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Immature stroma and high infiltration of CD15⁺ cells are predictive markers of poor prognosis in different subsets of patients with pancreatic cancer

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Abstract

Preoperative treatment is commonly carried out for borderline resectable pancreatic ductal adenocarcinoma (PDAC). However, the relationship between the combination of immune cells in the tumor microenvironment and their intratumoral heterogeneity along with their association with histological findings remains unclear, especially in patients receiving preoperative chemotherapy. We aimed to explore the therapeutic strategies for patients with PDAC with poor prognosis after receiving chemotherapy based on histological and immunological microenvironmental classifications. We investigated the correlation between the prognosis and histological immune microenvironmental factors of patients who initially underwent surgery ($n = 100$) and were receiving gemcitabine plus nab-paclitaxel (GEM + nabPTX) as preoperative chemotherapy ($n = 103$). Immune profiles were generated based on immune cell infiltration into the tumor, and their correlation with patient outcomes and histological features was analyzed. Tumor-infiltrating neutrophils (TINs) were identified as independent poor prognostic factors using multivariate analysis in both surgery-first and preoperative chemotherapy groups. The patients were further classified into four groups based on immune cell infiltration into the tumor. Patients with high CD15 infiltration into the tumor and immature stroma at the cancer margins showed the worst prognosis in the preoperative chemotherapy group. The analysis of mRNA expression and immunohistochemical features revealed that CXCR2, the receptor for CXCL8, was correlated with disease-free and overall survival. We inferred that patients with immature stroma at the margins and high infiltration of CD15⁺ neutrophils within the

Abbreviations: 5-FU, 5-fluorouracil; CAF, cancer-associated fibroblast; CI, confidence interval; CLR, Crohn-like lymphoid reaction; CT, computed tomography; DC, dendritic cell; DEG, differentially expressed gene; DFS, disease-free survival; DR, desmoplastic reaction; EUS, endoscopic ultrasound; FDR, false discovery rate; FOLFIRINOX, fluorouracil, leucovorin, irinotecan and oxaliplatin; GEM + nabPTX, gemcitabine plus nab-paclitaxel; GEPIA, gene expression profiling interactive analysis; G-MDSC, granulocyte-based myeloid-derived suppressor cell; GO, gene ontology; GSEA, gene set enrichment analysis; HR, hazard ratio; IHC, immunohistochemical; IRB, Institutional review board; MDSC, myeloid-derived suppressor cell; NAC, neoadjuvant chemotherapy; NK, natural killer; OS, overall survival; PDAC, pancreatic ductal adenocarcinoma; PDC, poorly differentiated cluster; PPI, protein-protein interaction; SF, surgery-first; TB, tumor budding; TIL, tumor-infiltrating lymphocyte; TIN, tumor-infiltrating neutrophil.

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tumor showed the worst prognosis and they could particularly benefit from treatment with inhibitors targeting CXCR2 or CXCL8.

KEYWORDS

neoadjuvant chemotherapy, neutrophils, pancreatic cancer, receptors, chemokine, tumor microenvironment

1 | INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal disease with poor OS rates.¹ Currently, the prognosis of patients with borderline resectable PDAC has considerably improved due to the development of NAC regimens, including FOLFIRINOX and GEM + nabPTX.² However, distinguishing between treatment-related and cancer-related fibrosis is difficult, as even untreated PDAC often shows extensive fibrosis associated with chronic pancreatitis. This difficulty in interpretation can lead to discrepancies in the assessment of tumor regression among observers. Furthermore, current treatment efficacy measures, including the Evans classification, are not correlated with prognosis.³ Moreover, the prognostic factors for PDAC resected after NAC remain unknown.

Recently, various pathological prognostic factors have been reported in digestive cancers. Tumor budding (TB) and poorly differentiated clusters (PDCs), which are associated with epithelial-mesenchymal transition (EMT), were reported as indicators of poor prognosis in various carcinomas.^{4–7} In colorectal cancer and other malignancies, the desmoplastic reaction (DR) at the invasive front is strongly correlated with prognosis.^{8–11} The presence of crohn-like lymphoid reaction (CLR) is also a favorable prognostic factor, as it enhances host immunity in the invasive area, regardless of TNM stage or microsatellite instability status.¹² PDAC is characterized by the infiltration of immunosuppressive cells, including regulatory T cells, M2-polarized macrophages, neutrophils, and myeloid-derived suppressor cells (MDSCs), which reportedly influence prognosis.^{13–17} Preoperative treatment induces various changes in the immune microenvironment in addition to its direct cytotoxic effects.¹⁸ Although some studies have focused on individual immune cells, the composition of immune cells within the microenvironment and its relationship with histological findings in PDAC patients remain unclear. Therefore, the present study aimed to identify the prognostic factors of neoadjuvant chemotherapy in PDAC patients and to explore the therapeutic targets by histological investigations, including previously reported prognostic factors and clustering patients with pancreatic cancer based on immunological microenvironmental factors.

2 | MATERIALS AND METHODS

2.1 | Study design and patient cohort

The present study was conducted in accordance with the principles stipulated in the Declaration of Helsinki and was approved by the Institutional review board (IRB) of Kyushu University (IRB number

2020–633). Clinicopathologic factors were collected from the data manually reviewed from an electronic medical record. The study cohort included consecutive patients who underwent curative resection for PDAC from January 2010 to December 2019 ($n=353$). Finally, the patients who received GEM + nabPTX were selected as the NAC group ($n=103$) and those who were preceded by surgery-first (SF) at the same time were selected as SF group ($n=100$) (Figure 1A). For clinicopathologic characteristics of the NAC and SF groups, see Table S1.

2.2 | Histological evaluation

There were no considerable changes in the method on how the specimens were processed during the study period. All available H&E-stained slides that included full-thickness sections of the tumor encompassing the deepest portion of the invasive front were reviewed. The deepest portion of PDAC was defined as the portion of the tumor that invaded into the peripancreatic adipose tissue. All histological factors were assessed as described previously.^{4–12} Briefly, DR was classified as mature, intermediate, or immature (Figure 1B). If the myxoid stroma was larger than the microscopic field of a 40× objective lens, DR was classified as immature. Conversely, among tumors that were not classified as immature, DR was classified as intermediate if keloid-like collagen was present. Desmoplastic reaction was classified as mature if the fibrous stroma contained no myxoid stroma or keloid-like collagen. When different types of desmoplastic stroma were present, DR was classified based on the area with the immature stroma as described previously.^{8–11} Tumor budding was defined as a single cancer cell or a cluster of fewer than five cancer cells at the invasive front and was graded as G1 (<5 buds), G2 (5–9 buds), or G3 (≥ 10 buds) based on the highest number of buds observed under an objective lens with a magnification of $\times 20$ (Figure 1B). Similarly, the number of PDCs defined as clusters of five or more cancer cells was then determined and graded as G1 (<5 clusters), G2 (5–9 clusters), or G3 (≥ 10 clusters) (Figure 1B). Crohn-like lymphoid reaction was defined as a minimum of three nodular lymphoid aggregates at the tumor periphery and was classified as present (three or more) or absent (fewer than three) (Figure 1B). These histological evaluations were independently carried out in a blinded manner for the clinical and pathological data by pathologists (Y.Y. and T.Y.). Table S2 presents the data on interobserver agreement for H&E staining review items. The kappa values for all markers were ranged 0.532–0.700, indicating moderate to substantial agreement.¹⁹

