyzed the associations of the infiltration levels of stromal NK cells, Gas6 expression, and a series of clinicopathological characteristics (Table 2). Patients were divided into low ($N = 223$) or high ($N = 55$) stromal NK cell infiltration groups, low ($N = 189$) or high ($N = 89$) stromal NK cell infiltration groups, and Gas6 negative ($N = 166$) or positive ($N = 112$) groups. We found that the infiltration level of stromal NK cells was significantly

Table 2. Correlations of stromal NK cells, TAM, Gas6 expression, prognostic risk scores and clinicopathological features

	Stromal NK cell		<i>P</i>	Gas6 expression		<i>P</i>	Stromal TAM		<i>P</i>	Prognostic risk score			<i>P</i>
	Low <i>N</i> = 223	High <i>N</i> = 55		Negative <i>N</i> = 166	Positive <i>N</i> = 112		Low <i>N</i> = 189	High <i>N</i> = 89		0 <i>N</i> = 38	1–2 <i>N</i> = 182	3 <i>N</i> = 58	
Age (years)													
<50	148	34	0.525	110	72	0.734	126	56	0.540	25	117	40	0.807
≥ 50	75	21		56	40		63	33		13	65	18	
Tumor size (cm)													
≤ 2	75	40	<0.001	74	41	0.186	91	24	0.001	26	77	12	<0.001
> 2	148	15		92	71		98	65		12	105	46	
Lymph node metastasis													
Negative	97	36	0.004	93	40	0.001	110	23	<0.001	26	92	15	<0.001
Positive	126	19		72	72		79	66		12	90	43	
TNM stage													
I,II	189	42	0.137	142	89	0.185	161	70	0.175	31	153	47	0.835
III	34	13		24	23		28	19		7	29	11	
Histological grade													
I, II	151	45	0.040	123	73	0.110	140	56	0.057	30	131	35	0.112
III	72	10		43	39		49	33		8	51	23	
P53 status													
Negative	71	32	<0.001	68	35	0.100	89	14	<0.001	22	75	6	<0.001
Positive	152	23		98	77		100	75		16	107	52	
Epidermal growth factor receptor													
Negative	65	19	0.435	52	32	0.624	59	25	0.596	13	57	14	0.494
Positive	158	36		114	80		130	64		25	125	44	
Stromal CD8 ⁺ TIL													
Low	110	25	0.607	80	55	0.881	93	42	0.754	18	89	28	0.984
High	113	30		86	57		96	47		20	93	30	
Stromal FOXP3 ⁺ TIL													
Low	150	44	0.065	112	82	0.306	133	61	0.756	31	121	42	0.162
High	73	11		54	30		56	28		7	61	16	

FOXP3, forkhead box protein 3; Gas6, growth-arrest specific gene 6; NK, natural killer; TAM, tumor-associated macrophage; TIL, tumor infiltrating lymphocyte.

correlated with tumor size ($P < 0.001$), LNM ($P = 0.004$), histological grade ($P = 0.040$) and P53 expression ($P < 0.001$). Gas6 expression was significantly correlated only with LNM ($P = 0.001$). Stromal TAM infiltration was correlated with tumor size ($P = 0.001$), LNM ($P < 0.001$) and P53 ($P < 0.001$). The prognostic risk score was significantly correlated with tumor size, LNM and P53 status ($P < 0.001$ for all). All the patients were Ki-67 index $>20\%$.

To study the clinical values of this stratification schema in early local disease recurrence and distant disease metastasis, 177 patients were screened out for further analysis. Disease-free survival (DFS) is an important index reflecting early disease recurrence and metastasis. Based on DFS, 90 patients fell within a DFS ≤ 3 years group and 87 patients into a DFS ≥ 5 years group. Patients' characteristics and stromal NK cell infiltration of the two groups are listed in Table 3. We found that the patients in DFS ≥ 5 years group had significantly higher stromal NK cell infiltration ($P < 0.001$) and lower prognostic risk scores ($P < 0.001$), stromal TAM ($P = 0.004$) and Gas6 expressions ($P < 0.001$).

