Ann Surg Oncol (2019) 26:635–643
https://doi.org/10.1245/s10434-018-6941-4

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – PANCREATIC TUMORS

Tumor-Infiltrating NETs Predict Postsurgical Survival in Patients with Pancreatic Ductal Adenocarcinoma

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ABSTRACT

Background. Tumor-infiltrating neutrophils (TINs) indicate poor prognosis for patients with pancreatic ductal adenocarcinoma (PDAC). Activated neutrophils can generate neutrophil extracellular traps (NETs). Little is known about the presence and prognostic significance of tumor-infiltrating NETs in PDAC.

Methods. This study enrolled 317 patients, in two independent sets (training and validation), who underwent curative pancreatectomy for PDAC in Shanghai Cancer Center. TINs and NETs were identified by immunohistochemical staining for CD15 and citrullinated histone H3, respectively. The relationship between clinicopathological features and outcomes was analyzed. Accuracy of prognostic prediction models was evaluated using concordance index (C-index) and Akaike information criterion (AIC).

Results. NETs were associated with OS (both, $P < 0.001$) and RFS (both, $P < 0.001$) in the training and validation sets. Tumor-infiltrating NETs predicted poor postsurgical survival of patients with PDAC. Moreover, multivariate analysis identified NETs and AJCC TNM stage as two independent prognostic factors for OS and RFS. Combination of NETs with the 8th edition TNM staging system (C-index, 0.6994 and 0.6669, respectively; AIC, 1067 and 1126, respectively) generated a novel model that improved the predictive accuracy for survival in both sets (C-index, 0.7254 and 0.7117, respectively; AIC, 1047 and 1102, respectively). The model combining presence of NETs with the 7th edition AJCC TNM staging system also had improved predictive accuracy.

Conclusions. NETs were an independent prognostic factor in PDAC and incorporation of NETs along with the standard TNM stating system refined risk-stratification and predicted survival in PDAC with improved accuracy.

Keywords Neutrophils · NETs · PDAC · Prognosis

Pancreatic ductal adenocarcinoma (PDAC), which has an extremely poor prognosis with a 5-year overall survival (OS) rate less than 8%, is a serious threat to global health.[1] Surgical resection is still the only potential curative treatment for approximately 20% of patients.[2] Despite the intense efforts made to improve clinical treatment, the prognosis of PDAC patients is still disappointing due to the high metastatic potential. To date, no effective biomarkers for predictive prognosis have been identified. Thus, it is critical to identify novel biomarkers to predict prognosis and aid the therapy of PDAC.

Electronic supplementary material The online version of this article (https://doi.org/10.1245/s10434-018-6941-4) contains supplementary material, which is available to authorized users.

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First Received: 29 May 2018;
Published Online: 29 October 2018

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It is well-known that cancer and the immune system are closely associated. Their interplay determines many aspects of clinical presentation and cancer progression.[3] The neutrophils are generally the most prominent inflammatory population, and promote metastasis, although under certain conditions they also kill disseminated cancer cells.[4–6] Recent studies demonstrated that the peripheral blood neutrophil to lymphocyte ratio (NLR) indicates a poor prognosis for patients with PDAC.[7,8] Moreover, our previous study showed that tumor-infiltrating neutrophils can predict recurrence and survival in PDAC patients.

Neutrophils perform their functions in three ways: (1) phagocytosis, by which bacteria and fungi are engulfed and digested; (2) degranulation of cytotoxic enzymes into the extracellular matrix; and (3) formation of neutrophil extracellular traps (NETs). NETs are generated from activated neutrophils through NETosis, a mechanism of cell death, characterized by cell-free DNA meshed with histones, and granular and cytoplasmic neutrophil proteins, which are released into the extracellular space.[9,10] NETs could be induced by multiple infectious and non-infectious stimuli.[11] Currently, the important roles of NETs in several pathophysiological processes, such as acute coronary syndrome, stroke, and venous thrombosis, have been identified besides its classical role in protection against pathogens. The detection of tumor-infiltrating NETs dates back to 2013 in Ewing's sarcoma tissue, and more recently, NETs have been detected in human liver and gastric cancers.[12–15] To date, the roles of neutrophils in enhancement of cancer cells survival, invasion, and metastasis have been identified, but the role of NETs in cancer progression remains unclear.[16–18]

We investigated the role of tumor-infiltrating NETs in PDAC and assessed their prognostic significance in clinical outcomes of PDAC patients. To our knowledge, this is the first study to demonstrate that tumor-infiltrating NETs can serve as biomarkers to predict postoperative prognosis in PDAC patients.

METHODS

Patients and Specimens

Two independent cohorts comprising 317 patients who underwent R0 resection for PDAC at Shanghai Cancer Center (Shanghai, China) were enrolled. The training set contained 170 patients who had undergone pancreatectomy from January 2011 to December 2012. The validation set contained 147 patients treated from January 2013 to December 2014. All specimens were pathologically assessed independently by two experienced pathologists according to the 7th and 8th editions of the American Joint Committee on Cancer (AJCC) TNM staging system. Pancreatic ductal adenocarcinoma patients were included in the study according to the following inclusion criteria: (a) complete clinicopathological and follow-up data; (b) underwent R0 resection for PDAC identified by pathology; (c) without any record of another malignant tumor; (d) without any history of antitumor treatments; (e) without death within 30 days of surgery caused by postoperative complications.

Overall survival was calculated as the interval between surgery and the date of death. Recurrence-free survival (RFS) was defined as the interval between surgery and the date of first recurrence. All patients were followed up until December 2017. In the present study, we included patients with completed information of follow-up. The Research Ethics Committees at the associated pancreatic centers approved the use of human tissues. Informed consent was obtained from all patients according to the committees' regulations.