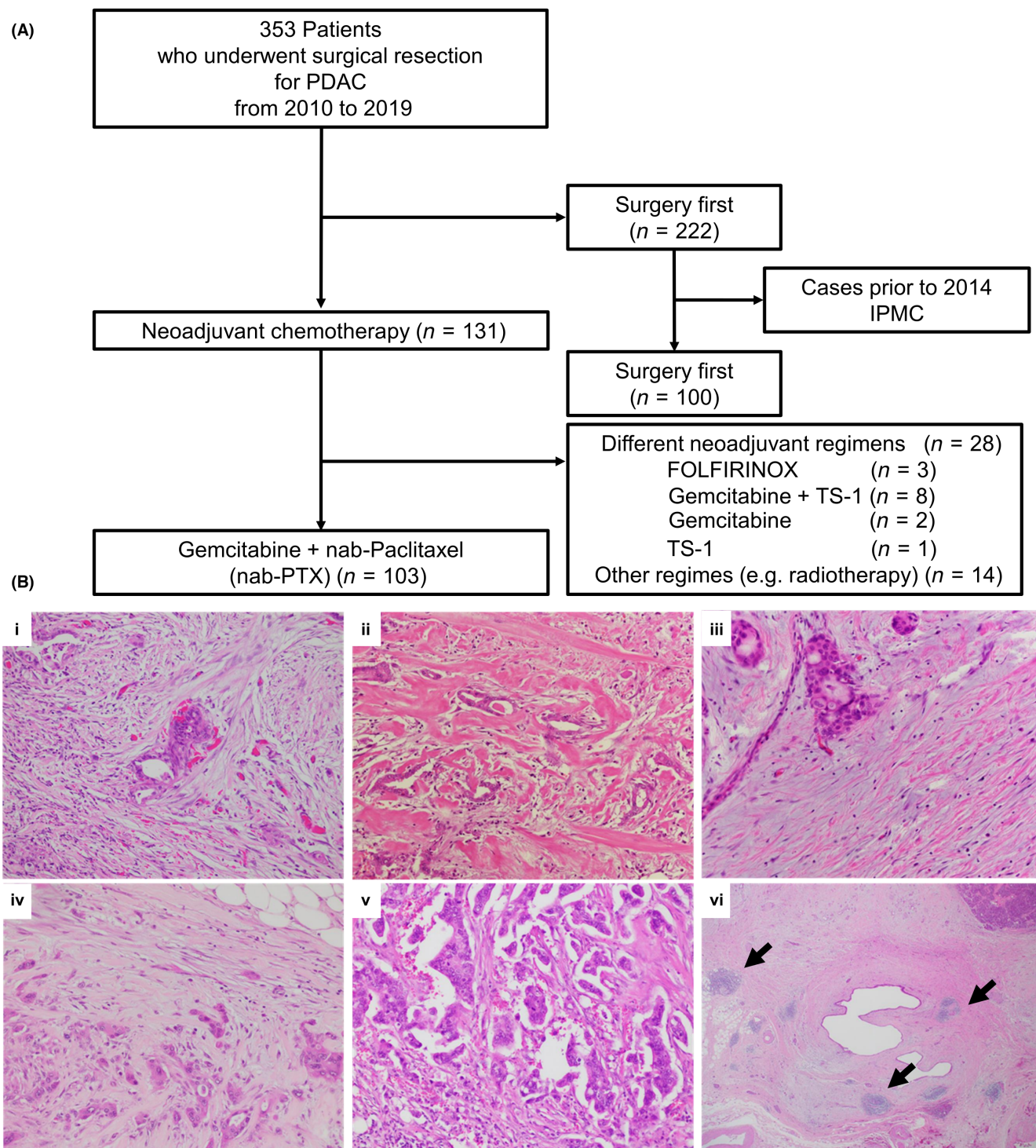


FIGURE 1 Overview of patient selection and histological findings. (A) Flowchart of sample selection for neoadjuvant chemotherapy and surgery-first groups. (B) (i–iii) Histological findings. Desmoplastic reaction: (i) mature, (ii) intermediate, (iii) immature. (iv) Tumor budding (single cancer cells or clusters comprising <5 cancer cells). (v) Poorly differentiated clusters (≥ 5 cancer cells that lacked glandular formation). (vi) Crohn-like lymphoid reaction (several nodular lymphoid aggregates at the tumor microenvironment). FOLFIRINOX, fluorouracil, leucovorin, irinotecan and oxaliplatin; IPMC, intraductal papillary mucinous carcinoma; PDAC, pancreatic ductal adenocarcinoma.

2.3 | Evaluation of immune cell infiltration

In this study, T cells (CD4, CD8, FOXP3), B cells (CD20), plasma cells (CD138), antigen-presenting cells, such as DCs (CD155), macrophages (M1, CD68; M2, CD163), NK cells (CD56), and neutrophils (CD15) were evaluated (Table S3). Each respective area of the tumor

nest was measured at 400x high-power magnification to evaluate the density of each type of immune cell. We evaluated this by using an average of five high-power fields.

Based on the median number of immune cells, the patients were separated into the high and low groups. Tumor-infiltrating neutrophils were classified as low (0–19) and high (≥ 20) TINs

based on previous reports.²⁰ In the present study, we did not assess the TILs or TINs in the tumor marginal zone, as this would have obscured whether the invasion was due to secondary effects, such as pancreatitis, or tumor necrosis, rather than due to the tumor itself.

2.4 | Hierarchical cluster analysis and immune subtype classification

For the hierarchical cluster analysis of immune cell infiltration, Z score was calculated using the data of each immune cell infiltration, and hierarchical cluster analysis (distance metric, Pearson correlation; linkage method, average linkage) and heat map generation was carried out using Multi Experimental Viewer version 4.9.^{21,22}

2.5 | RNA extraction

According to the manufacturer's protocol, total RNA was extracted from only the FFPE specimens using RNAsort FFPE Kit (Cell Data Science). The samples were collected from carcinoma cells, excluding normal pancreatic tissue by macrodissection.

2.6 | mRNA expression analysis

We assessed the comprehensive mRNA expression using the nCounter® Gene Expression Assay (Nanostring Technology). All 12 samples were extracted from FFPE. The extracted mRNA samples were enriched for target regions for the nCounter® (Human PanCancer IO 360 Panel) and measured using the nCounter® Analysis System (Nanostring Technology). The raw data were normalized using nSolver Analysis Software (version 4.0; Nanostring Technology).