Predictive significance of stromal natural killer cell, growth arrest-specific protein 6 expression and the prognostic risk model in triple-negative breast cancer patients. Multivariate survival analysis was used to evaluate the prognostic risk scores and clinicopathological characteristics with respect to prognosis in TNBC patients. We found that TNM stage (HR = 0.432, 95% CI = 0.281–0.665, $P = 0.003$), FOXP3 positive tumor infiltrating lymphocytes (TIL) (HR = 1.712, 95% CI = 1.085–2.702, $P = 0.021$) and prognostic risk scores (HR = 1.340, 95% CI = 1.192–1.644, $P = 0.005$) were independent prognostic factors for OS (Table 4). The Kaplan–Meier 5-year survival curves stratified for stromal NK cell infiltrations, Gas6 expressions and prognostic risk scores in TNBC patients are shown in Figure 6. Among the 278 TNBC patients, the infiltration level of stromal NK cells ($P = 0.018$), Gas6 expression ($P = 0.002$) and prognostic risk score ($P = 0.001$) showed significant effects on OS. These data indicate that low infiltration levels of NK cells, high Gas6 expression and high prognostic risk score were associated with worse OS in TNBC. Compared with stromal NK cells, TAM or Gas6 alone, the prognostic risk model demonstrated a stronger prognostic value for TNBC patients.

Correlations of stromal tumor-associated macrophages, natural killer cell infiltrations and growth arrest-specific protein 6 expression. We detected high TAM infiltration in 23 of 166 (13.9%) patients in the Gas6-negative group versus 66 of 112 (58.9%) patients in the Gas6-positive group, respectively. The infiltration level of stromal TAM correlated positively with Gas6 expression (correlation coefficient 0.474, $P < 0.001$). Stromal NK cells were highly infiltrated in 51 of 166 (30.7%) patients of the Gas6-negative group and 4 of 112 (3.6%) patients of the Gas6-positive group (correlation coefficient -0.312 , $P < 0.001$).

Discussion

In this study we showed that the infiltration level of stromal NK cells was associated with a series of good clinicopathological characteristics and longer survival in patients with TNBC, but negatively influenced by Gas6 expression (correlation coefficient -0.312 , $P < 0.001$). Low stromal NK cell infiltration in TNBC samples was associated with higher Gas6 expression and shorter survival. NK cells are an important component of innate immune defense cancer, particularly in the elimination

Table 3. Clinicopathological features, prognostic risk scores, infiltration levels of stromal NK cells and TAM between DFS ≤ 3 years group and DFS ≥ 5 years group

Characteristics	DFS ≤ 3 years group (N = 90)	DFS ≥ 5 years group (N = 87)	P-value
Age (years)			
<50	57	63	0.196
≥ 50	33	24	
Tumor size (cm)			
≤ 2	37	43	0.267
> 2	53	44	
Lymph node metastasis			
Negative	39	50	0.060
Positive	51	37	
TNM stage			
I, II	66	78	0.005
III	24	9	
Histological grade			
I, II	63	69	0.155
III	27	18	
P53 status			
Negative	28	37	0.115
Positive	62	50	
Epidermal growth factor receptor			
Negative	20	40	0.001
Positive	70	47	
Stromal CD8+ TIL			
Low	52	36	0.029
High	38	51	
Stromal FOXP3+ TIL			
Low	68	53	0.036
High	22	34	
Stromal NK cell			
Low	79	61	<0.001
High	11	26	
Gas6 expression			
Negative	44	66	<0.001
Positive	46	21	
Stromal TAM			
Low	50	66	0.004
High	40	21	
Prognostic risk score			
0	6	21	<0.001
1–2	56	57	
3	28	9	

DFS, disease-free survival; FOXP3 forkhead box protein 3; Gas6, growth-arrest specific gene 6; NK, natural killer; TAM, tumor-associated macrophage; TIL, tumor infiltrating lymphocyte.