Immunohistochemistry

Immunostaining was performed using 96 full-face pathological sections of formalin-fixed, paraffin-embedded, surgical specimens. We enrolled another 74 patients and constructed the training set using tissue microarrays (TMAs; Shanghai Biochip Company, Shanghai, China). The validation set TMA contained specimens obtained from 147 patients. TMAs were constructed as previously described.[19–21]

Immunohistochemical staining was performed as described.[22] Briefly, the staining was performed with a CitH3 antibody (diluted 1:100, Anti-Histone H3 [citrulline R2 + R8 + R17]; ab5103; Abcam, Cambridge, MA) and CD15 antibody (diluted 1:200, Anti-CD15; sc-19648; Santa Cruz Biotechnology, Santa Cruz, CA). Different parts of the tumor have different distinct morphological features. To minimize the influence of tumor heterogeneity on the results, low-power microscopes were used to observe the overall immunohistochemical staining samples. We selected five random fields in the hot spot area under a high-power microscope to evaluate the expression of CitH3 and CD15 in the same tumor. An average scoring of the CitH3 and CD15 staining from five different points was calculated. Two patient data sets, representative of the training and validation cohorts, were collected at different times to verify these data. Finally, the expressions of CitH3 and CD15 in all tissue specimens were evaluated by two independent experienced pathologists who were blinded to the clinical data.

Statistical Analysis

The OS and RFS were analyzed using Kaplan–Meier survival curves with 95% confidence intervals (CIs), and the differences between subgroups were compared using the log-rank test. Continuous variables were compared by an unpaired *t* test and one-way analysis of variance (ANOVA). Associations between immunohistochemical variables and clinicopathological features were evaluated using the χ^2 test or Fisher's exact test. To identify independent prognostic factors, univariate and multivariate regression analyses were used. A value of $P < 0.05$ was set as the criterion for variable deletion to perform backward stepwise selection. The accuracies of predictive factors were analyzed using the concordance index (C-index) and Akaike information criterion (AIC). All tests were two-sided, and statistical significance was set at values of $P < 0.05$.

RESULTS

Identification of Tumor-Infiltrating NETs in PDAC Patients

To identify the existence and location of NETs in PDAC tumors, hematoxylin–eosin and immunohistochemical analysis of CitH3 was performed in full-face tissue sections. The results showed that NETs were mainly detected in the intratumoral stroma rather than tumor nests in a diffused manner, and NETs could not be detected in normal adjacent pancreatic tissues (Fig. 1a). As NETs were generated from neutrophils, we assessed the overlap between the presence of NETs and neutrophils. NETs were mainly located in neutrophil-rich areas, while not all neutrophil-positive tissues had NETs present (Fig. 1b).

The positivity rates of CD15 were 58.8% and 60.1% among the training and validation sets, respectively, and the CitH3 positivity rates were only 27.1% and 23.8%, respectively. The differences among the positivity rates of CD15 and CitH3 among the two data sets were not statistically significant (Supplementary Table 1).

The relationships between CD15/CitH3 expression and patients' clinical features are listed in Supplementary Tables 2 and 3, respectively. CitH3 expression in the two sets were positively correlated with the 8th edition TNM stage ($P < 0.001$ and $P = 0.001$, respectively), the 8th edition T classification ($P < 0.001$ and $P = 0.002$, respectively), the 8th edition N classification ($P < 0.001$ and $P = 0.001$, respectively), and CD15 expression (both, $P < 0.001$).

Presence of NETs was Associated with Poor OS and RFS in PDAC Patients

To investigate the prognostic value of neutrophils and NETs, we conducted Kaplan–Meier analysis of patient subgroups differentiated according to the intratumoral CD15 and CitH3 expression. Results of the log-rank test showed that patients without tumor-infiltrating NETs had significantly improved OS and RFS compared with those with tumor-infiltrating NETs in the training set (both, $P < 0.001$) and the validation set (both, $P < 0.001$; Fig. 2a–d). These data also showed that patients with tumor-infiltrating neutrophils had worse OS and RFS than patients without neutrophil infiltration in the training set (OS, $P = 0.006$; RFS, $P = 0.002$) and the validation set (OS, $P = 0.049$; RFS, $P = 0.011$; Fig. 2e–h), which were consistent with the findings of our previous study.[19] Because NETs are a functional form of neutrophils, none of the PDAC tumor tissues tested were NETs-positive and neutrophil-negative. Thus, the patients could be divided into three groups: $CD15^{-}NETs^{-}$ (lacking neutrophils and NETs), $CD15^{+}NETs^{-}$ (neutrophils-positive, lacking NETs), and $CD15^{+}NETs^{+}$ (neutrophils-positive, NETs-positive; Fig. 1b). Further analysis showed that in NETs-negative subgroups, the OS and RFS of the training (OS, $P = 0.665$; RFS, $P = 0.226$) and the validation (OS, $P = 0.507$; RFS, $P = 0.687$) sets were comparable, regardless of the neutrophil infiltration state (Fig. 2i–l). These data indicated that if the tumor was neutrophil-infiltrated, but lacked the activated form-NETs, the neutrophils alone would not predict the patient's prognosis. Furthermore, the NETs had a higher C-index (0.6193 vs. 0.5789) and a lower AIC (1087 vs. 1113) than neutrophils, indicating that NETs could predict prognosis with higher accuracy. These results were confirmed in the validation set (Supplementary Table 5).