2.7 | Bioinformatic DEG analysis

For the analyses of differentially expressed genes (DEGs) among groups, we used Tag Count Comparison (TCC)-GUI (<https://github.com/swsoyee/TCC-GUI>)²³ with default parameters (number of iterations=3, cut-off FDR=0.1, elimination of potential DEGs=0.05) using edgeR estimation. We undertook these analyses to obtain an overview of the DEGs using the Database for Annotation Visualization and Integrated Discovery available online for gene ontology (GO) analysis and using ReactomePA package in R for Reactome pathway analysis. Additionally, to find the hub genes, we developed a protein-protein interaction (PPI) network using stringApp, an application on Cytoscape (version 3.9.1). Next, a topological analysis on the network was carried out using NetworkAnalyzer, a Cytoscape application. Then the degree centrality was calculated.

Finally, the top 20 genes with the largest degree of centrality were identified as the candidate for hub genes.

2.8 | Gene set enrichment analysis (GSEA)

Gene set enrichment analysis was undertaken to elucidate the molecular mechanisms of the prognostic gene signature. Gene set enrichment analysis was carried out in Java GSEA version 3.0 based on the Molecular Signatures Database c5.all.v2022.1.Hs.symbols.gmt [gene ontology] to identify biological processes, cellular components, molecular functions, and dysregulated oncogenic signatures associated with poor survival of the high-risk group. |normalized enrichment score (NES)| > 1, *p* value < 0.05, and FDR < 0.25 were considered statistically significant.

2.9 | Gene expression profiling interactive analysis

GEPIA (<http://gepia2.cancer-pku.cn>, version 2) is an open-access online tool for the interactive exploration of RNA sequencing data of 9736 tumors and 8587 normal samples from The Cancer Genome Atlas and Genotype-Tissue Expression programs.²⁴ The GEPIA2 was used to compare the expression levels at the mRNA level between the normal and pancreatic cancer tissues. *p* < 0.001 was considered statistically significant. Moreover, the correlation between the CAF markers (FAP/ACTA2/POSTN) and CXCR2/CXCL8 was also analyzed using this tool.

2.10 | Multiplex IHC staining

We established the panel to assess the relationship between immune cell infiltration and proliferative activity. The panel comprised neutrophils (CD15 and CXCR2) and cytotoxic cell marker (CD8). Multiple IHC was carried out as described previously.²⁵ Automatic digital slide scanning of stained tissue was undertaken by fluorescence microscopy (BZ-X800; Keyence) at 200× magnification. Each immune marker signal was then converted into an indicated pseudo-colored image. Finally, multiple-colored images were merged with the BZ-X800 analyzer, in which different pseudo-colors represent different cell types.

2.11 | Staging and surveillance protocol

Preoperative staging was determined by contrast-enhanced computed tomography (CT) of the chest, abdomen, and pelvis, and colonoscopy. Adjuvant chemotherapy was recommended for all patients. The general practice for postoperative surveillance of pancreatic cancer was according to the National Comprehensive Cancer Network guidelines (<https://www.nccn.org/professionals/>

physician_gls/pdf/pancreatic.pdf). Radiographic imaging reports were reviewed, and definitive diagnoses of recurrence were established based on the appearance of new lesions on CT, MRI, and/or PET images and/or biopsy-based histological confirmation of recurrence.

2.12 | Statistical analysis

The primary outcomes were DFS and OS following surgical resection. Patients were monitored from the date of surgery until recurrence, death, or last follow-up. Disease-free survival was estimated using the Kaplan–Meier method. Patients who died or had a recurrence during the study period represented the events in the analysis. Patients who were alive without recurrence at the last follow-up were censored. Each pathological parameter was assessed for the association with DFS using the log-rank test. Hazard ratios (HR) along with 95% confidence intervals (CIs) were estimated using Cox proportional hazards regression modeling. Categorical variables were compared using the χ^2 -test. All statistical analyses were carried out using JMP version 10.1.2 software (SAS Institute Inc.), SAS version 9.4 (SAS Institute Inc.), or R version 3.2.4 (www.R-project.org). All tests were two-sided, and p values < 0.05 were considered significant.

3 | RESULTS

3.1 | Immature stroma and high TINs associated with poor prognosis

First, we examined the data of the SF group. In the multivariate analysis, immature DR (HR 2.20, 95% CI 1.30–4.15, $p = 0.0035$) and high TINs (HR 2.01, 95% CI 1.20–3.37, $p = 0.0085$) were significantly correlated with recurrence risk (Table S4). Regarding OS, in the multivariate analysis, immature DR (HR 2.15, 95% CI 1.12–4.14, $p = 0.0216$), high-grade PDC (HR 3.57, 95% CI 1.32–9.66, $p = 0.0121$), and high TINs (HR 1.97, 95% CI 1.01–3.81, $p = 0.0453$) were significantly correlated with mortality risk (Table S4).

Next, we examined the data of the NAC group. Figure 2 shows Kaplan–Meier curves of DFS and OS based on each pathological factor. Multivariate analysis showed a significantly higher recurrence risk for among those with high TINs (HR 3.07, 95% CI 1.71–5.37, $p = 0.0001$) (Table S4). In terms of OS, multivariate analysis showed a significantly higher mortality risk for patients with high-grade TB (HR 3.22, 95% CI 1.59–6.51 $p = 0.0011$) and high TINs (HR 2.83, 95% CI 1.44–5.58, $p = 0.0026$) (Table S4). Figure S1 shows a comparison of pathological factors examined in this study between the two groups. Tumor budding, PDC, and CLR showed no significant difference between the two groups (Figure S1L–N). Contrarily, the proportion of immature stroma, TINs (CD15), TILs (FOXP3), M1 (CD68), and M2 (CD163) were significantly lower in the NAC group (Figure S1A,B,E,F,K). However, the CD8, CD20, CD4, and CD56 levels were not significantly different between the two groups (Figure S1C,D,I,J).

3.2 | Distinct immune subtypes in PDAC revealed by cluster analysis in NAC group

We undertook a cluster analysis of the data to classify the patterns within the immune microenvironment in the NAC group. Cluster analysis revealed four distinct patterns of immune cell infiltration (Figure 3A). Subtype A comprised patients with predominantly CD56⁺ and CD68⁺ immune cells. Subtype B included patients with predominantly high CD20⁺ and CD155⁺ immune cells. Subtype D comprised patients with high neutrophil infiltration and low immune cell infiltration. The proportion of immature stroma was significantly higher in cases with high TINs (Figure 3B,C). Subtype C included patients with high infiltration with various types of immune cells and significantly increased exhaust markers PD-1 and CTLA4 (Figure S2).

3.3 | High infiltration of CD15⁺ neutrophils within tumor and myxoid stroma at margins is associated with poor prognosis

Among the subgroups, group D2 showed a poor prognosis in both DFS and OS (Figures 3D and S3A). The D2 subtype correlated significantly with lymphatic, vascular, and perineural invasion and immature stroma (Table 1). Therefore, we grouped the patients into three groups according to CD15 and immature stroma status; the group with high CD15 levels and immature stroma had the worst prognosis (Figure S3B).