of cancer metastases and small tumors. High numbers of immune cells, such as NK cells or CD8-positive T cells, infiltrating the microenvironment of tumors have been reported to correlate with prolonged survival in patients with various cancers.⁽⁴¹⁾ Our results are comparable to those reported in previous studies. Researchers have observed a significant association of NK cell infiltration and patient survival in breast cancer. During breast cancer progression, expression of activating NK cell receptors decreased, while expression of NK cell inhibitory receptors increased.⁽⁴²⁾ Survival analyses performed on a larger number of breast cancer patients ($n = 115$), derived from four publicly available breast cancer datasets of gene expression, revealed significantly ($P < 0.03$) increased survival

Table 4. Prognostic factors in the Cox proportional hazards model

Variables	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Age (years)						
<50/50	0.854	0.585–1.247	0.414			
Tumor size (cm)						
≤2.0>2.0	0.915	0.628–1.331	0.641			
Lymph node metastasis						
Negative/positive	0.812	0.561–1.175	0.269			
TNM stage						
I/II and III	0.484	0.317–0.738	0.001	0.432	(0.281–0.665)	0.003
Histological grade						
I/II and III	1.358	0.891–2.070	0.155			
P53 status						
Negative/positive	0.679	0.456–1.011	0.057	0.886	(0.582–1.348)	0.571
Epidermal growth factor receptor						
Negative/positive	0.591	0.382–0.915	0.018	0.660	(0.424–1.027)	0.650
Stromal CD8+ TIL						
Low/high	1.453	1.004–2.102	0.047	1.365	(0.937–1.987)	0.105
Stromal FOXP3+ TIL						
Low/high	1.743	1.111–2.734	0.016	1.712	(1.085–2.702)	0.021
Prognostic risk score						
0–3	1.857	1.347–2.560	<0.001	1.340	(1.192–1.644)	0.005

CI, confidence interval; FOXP3, forkhead box protein 3; TIL, tumor infiltrating lymphocyte.

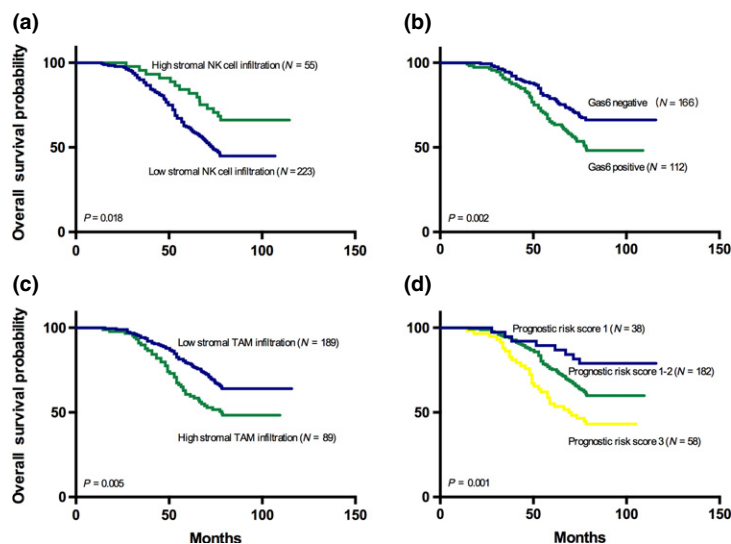


Fig. 6. Kaplan-Meier (K-M) analysis for overall survival (OS). (a) K-M analysis of OS based on stromal NK cells infiltration ($P = 0.018$) (b) K-M analysis of OS based on Gas6 expressions ($P = 0.002$) (c) K-M analysis of OS based on stromal TAM ($P = 0.005$) (d) K-M analysis of OS based on prognostic risk scores ($P = 0.001$).

in patients that showed higher expression of the NCR3 (NKp30), NCR1 (NKp46), CD96, CRTAM, DNAM1 and NKG2D.^(43–45) It has been reported that tumor stromal tissues from the good-outcome cluster overexpressed an obvious set of immune-related genes, including T cell and NK cell markers.⁽⁴⁶⁾