Tumor-Infiltrating NETs were an Independent Prognostic Factor for PDAC Patients

Univariate Cox regression analysis in the two data sets identified the 8th edition *T* classification (OS, all $P < 0.001$; RFS, all, $P < 0.001$, respectively), the 8th edition *N* classification (OS, all, $P < 0.001$; RFS, all, $P < 0.001$), the 8th edition TNM stage (OS, all, $P < 0.001$; RFS, all, $P < 0.001$), chemotherapy (OS, all, $P < 0.001$; RFS, all, $P < 0.001$), neutrophil–lymphocyte ratio (training set: OS, $P = 0.049$; RFS, $P = 0.036$ and validation set: OS, $P = 0.045$; RFS, $P = 0.016$), tumor-infiltrating NETs (OS, all $P < 0.001$; RFS: all $P < 0.001$), and tumor-infiltrating neutrophils (training set: OS, $P = 0.006$; RFS, $P = 0.002$ and validation set: OS, $P = 0.026$; RFS,

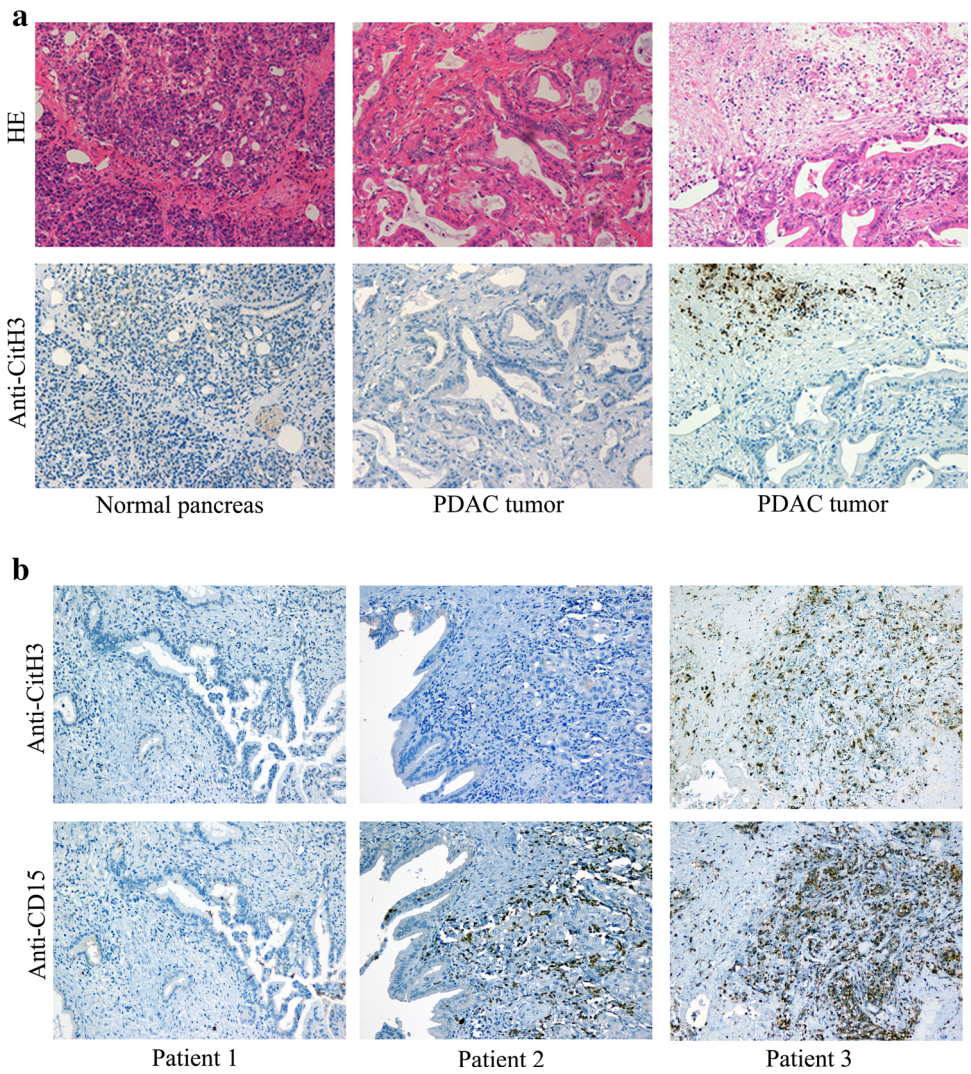

FIG. 1 Representative microphotographs of CitH3 and CD15 immunostainings. (**a**) Hematoxylin–eosin (HE) stained and CitH3 immunostained PDAC tissue (200×). Left, normal pancreas without CitH3 staining. Middle, tumor tissue negative for CitH3. Right, tumor tissue stained positive for CitH3. (**b**) Immunostainings of CitH3 and CD15 in PDAC tissue (200×). Left, tissue negative for both CD15 and CitH3. Middle, tissue stained positive for CD15 and negative for CitH3. Right, tissue stained positive for both CD15 and CitH3

$P = 0.022$) as clinical factors that were associated with OS and RFS in PDAC patients (Table 1).

The multivariate analysis included all the variables that demonstrated a meaningful effect on outcomes in the univariate analyses and showed that the 8th edition TNM stage (both, $P < 0.001$), chemotherapy (both, $P < 0.001$), and NETs ($P < 0.001$ and $P = 0.002$, respectively) were independent prognostic factors associated with OS in both sets. Also, multivariate analyses of the two data sets identified the 8th edition TNM stage (both, $P < 0.001$), chemotherapy (both, $P < 0.001$) and NETs ($P < 0.001$ and $P = 0.010$, respectively) as independent prognostic factors associated with RFS. We validated the results by performing similar analyses through the 7th edition TNM staging system. Analyses of the validation set using the 7th edition TNM staging system produced similar results (Table 2).