3.4 | Upregulation of chemokine genes associated with neutrophil migration

DEGs were studied between D2 (four cases) and other groups (group A, four cases; group B, two cases; group C, one case; D1, one case). We extracted 65 DEG candidates significantly upregulated or downregulated by $|\log_2(\text{fold change})| > 1$ in independent samples (Figure 4A, Table S5). The chemokines CXCL1, CXCL6, and CXCL8 are particularly upregulated in the D2 group. The GO and Reactome pathway enrichment analyses were applied to identify the functions of the DEGs (Figure S4A–C). The DEGs were significantly enriched in the biological processes related to neutrophil chemotaxis. Significantly enriched biological processes included inflammatory response and positive angiogenesis regulation. Moreover, the molecular function related to cytokine activity and CXCR chemokine receptor binding was included. The pathways by interleukins including IL1A, IL11, CXCL1, and CXCL8 were activated (Figure S4C). In the PPI network analysis, we identified 20 candidates for the hub genes (MYC, EZH2, UBE2C, CEP55, HIF1A, CCNB1, SOX2, ANLN, EGF, MKI67, RRM2, MELK, CXCL8, BIRC5, CENPF, CX3CR1, CCL2, CXCL1, CDC20, and IL1A) with a higher degree of centrality in the topology analysis (Figure 4B). The GSEA was compared with the D2 group and other immune subgroups.

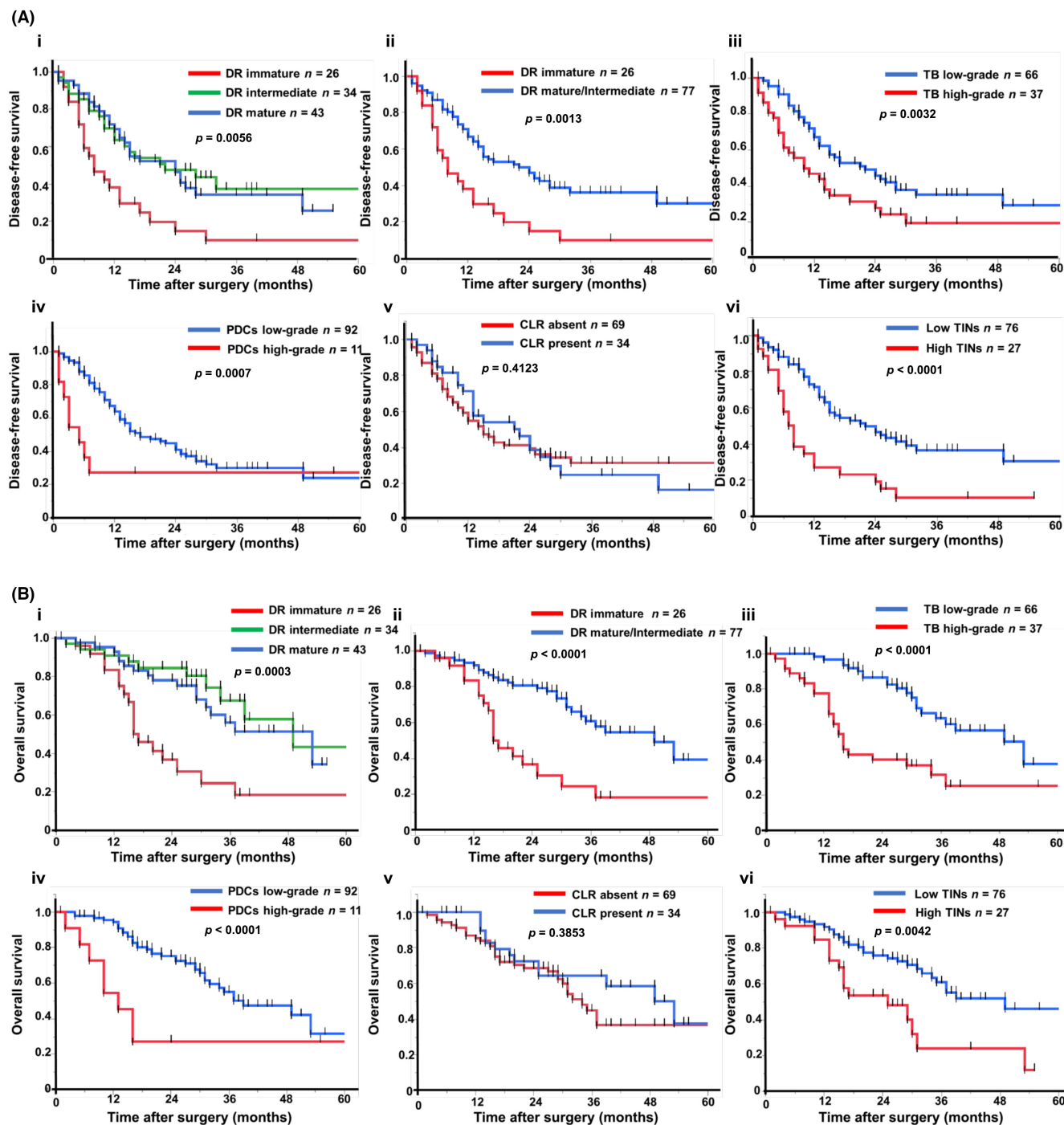
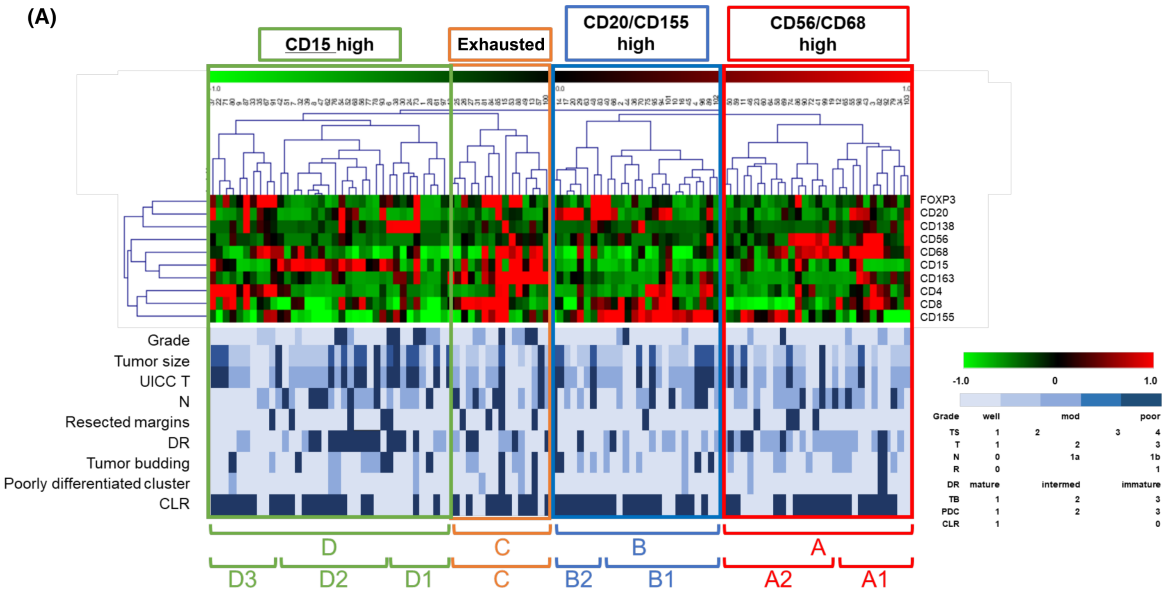


FIGURE 2 Kaplan–Meier curves of (A) disease-free survival and (B) overall survival associated with histological findings (neoadjuvant chemotherapy group) in patients with pancreatic ductal adenocarcinoma. (i, ii) Desmoplastic reaction (DR). (iii) Tumor budding (TB). (iv) Poorly differentiated clusters (PDC). (v) Crohn-like lymphoid reaction (CLR). (vi) Tumor-infiltrating neutrophils (TINs).