Little is known about the role of Gas6 in breast cancer, especially in TNBC. Previous studies have shown that TAM were induced by the tumor microenvironment to produce elevated levels of Gas6 in tumor stroma and Gas6 was prominently expressed by leukocytes, in particular by macrophages.⁽³⁰⁾ Our study found that Gas6 expression positively correlated with the infiltration level of stromal TAM (correlation coefficient 0.474, $P < 0.001$) in TNBC

environment, and Gas6 expression is a predictor of poor survival in patients with TNBC. This is the first documented positive relationship between TAM and Gas6 expression in human TNBC. Our results seem to contradict those reported in previous studies. Researchers had observed that Gas6 correlated positively with a number of favorable prognostic variables, including lymph node negativity ($P = 0.0002$), smaller size of tumors ($P = 0.02$), low Nottingham prognostic index scores ($P = 0.03$) and low nuclear morphology ($P = 0.03$) in human breast cancer.⁽²³⁾ However, this phenomenon may be explained as follows. Approximately 60–80% of breast cancer cases are hormone receptor positive, and TNBC accounts for only 15–20%. However, the former study did not include differentiation via immunohistochemical characteristics. Moreover, Axl is

overexpressed in highly invasive breast cancer.⁽²⁴⁾ Therefore, the research above applies primarily only to hormone receptor positive breast cancers, not TNBC. For TNBC, Gas6 expression is a predictor of poor prognosis.

We propose that there exists a TAM-Gas6-NK cells axis in the breast cancer microenvironment, in which stromal TAM negatively influence the infiltration of NK cells by producing Gas6 protein. It has been proven that stromal TAM are a prognostic factor for reduced breast cancer-specific survival.⁽³³⁾ In this study we found that stromal NK cell infiltration was a positive prognostic factor, and Gas6 protein was a negative prognostic factor in TNBC. A risk model combining the three factors of the TAM-Gas6-NK cells axis has higher prognostic value than any single factor. Patient groups expressing the combination of high TAM, high Gas6 and low NK cells (risk score = 3) exhibited the lowest survival rates. Conversely, groups with low TAM, low Gas6 and high NK cell infiltration (risk score = 0) exhibited the highest survival rates. The survival of patients with an intermediate risk (score = 1–2) was between these extremes.

Tumor infiltrating lymphocytes as an expression of immune response are associated with patient survival in a wide variety of tumor types.⁽⁴⁷⁾ CD8-positive lymphocytes ($P = 0.036$)⁽⁴⁰⁾ and FOXP3-positive lymphocytes⁽⁴⁸⁾ were significantly associated with prognosis in TNBC. In this study, CD8-positive ($P = 0.047$) and FOXP3-positive ($P = 0.016$) lymphocytes were significantly correlated with patients' survival, and FOXP3-positive lymphocyte ($P = 0.021$) was a prognostic factor independent of prognostic risk score and TNM stage.

A recent study suggested that Axl expression was associated with epithelial-mesenchymal transition (EMT).⁽²²⁾ EMT was associated with acquired resistance to EGFR-specific tyrosine

kinase inhibitors (TKI).^(49,50) EGFR is overexpressed in more than 50% of TNBC, yet EGFR TKI remain ineffective for their treatment.^(51,52) An overexpressed/overactivated Gas6/Axl axis may be one of the mechanisms that promote resistance to EGFR targeted therapies in TNBC. In addition, the Gas6/Axl pathway plays a negative role in the regulation of cancer metastases via the modulation of NK cell activity and proliferation.⁽³²⁾ Could inhibitors of the Gas6/Axl axis have beneficial anti-cancer effects through NK cell activation? This is worthy of further investigation.

In summary, we established a predictive risk model, yielding scores with significant prognostic value for patients with TNBC. There are limitations to the scope of this study. We observed the prognostic value of the predictive model in invasive ductal carcinomas of TNBC. This observation needs to be studied in all histological subtypes of breast cancers, including at the molecular level. These problems will be resolved in future studies. We believe that our data will provide the foundation for developing new prognostic biomarkers and improving the treatment outcomes of TNBC patients.

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Disclosure Statement

The authors have no conflict of interest to declare.

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