Tumor-Infiltrating NETs were Associated with Survival of Patients Received Gemcitabine-based Adjuvant Chemotherapy

The proportion of adjuvant chemotherapy was 81.8% and 84.8% in the training and validation sets, respectively. Patients who had received adjuvant chemotherapy had a longer median OS than those who did not receive any adjuvant chemotherapy (14.4 vs. 5.6 months for the training set, $P < 0.001$; 20.0 vs. 4.0 months for the validation set, $P < 0.001$; Supplementary Figs. 1a and b). Among patients who were CitH3-positive, patients who had received adjuvant chemotherapy had a longer median OS time than those who did not receive any adjuvant chemotherapy (9.6 vs. 4.8 months for the training set, $P < 0.001$; 12.0 vs. 4.0 months for the validation set, $P < 0.001$; Supplementary Figs. 1c and d). Among patients who received adjuvant chemotherapy, the median OS time of patients with CitH3-positive tumors was significantly

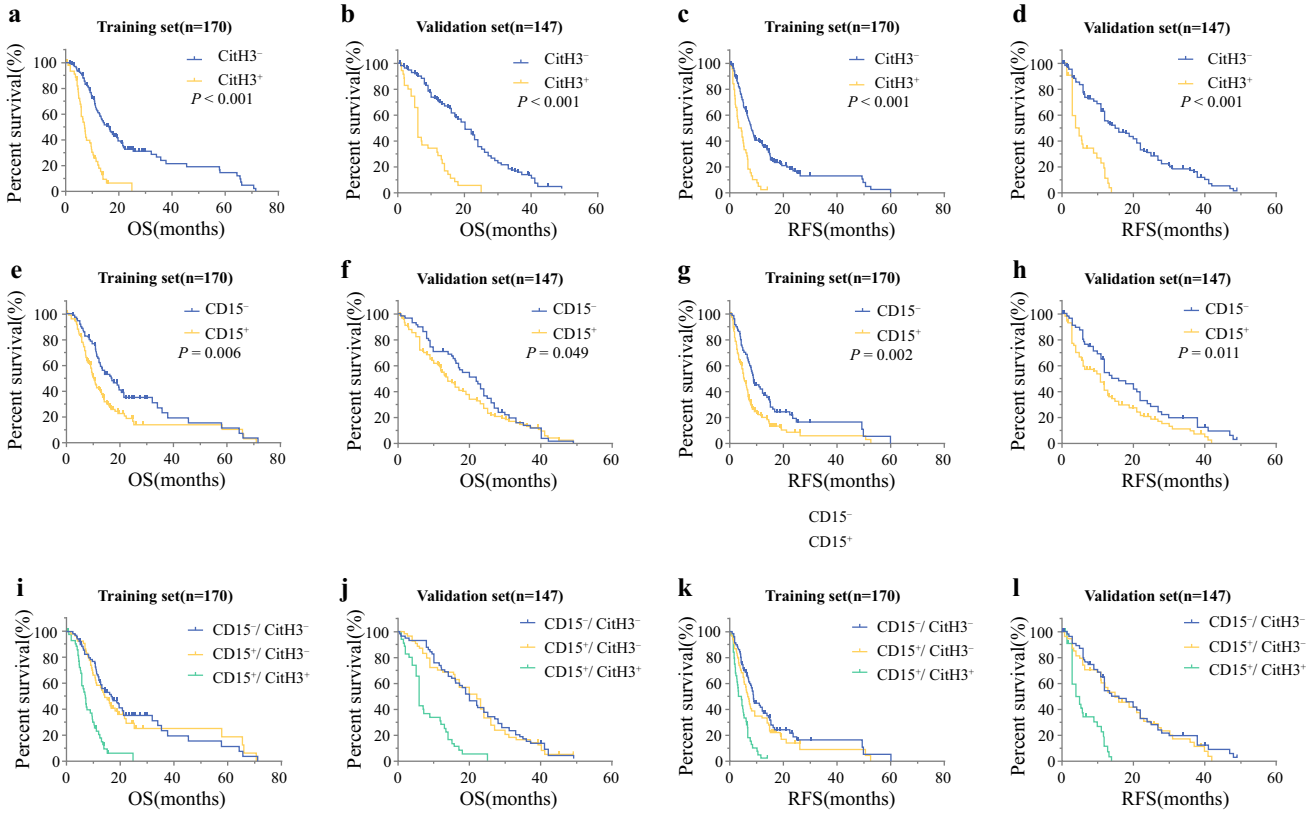

FIG. 2 Kaplan-Meier analysis of OS and RFS according to the presence of neutrophils and NETs in patients with PDAC. (**a**, **c**) OS and RFS in the Training Set according to the presence of NETs. Training Set, OS ($P < 0.001$), RFS ($P < 0.001$). (**b**, **d**) OS and RFS in the Validation Set according to the presence of NETs. Validation Set, OS ($P < 0.001$), RFS ($P < 0.001$). (**e**, **g**) OS and RFS in the Training Set according to the presence of neutrophils. Training Set, OS ($P = 0.006$), RFS ($P = 0.002$). (**f**, **h**) OS and RFS in the Validation Set according to the presence of neutrophils. Validation Set, OS ($P = 0.049$), RFS ($P = 0.011$). *P* values were calculated using the log-rank test. (**i**, **k**) OS and RFS of the three subgroups in the Training Set. $CD15^{-}NETs^{-}$ vs. $CD15^{+}NETs^{-}$ (OS, $P = 0.665$; RFS, $P = 0.226$); $CD15^{+}NETs^{-}$ vs. $CD15^{+}NETs^{+}$ (both, $P < 0.001$); and $CD15^{-}NETs^{-}$ vs. $CD15^{+}NETs^{+}$ (both, $P < 0.001$). (**j**, **l**) OS and RFS of three subgroups in the validation set. $CD15^{-}NETs^{-}$ vs. $CD15^{+}NETs^{-}$ (OS, $P = 0.507$; RFS, $P = 0.687$); $CD15^{+}NETs^{-}$ vs. $CD15^{+}NETs^{+}$ (both, $P < 0.001$); and $CD15^{-}NETs^{-}$ vs. $CD15^{+}NETs^{+}$ (both, $P < 0.001$)