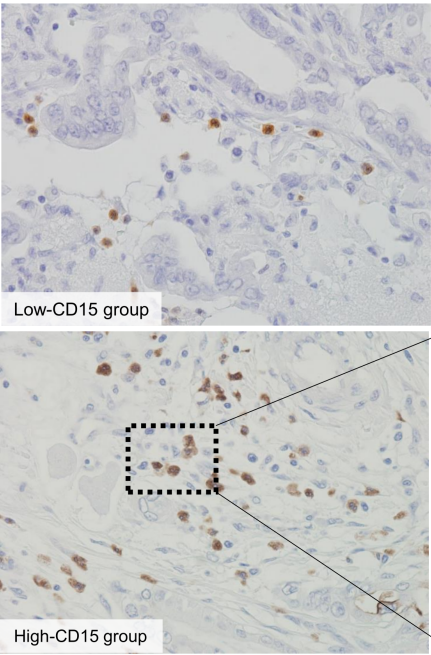
The results of the GSEA are shown in Table S6. The data also showed that the genes were related to cell cycle, tumor growth activity, and neutrophil migration into tissues, and chemokines were significantly upregulated (Figure 4C). We focused on 11 genes

commonly detected in those extracted by both GSEA and PPI network analysis in this study (Figure 4D). Among the 11 genes, *ANLN*, *CCNB1*, *CDC20*, *CENPF*, *CEP55*, *CXCL1*, *CXCL8*, *MKI67*, and *UBE2C* were significantly expressed in pancreatic cancer tissues,

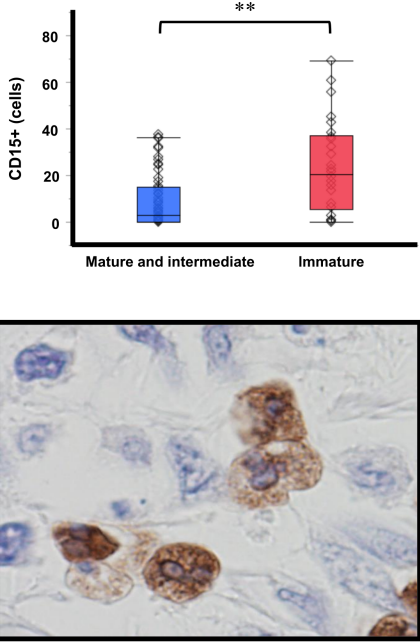
FIGURE 3 Hierarchical cluster analysis of immune cell infiltration in pancreatic ductal adenocarcinoma (PDAC). (A) Heat map results of the cluster analysis ($n = 103$). Histopathological findings are shown in the lower sections. (B) Representation of CD15-high and low groups. (C) Correlation with immature stroma. (D) Kaplan–Meier curves of disease-free survival and overall survival analyses for D2 subtype versus other subtypes. $**p < 0.01$. CLR, Crohn-like lymphoid reaction; DR, desmoplastic reaction.



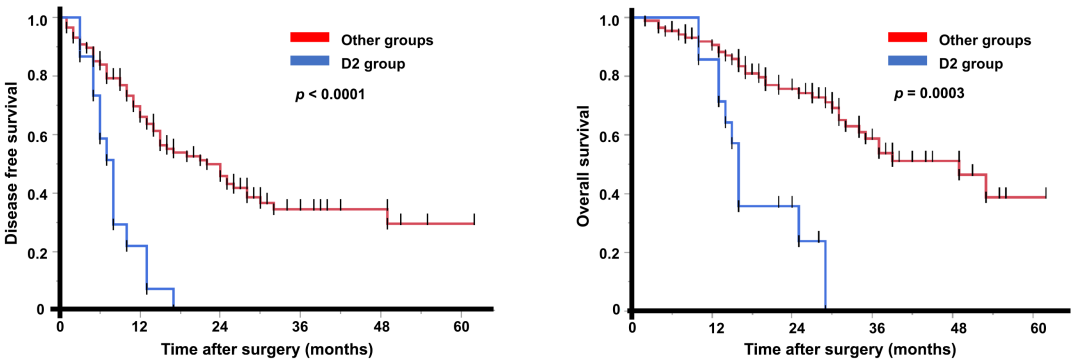
(B)



(C)



(D)



Variable		D2 subgroup	Other subgroups	p value
Tumor size, median (cm)	≤2.8	4 (25.0)	50 (57.5)	0.0168
	>2.8	12 (75.0)	37 (42.5)	
Lymph nodes	Negative	4 (25.0)	37 (42.5)	0.1880
	Positive	12 (75.0)	50 (57.5)	
Resected margin	Negative	13 (81.1)	75 (86.2)	0.6054
	Positive	3 (18.9)	12 (13.8)	
Lymphatic invasion	Absent	8 (50.0)	72 (82.8)	0.0038
	Present	8 (50.0)	15 (17.2)	
Vascular invasion	Absent	4 (25.0)	57 (65.5)	0.0024
	Present	12 (75.0)	30 (34.5)	
Perineural invasion	Absent	0 (0.0)	22 (25.3)	0.0233
	Present	16 (100.0)	65 (74.7)	
Desmoplastic reaction	Mature/intermediate	3 (18.8)	74 (85.1)	<0.0001
	Immature	13 (81.2)	13 (14.9)	
Tumor budding	Grade 1	9 (56.3)	57 (65.5)	0.4776
	Grade 2/3	7 (43.7)	30 (34.5)	
Poorly differentiated clusters	Grade 1	14 (87.5)	78 (89.7)	0.7975
	Grade 2/3	2 (12.5)	9 (10.0.3)	
Crohn-like lymphoid reaction	Absent	9 (56.3)	60 (69.0)	0.3202
	Present	7 (43.7)	27 (31.0)	

Note: Data are shown as n (%). $p < 0.05$ is statistically significant.

TABLE 1 D2 subtype and tumor characteristics in relation to histological findings in patients with pancreatic cancer.

as compared to the normal tissues, according to the published GEPIA2 data (Figure S5A).

3.5 | High infiltration of CXCR2⁺ CD15⁺ neutrophils reduced CD8 infiltration

In the D2 group, we observed neutrophils with high expression of CXCR2, which is a receptor for CXCL1 and CXCL8 (Figure S5B). High expression of CXCR2 is related to a poor prognosis (Figure 5A). Moreover, in the same fields, the D2 group showed less CD8 infiltration, and the CXCR2⁺CD15⁺ cell/CD8 cell ratio was significantly higher in the D2 group (Figure 5B,C).

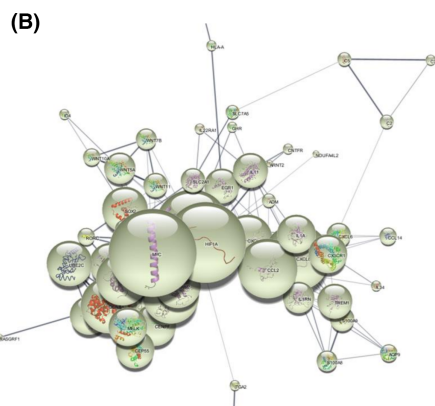
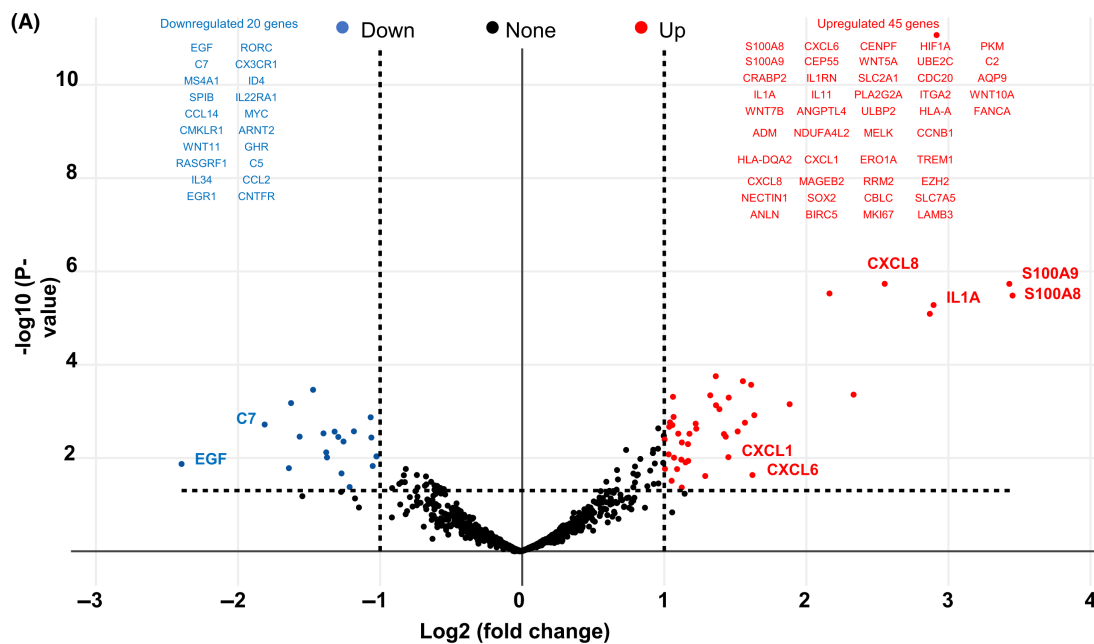
4 | DISCUSSION

In this study, we undertook histological investigations, including previously reported prognostic factors, and attempted to cluster pancreatic cancer patients in the preoperative chemotherapy

group based on their immunological microenvironmental factors. The group with a high infiltration of CD15⁺ neutrophils at the tumor center and with the least infiltration of other immune cells had the worst prognosis. This patient group was associated with the presence of immature stroma at the tumor margins.

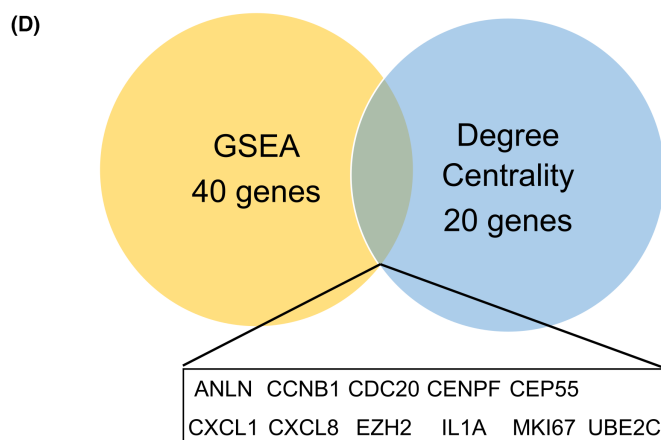
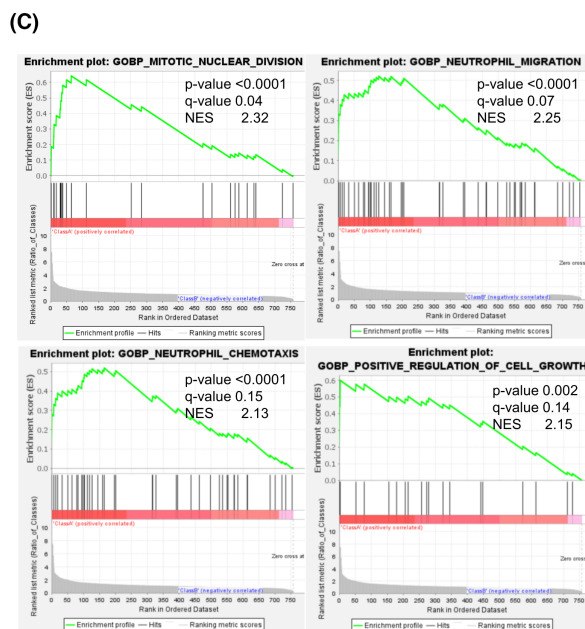
Clustering analysis based on various immune cell infiltrates in the NAC group revealed four major groups, with each group having distinct characteristics. Subtype D, characterized by a high neutrophil count and reduced infiltration of other immune cells, particularly in the D2 subgroup, had the worst prognosis in the present study. Subtype C showed high infiltration of various immune cells and significantly higher levels of PD-1 and CTLA4, which are markers of immune cell exhaustion (Figure S2). This suggests that immune cell exhaustion and self-tolerance are likely to progress in the C group,²⁶ and targeting PD-1 or CTLA4 could potentially reactivate T cell cytotoxicity and have a tumor-reducing effect. Subtypes A and B exhibited predominant expression of M1 macrophages (CD68), DCs (CD155), B cells (CD20) with antigen-presenting function, and NK cells (CD56) with direct cytotoxicity. This indicates that the A+B group had more activated antitumor immunity as compared

FIGURE 4 Identification of differentially expressed genes in the D2 subgroup of patients with pancreatic ductal adenocarcinoma. (A) Red and blue colors represent upregulated and downregulated genes, respectively. Black represents genes with no significant difference. (B) Protein–protein interaction (PPI) network analysis and identification of hub genes. (C) Oncological signatures significantly enriched in the D2 subgroup identified through a gene set enrichment analysis (GSEA) ($p < 0.05$ and $q < 0.25$). (D) Venn diagram of genes detected by GSEA and PPI network analysis. NES, normalized enrichment score.