shorter than those with CitH3-negative tumors (12.0 vs. 22.0 months for the training set, $P < 0.001$; 9.0 months vs. 18.2 months for the validation set, $P < 0.001$; Supplementary Figs. 1e and f).

Extension of the TNM Stage Prognostic Model with Tumor-Infiltrating NETs

To increase the accuracy of the current prognostic model, we generated a novel predictive model for patients with PDAC by combining the TNM staging system with tumor-infiltrating NETs. The new model included both the 8th edition TNM stage and NETs, which had a higher C-index (0.7254 vs. 0.6994) and a lower AIC (1047 vs. 1067) than the model based on 8th edition TNM stage alone. The analysis of validation data set produced similar results.

The novel model was validated by combining the 7th edition TNM stage and NETs, and the results showed that the combined model had a higher C-index (0.7232 vs. 0.6872) and a lower AIC (1048 vs. 1071) than the standard model, which was based on the 7th edition TNM stage alone, in the training set. The analysis of the validation data set produced similar results (Table 3).

DISCUSSION

In this study, we identified the existence of tumor-infiltrating NETs in PDAC and analyzed the association of NETs with patients' prognosis. As an independent prognostic factor, association between NETs and poor prognosis led us to investigate the potential role of NETs in cancer progression. Since, NETs have been detected in blood and tissue samples in several of cancers.[15,23,24] NETs constitute fibers of chromatin DNA released from neutrophils in a process defined as NETosis.[25] The nuclear and cell membrane disappears, DNA meshes are released into the extracellular matrix, decorated with various

TABLE 1 Univariate cox regression analyses of OS and RFS in the training and validation sets of patients with PDAC

Factors	OS				RFS			
	Training set (n = 170)		Validation set (n = 147)		Training set (n = 170)		Validation set (n = 147)	
	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Age (> 62 yr/≤ 62 yr)	1.011 (0.988–1.034)	0.339	0.995 (0.979–1.012)	0.575	1.057 (0.755–1.479)	0.746	1.054 (0.732–1.517)	0.778
Gender (male/female)	1.282 (0.892–1.843)	0.180	0.990 (0.704–1.391)	0.953	1.234 (0.880–1.730)	0.223	1.122 (0.776–1.624)	0.540
Tumor location (head/body, tail)	1.271 (0.899–1.796)	0.175	0.994 (0.711–1.389)	0.972	1.170 (0.843–1.624)	0.347	0.898 (0.628–1.285)	0.556
Neural invasion (yes/no)	1.096 (0.736–1.632)	0.651	1.204 (0.841–1.723)	0.311	1.097 (0.750–1.605)	0.633	1.119 (0.762–1.643)	0.567
Microvascular invasion (yes/no)	1.328 (0.892–1.978)	0.163	0.858 (0.579–1.270)	0.443	1.152 (0.789–1.683)	0.464	1.192 (0.805–1.765)	0.379
Tumor differentiation (well, moderate/poor)	1.038 (0.735–1.466)	0.833	0.721 (0.511–1.019)	0.064	0.850 (0.611–1.182)	0.334	0.700 (0.482–1.015)	0.060
8th edition TNM stage (I/II/III)	2.849 (2.160–3.759)	< **0.001**	2.152 (1.693–2.735)	< **0.001**	2.095 (1.604–2.735)	< **0.001**	1.864 (1.442–2.409)	< **0.001**
8^{th} edition T classification (T1 + T2/T3)	3.936 (2.726–5.682)	< **0.001**	2.691 (1.905–3.803)	< **0.001**	2.539 (1.809–3.563)	< **0.001**	2.130 (1.464–3.098)	< **0.001**
8th edition N classification (N0/N1/N2)	2.643 (2.063–3.386)	< **0.001**	2.017 (1.601–2.540)	< **0.001**	2.034 (1.602–2.581)	< **0.001**	1.854 (1.449–2.371)	< **0.001**
7th edition TNM stage (I/IIa/IIb)	2.306 (1.792–2.967)	< **0.001**	1.875 (1.499–2.345)	< **0.001**	1.696 (1.366–2.107)	< **0.001**	1.738 (1.375–2.196)	< **0.001**
7th edition T classification (T1 + T2/T3)	1.913 (1.327–2.756)	**0.001**	2.193 (1.560–3.084)	< **0.001**	1.687 (1.194–2.384)	**0.003**	2.088 (1.439–3.029)	< **0.001**
7th edition N classification (N0/N1)	3.550 (2.413–5.221)	< **0.001**	2.287 (1.612–3.244)	< **0.001**	2.107 (1.500–2.960)	< **0.001**	1.978 (1.364–2.870)	< **0.001**
Preoperative CA19-9 (U/mL, > 37/≤ 37)	1.490 (0.921–2.410)	0.105	1.538 (0.978–2.325)	0.119	1.488 (0.941–2.353)	0.089	1.35555 (0.947–1.938)	0.096
Chemotherapy	3.593 (2.401–5.375)	< **0.001**	4.080 (2.669–6.239)	< **0.001**	2.860 (1.939–4.220)	< **0.001**	3.935 (2.463–6.285)	< **0.001**
Neutrophil–lymphocyte ratio (< 2 vs ≥ 2)	1.429 (1.000–2.043)	**0.049**	1.407 (1.007–1.965)	**0.045**	1.321 (1.020–1.991)	**0.036**	1.657 (1.097–2.502)	**0.016**
CitH3 expression (positive/negative)	3.593 (2.401–5.375)	< **0.001**	4.080 (2.669–6.239)	< **0.001**	2.860 (1.939–4.220)	< **0.001**	3.935 (2.463–6.285)	< **0.001**
CD15 expression (positive/negative)	1.651 (1.155–2.358)	**0.006**	1.473 (1.046–2.073)	**0.026**	1.723 (1.228–2.417)	**0.002**	1.548 (1.065–2.252)	**0.022**