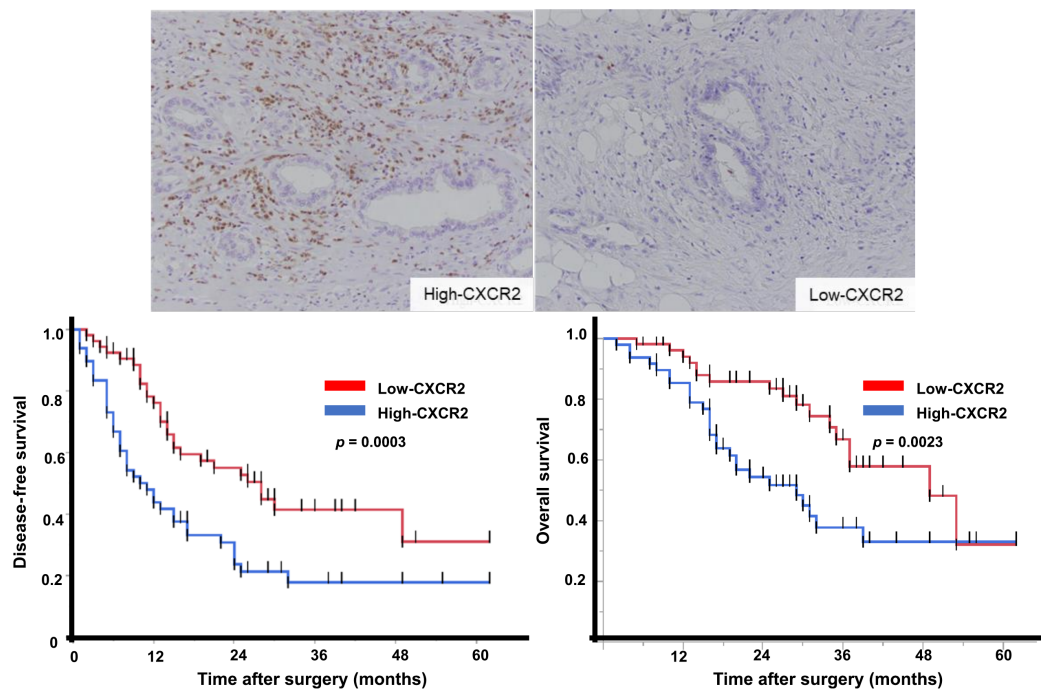


Degree Centrality top 20

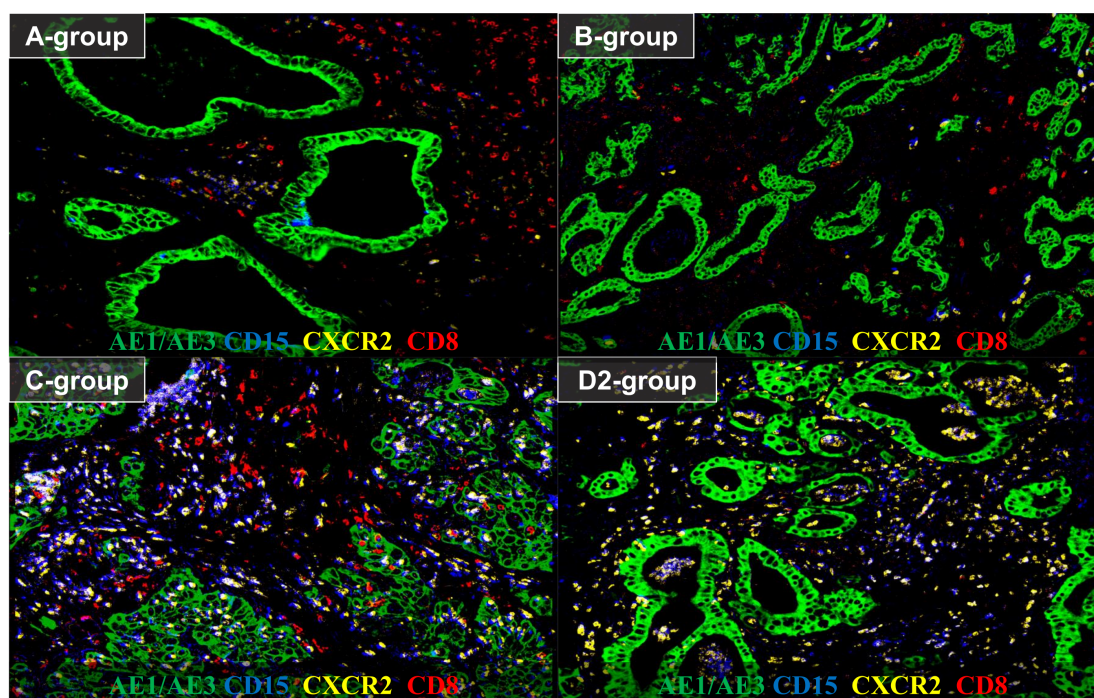
MYC	EZH2	UBE2C	CEP55
HIF1A	CCNB1	SOX2	ANLN
EGF	MKI67	RRM2	MELK
CXCL8	BIRC5	CENPF	CX3CR1
CCL2	CXCL1	CDC20	IL1A



(A)



(B)



(C)

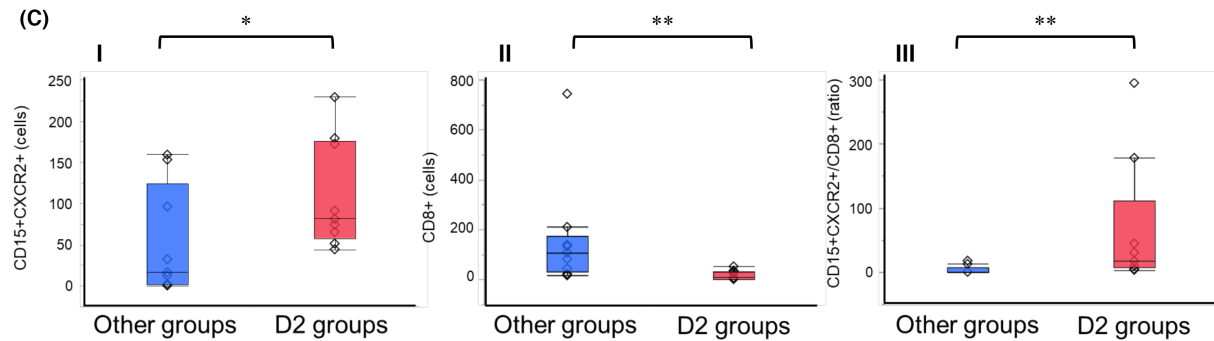


FIGURE 5 Immunohistochemical and multiple immunostaining analyses in samples from patients with pancreatic ductal adenocarcinoma. (A) Immunohistochemical results for CXCR2 and Kaplan–Meier curves of disease-free survival and overall survival analyses. (B) Multiple immunostaining in each group (A, B, C, and D2 groups) (green, AE1/AE3; blue, CD15; yellow, CXCR2; red, CD8). (C) Numbers of each cell (CD15⁺CXCR2⁺, CD8) and ratio (CD15⁺CXCR2⁺/CD8). D2 (*n* = 9) versus other groups (*n* = 9): (i) CD15⁺CXCR2⁺, (ii) CD8, and (iii) CD15⁺CXCR2⁺/CD8 ratio. **p* < 0.05, ***p* < 0.01.

to the C+D group. Importantly, the A+B group had a better prognosis than the C+D group (Figure S6). Further accumulation of cases in the future could lead to the development of personalized immunotherapies.

The subgroup with a high infiltration of CD15⁺ neutrophils was associated with the worst prognosis. Neutrophils play a crucial role in cancer proliferation, metastasis, antitumor immunity suppression, and ECM reconstitution.²⁷ Although neutrophils have been reported as a poor prognostic factor in various cancers, disagreements regarding their prognostic impact exist.^{28–30} CD15, commonly used as a neutrophil marker, is a marker for G-MDSCs,^{31,32} which are a heterogeneous population of bone marrow-derived precursor cells or immature myeloid cells that possess immunosuppressive functions and are involved in tumor progression and metastasis.³³ This study suggests that CD15⁺ neutrophils might reflect G-MDSCs. There are ongoing reports on anticancer agents that directly target MDSCs, showing effectiveness in reducing their population.^{34–36} Specifically, 5-FU selectively reduces tumor-associated MDSCs, leading to tumor control.³⁴ Chemotherapies, such as those including 5-FU, may contribute to improved prognosis in this subgroup.

The group of patients with high infiltration of CD15⁺ neutrophils showed significantly upregulated expression of chemokines CXCL8 and CXCL1 in the mRNA analysis of pancreatic cancer tissue. CXCL1 and CXCL8 are known to be involved in MDSC induction, which suppresses CD8⁺ T cells, DCs, and NK cells through the CXCL1/CXCL8–CXCR2 axis while enhancing their function.³⁷ CXCR2, the receptor for CXCL1 and CXCL8, is expressed on cancer cells, neutrophils, and MDSCs, promoting tumor growth, invasion, and metastasis.³⁸ In the present study, CXCR2 was upregulated in CD15⁺ neutrophils but rarely in cancer cells, suggesting that CXCL1 and CXCL8 may induce CD15⁺ neutrophils through CXCR2. The group of patients with high infiltration of CD15⁺CXCR2⁺ cells also showed reduced infiltration of CD8⁺ T cells into the tumor, which could be an additional factor indicating a poor prognosis. Recently, CXCR2-targeted therapies have been investigated, showing promise in suppressing neutrophil migration, increasing effector T cells, and enhancing antitumor immunity and chemotherapeutic response in a mouse model of PDAC.^{38–40} A phase I/II clinical trial evaluating CXCL8 inhibitors in combination with nivolumab or other agents (NCT03400332) is currently underway. Depending on the results, CXCR2 or CXCL8 inhibitors, in combination with conventional therapies, could improve the prognosis of high-risk patients.

In this multivariate analysis of histological prognostic factors in the SF group, the immature stroma was identified as an independent predictor of DFS and OS. However, no significant prognostic

correlation was found in the NAC group. Previous reports have evaluated the tumor/interstitial ratio in PDAC, but no study has undertaken qualitative assessments.⁴¹ Furthermore, GEM+nabPTX, especially nab-paclitaxel, can induce qualitative and quantitative changes in the stroma, and EUS elastography has revealed a decrease in stiffness postchemotherapy.⁴² The percentage of myxoid stroma was significantly lower in the NAC group than in the SF group, suggesting that preoperative chemotherapy might have affected the stromal characteristics.

This study also revealed a correlation between myxoid stroma at the cancer margins and high infiltration of CD15⁺ neutrophils. We further investigated the correlation between chemokines (IL1a, CXCL8, and CXCL1), which were significantly upregulated in the D2 subgroup, and CAF markers (FAP/ACTA2/TDPN/POSTN).²⁴ POSTN and CXCL8 (*p* 1.2e-08 *R*=0.41) showed a significant positive correlation (Figure S7). POSTN also activates the nuclear factor- κ B pathway, induces CXCL8 and other factors, promotes cancer stem cell self-renewal, and enhances metastasis.⁴³ POSTN mRNA and protein expression levels have also been reported to be significantly higher in the immature stroma of colorectal cancer.⁴⁴ This interaction between chemokines and CAFs could contribute to the poor prognosis observed in the D2 subgroup. Further research is needed to elucidate the interrelationship between CAFs and immunological factors.

This study has several limitations. First, the number of EUS-guided fine-needle aspiration specimens was insufficient to allow comparisons within the same patients. Second, this study was limited to cases treated with GEM+nabPTX only, excluding patients treated with FOLFIRINOX, chemoradiotherapy, or GEM + TS-1. Further studies are required to examine the effect of each preoperative chemotherapy regimen on the pancreatic cancer microenvironment. Third, the qualitative changes caused by preoperative chemotherapy and the relationship between the immune microenvironment and CAFs were not adequately investigated in the present study. Therefore, further studies are required to clarify these issues. Finally, some of our cases had decreased cancer cell density due to chemotherapy. As a result, the number of samples available was insufficient to ensure adequate quality in the analysis of mRNA expression levels, leading to a bias in the submitted sample group. Further studies with larger populations are needed in the future to validate our findings.

In conclusion, to the best of our knowledge, this is the first study to identify the subtypes in pancreatic cancer after preoperative chemotherapy by clustering based on various immune cell infiltrations. Our study results showed a correlation with prognosis in each group, suggesting a new therapeutic strategy. Patients with immature stroma at the margins and high infiltration of CD15⁺ neutrophils

within the tumor could particularly benefit from treatment with CXCR2 or CXCL8 inhibitors.

AUTHOR CONTRIBUTIONS

Yoshinao Oda: Conceptualization; project administration; resources; supervision; writing – review and editing. **Yutaka Yamada:** Conceptualization; data curation; formal analysis; investigation; methodology; resources; validation; visualization; writing – original draft. **Takeo Yamamoto:** Conceptualization; investigation; project administration; resources; supervision; writing – review and editing. **Chikanori Tsutsumi:** Methodology; resources. **Takashi Matsumoto:** Resources. **Shoko Noguchi:** Resources. **Yuki Shimada:** Resources. **Kohei Nakata:** Resources. **Kenoki Ohuchida:** Resources. **Masafumi Nakamura:** Resources; supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

Yoshinao Oda is an editorial board member of *Cancer Science*. The other authors declare no conflict of interest.

ETHICS STATEMENT

Approval of the research protocol by an institutional review board: This study was approved by the Ethics Committee of Kyushu University (IRB: 2020–633).

Informed consent: N/A.

Registry and the registration no. of the study/trial: N/A.

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SUPPORTING INFORMATION

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