Bold values are statistically significant ($P < 0.05$)

antimicrobial proteins from the neutrophil granules, including CitH3, neutrophil elastase myeloperoxidase, proteinase 3, cathepsin G, matrix metalloproteinase-9, and bactericidal permeability increasing protein.[26,27] NETs can be identified by markers, such as cell-free DNA, nucleosomes, and CitH3.[28] CitH3 has been a reliable biomarker for diagnosis and treatment of NETs.[29–31] Cancer cells can change the ability of neutrophils to eradicate pathogens and aid metastatic spread by formation of NETs. In breast cancer, cancer cell-induced NET-formation facilitates the metastatic seeding of the liver.[27] Tumor-derived cytokines, such as granulocyte colony-stimulating factor (G-SCF) and interleukin 8, contribute to induction of NETs.[32] Blocking antibodies against G-SCF or treatment with DNase I were reported to reduce metastasis through an unknown mechanism.[27] DNase I has been approved by the FDA for the treatment of cystic fibrosis to decrease the mucus viscosity induced by accumulation of NETs.[33] In our study, tumor-

TABLE 2 Multivariate cox regression analyses for postsurgical OS and RFS in the training and validation sets of patients with PDAC

Factors	OS			RFS		
	HR	95% CI	*P*	HR	95% CL	*P*
8th edition TNM stage						
Training set (n = 170)						
NETs	2.707	1.686–4.395	< 0.001	2.084	1.334–3.257	< 0.001
TNM stage	2.095	1.564–2.807	< 0.001	1.148	1.070–1.711	< 0.001
Chemotherapy	0.136	0.076–0.244	< 0.001	0.300	0.184–0.489	< 0.001
Validation set (n = 147)						
NETs	2.330	1.362–3.985	0.002	2.134	1.199–3.797	0.010
TNM stage	2.014	1.572–2.579	< 0.001	1.645	1.258–2.152	< 0.001
Chemotherapy	0.120	0.064–0.228	< 0.001	0.188	0.099–0.360	< 0.001
7th edition TNM stage						
Training set (n = 170)						
NETs	2.969	1.848–4.770	< 0.001	2.075	1.331–3.237	0.001
TNM stage	2.090	1.600–2.730	< 0.001	1.482	1.177–1.866	0.001
Chemotherapy	0.118	0.067–0.209	< 0.001	0.292	0.180–0.472	< 0.001
Validation set (n = 147)						
NETs	2.366	1.408–3.978	< 0.001	3.037	1.809–5.098	< 0.001
TNM stage	1.795	1.424–2.264	< 0.001	1.634	1.277–2.091	< 0.001
Chemotherapy	0.119	0.063–0.225	< 0.001	0.193	0.101–0.368	< 0.001

OS overall survival, *RFS* recurrence-free survival, *HR* hazard ratio, *CI* confidence interval, *TNM* tumor node metastasis, *NETs* neutrophil extracellular traps

TABLE 3 Comparison of the prognostic accuracies of TNM stage, NETs, and the combined model

Model	TNM^{8th}		TNM^{7th}	
	C-index	AIC	C-index	AIC
Training set (n = 170)				
NETs	0.6193	1087	0.6193	1087
TNM	0.6994	1067	0.6872	1071
TNM + NETs	0.7254	1047	0.7232	1048
Validation set (n = 147)				
NETs	0.6190	1128	0.6190	1128
TNM	0.6669	1126	0.6487	1131
TNM + NETs	0.7117	1102	0.7075	1106

C-index concordance index, *AIC* Akaike information criterion, TNM^{8th} AJCC TNM staging system 8th edition, TNM^{7th} AJCC TNM staging system 7th edition, *NETs* neutrophil extracellular traps

infiltrating NETs were associated with worse OS and RFS, indicating that NETs play important roles in the procession of PDAC. Thus, NETs could be an exciting and feasible new target for PDAC therapy.

Further analysis indicated that NETs were an independent prognostic factor and could predict prognosis with better predictive accuracy than neutrophils. Although compelling evidence suggests that tumor-infiltrating neutrophils participate in tumor progression, the correlation between neutrophils and prognosis remains controversial.[34,35] Neutrophils can exhibit a pro-tumorigenic N2 phenotype and an antitumorigenic N1 phenotype, analogous to the effects of macrophages.[36] In general, the N1 and N2 neutrophils can be identified based on their function; the N1 neutrophils are mainly characterized by cytotoxicity to cancer cells and an immunostimulatory profile, such as high expression of tumor necrosis factor α, intercellular cell adhesion molecule-1, chemokine (C–C motif) ligand (CCL) 3, and low expression of arginase, whereas N2 are characterized by upregulation of chemokines, such as chemokine (C–X–C motif) ligands (CXCLs) 1, 2, 8, and 16 and CCL2, 3, 4, 8, 12, and 17.[36,37] Until now, no specific marker has been identified to distinguish the N1/N2 subgroups in clinical research and diagnosis.[37–39] The phenotypic subtype may be responsible for the opposite correlations between tumor-infiltrating neutrophils and survival. NETs are generated from activated neutrophils, and no phenotypic subtypes have been described in NETs to date.[40,41] Thus, NETs may be easier to identify in pathological tissue specimens and a more suitable parameter as a clinical predictor of prognosis. Our experiments also suggested that NETs were related to the effect of gemcitabine-based adjuvant chemotherapy, and it might serve as a predictor of adjuvant treatment; however, there is an urgent need for multicenter and high-evidence-level clinical trials to validate these results.

In addition, the combination of NETs with the TNM staging system generated a novel prognostic model with better predictive accuracy for prognosis. Traditional prognostic models for patients with PDAC depend on the TNM staging system alone, which only assesses the objective state of the tumor without consideration of the tumor microenvironment. The new prognostic model that we describe in this study, which combines NETs with the TNM staging system, takes information regarding the immune tumor microenvironment into consideration to better identify patients with different outcomes.

Our study has some limitations. First, the findings and model in this study need to be validated in a larger, prospective, multicentered data set. Second, our study does not provide information about the mechanism by which NETs affect the prognosis of PDAC patients. Because the results showed that NETs were mainly distributed in PDAC stroma, NETs do not have direct contacts with cancer cells. The mechanism through which NETs promote PDAC progression warrants further study.

CONCLUSIONS

Tumor-infiltrating NETs are better markers, as an independent prognostic factor, to predict PDAC progression and clinical outcomes than neutrophils. Combination of NETs with an established TNM stage-based prognostic model was employed to generate a novel predictive model that could stratify patients with different tumor immune microenvironments and prognoses. These findings pave the way for customizing management of PDAC patients based on the presence of tumor-infiltrating NETs and have implications in treating PDAC patients with immunotherapy.

ACKNOWLEDGMENTS This work was supported by grants from the National Science Foundation for Distinguished Young Scholars of China (81625016), the National Natural Science Foundation of China (81472670, 81871941, 81872366, 81802380, and 81802675), the Outstanding Academic Leader Program of the "Technological Innovation Action Plan" in Shanghai Science and Technology Commission (18XD1401200), and the Young Talented Specialist Training Program of Shanghai.

DISCLOSURE The authors declare no conflicts of interest in this study.

REFERENCES

1. Wolfgang CL, Herman JM, Laheru DA, et al. Recent progress in pancreatic cancer. *Ca Cancer J Clin* 2013;63(5):318.
2. Kamisawa T, Wood LD, Itoi T, Takaori K. Pancreatic cancer. *Lancet.* 2016;388(10039):73.
3. Diakos CI, Charles KA, Mcmillan DC, Clarke SJ. Cancer-related inflammation and treatment effectiveness. *Lancet Oncol.* 2014; 15(11):e493–503.
4. Wculek SK, Malanchi I. Neutrophils support lung colonization of metastasis-initiating breast cancer cells. *Nature.* 2015;528(7582): 413–7.
5. Spiegel A, Brooks MW, Houshyar S, et al. Neutrophils suppress intraluminal NK cell-mediated tumor cell clearance and enhance extravasation of disseminated carcinoma cells. *Cancer Discovery.* 2016;6(6):630.
6. Granot Z, Henke E, Comen EA, King TA, Norton L, Benezra R. Tumor entrained neutrophils inhibit seeding in the premetastatic lung. *Cancer Cell.* 2011;20(3):300–14.
7. Yang JJ, Hu ZG, Shi WX, Deng T, He SQ, Yuan SG. Prognostic significance of neutrophil to lymphocyte ratio in pancreatic cancer: a meta-analysis. *World J Gastroenterol.* 2015;21(9): 2807–15.
8. Cheng H, Luo G, Lu Y, et al. The combination of systemic inflammation-based marker NLR and circulating regulatory T cells predicts the prognosis of resectable pancreatic cancer patients. *Pancreatology.* 2016;16(6):1080–4.
9. Pulze L, Bassani B, Gini E, et al. NET amyloidogenic backbone in human activated neutrophils. *Clin Exp Immunol.* 2016;183(3): 469–79.
10. Korabecna M, Tesar V. NETosis provides the link between activation of neutrophils on hemodialysis membrane and comorbidities in dialyzed patients. *Inflamm Res.* 2017;66(5): 1–10.
11. Bonaventura A, Liberale L, Carbone F, et al. The pathophysiological role of neutrophil extracellular traps in inflammatory diseases. *Thromb Haemost.* 2018;118(1):6.
12. Berger-Achituv S, Brinkmann V, Abed UA, et al. A proposed role for neutrophil extracellular traps in cancer immunoediting. *Frontiers Immunol.* 2013;4:48.
13. Tohme S, Yazadani HO, Al-Khafaji AB, et al. Neutrophil extracellular traps promote the development and progression of liver metastases after surgical stress. *Cancer Res.* 2016;76(6): 1367.
14. Merza M, Hartman H, Rahman M, et al. Neutrophil extracellular traps induce trypsin activation, inflammation, and tissue damage in mice with severe acute pancreatitis. *Gastroenterology.* 2015; 149(7):1920–31.
15. Yang C, Sun W, Wu C, et al. Procoagulant role of neutrophil extracellular traps in patients with gastric cancer. *Int J Clin Exp Pathol.* 2015;8(11):14075–86.
16. Cools-Lartigue J, Spicer J, Najmeh S, Ferri L. Neutrophil extracellular traps in cancer progression. *Cell Molec Life Sci.* 2014; 71(21):4179–94.
17. Zhao J, Pan K, Wang W, et al. The prognostic value of tumor-infiltrating neutrophils in gastric adenocarcinoma after resection. *Plos One.* 2015;7(3):e33655.
18. Perisanidis C, Kornek G, Pöschl PW, et al. High neutrophil-to-lymphocyte ratio is an independent marker of poor disease-specific survival in patients with oral cancer. *Med Oncol.* 2013;30(1):1–8.
19. Wang WQ, Liu L, Xu HX, et al. Infiltrating immune cells and gene mutations in pancreatic ductal adenocarcinoma. *Br J Surg.* 2016;103(9):1189–99.
20. Wang WQ, Liu L, Xu HX, et al. The combination of HTATIP2 expression and microvessel density predicts converse survival of hepatocellular carcinoma with or without sorafenib. *Oncotarget.* 2014;5(11):3895–906.
21. Wang WQ, Liu L, Xu HX, et al. Intratumoral α-SMA enhances the prognostic potency of CD34 associated with maintenance of microvessel integrity in hepatocellular carcinoma and pancreatic cancer. *Plos One.* 2013;8(8):e71189.
22. Wei J, Liao X, Lv Y, et al. MUC1 induces acquired chemoresistance by upregulating ABCB1 in EGFR-dependent manner. *Cell Death Dis.* 2017;8(8):e2980.

23. Arelaki S, Arampatzioglou A, Kambas K, et al. Gradient infiltration of neutrophil extracellular traps in colon cancer and evidence for their involvement in tumour growth. *Plos One*. 2016;11(5):e0154484.
24. Thålin C, Demers M, Blomgren B, et al. NETosis promotes cancer-associated arterial microthrombosis presenting as ischemic stroke with troponin elevation. *Thromb Res.* 2016;139:56.
25. Branzk N, Papayannopoulos V. Molecular mechanisms regulating NETosis in infection and disease. *Sem Immunopathol.* 2013;35(4):513.
26. Volker B, Arturo Z. Neutrophil extracellular traps: Is immunity the second function of chromatin? *J Cell Biol.* 2012;198(5): 773–83.
27. Park J, Wysocki RW, Amoozgar Z, et al. Cancer cells induce metastasis-supporting neutrophil extracellularDNA traps. *Sci Transl Med.* 2016;8(361):361ra138.
28. Vallés J, Lago A, Santos MT, et al. Neutrophil extracellular traps are increased in patients with acute ischemic stroke: prognostic significance. *Thromb Haemost.* 2017;117(10):1919–29.
29. Chowdhury CS, Giaglis S, Walker UA, Buser A, Hahn S, Hasler P. Enhanced neutrophil extracellular trap generation in rheumatoid arthritis: analysis of underlying signal transduction pathways and potential diagnostic utility. *Arthritis Res Ther.* 2014;16(3):R122.
30. Stoikou M, Grimolizzi F, Giaglis S, et al. Gestational diabetes mellitus is associated with altered neutrophil activity. *Frontiers Immunol.* 2017;8.
31. Pan B, Alam HB, Wei C, et al. CitH3: a reliable blood biomarker for diagnosis and treatment of endotoxic shock. *Scientific Rep.* 2017;7(1):8972.
32. Cedervall J, Zhang Y, Huang H, et al. Neutrophil extracellular traps accumulate in peripheral blood vessels and compromise organ function in tumor-bearing animals. *Cancer Res.* 2015;75(13):2653–62.
33. Shak S, Capon DJ, Hellmiss R, Marsters SA, Baker CL. Recombinant Human DNase I Reduces the Viscosity of Cystic Fibrosis Sputum. *Proc Natl Acad Sci U S A.* 1990;87(23):9188.
34. Li YW, Qiu SJ, Fan J, et al. Intratumoral neutrophils: a poor prognostic factor for hepatocellular carcinoma following resection. *J Hepatol.* 2011;54(3):497.
35. Caruso RA, Bellocco R, Pagano M, Bertoli G, Rigoli L, Inferrera C. Prognostic value of intratumoral neutrophils in advanced gastric carcinoma in a high-risk area in northern Italy. *Mod Pathol.* 2002;15(8):831–7.
36. Fridlender ZG, Albelda SM. Tumor-associated neutrophils: friend or foe? *Carcinogenesis.* 2012;33(5):949–55.
37. Sionov RV, Fridlender ZG, Granot Z. The multifaceted roles neutrophils play in the tumor microenvironment. *Cancer Microenviron.* 2015;8(3):125–58.
38. Zhang X, Zhang W, Yuan X, Fu M, Qian H, Xu W. Neutrophils in cancer development and progression: Roles, mechanisms, and implications (Review). *Int J Oncol.* 2016;49(3):857.
39. Zhang H, Liu H, Shen Z, et al. Tumor-infiltrating neutrophils is prognostic and predictive for postoperative adjuvant chemotherapy benefit in patients with gastric cancer. *Ann Surg.* 2016:1.
40. Powell DR, Huttenlocher A. Neutrophils in the tumor microenvironment. *Trends Immunol.* 2016;37(1):41–52.
41. Sørensen OE, Borregaard N. Neutrophil extracellular traps—the dark side of neutrophils. *J Clin Invest.* 2016;126(5):1612–20.