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Pathology

Neoadjuvant Therapy-Induced Remodeling in Pancreatic Ductal Adenocarcinoma: Multimodal Spatial Analysis and Prognosis

Xiaofei Zhang¹  | Ruoxin Lan² | Danting Li² | Yongjun Liu³ | Sonu Kalyan¹ | Momin Iqbal¹ | Nancy Liu⁴ | Jerry Zhang⁵ | Iman Hanna¹ | Mala Gupta¹ | Chaohui L. Zhao¹ | Weiguo Liu¹ | Jonathan Melamed¹ | Michael Shusterman⁶ | Jessica Widmer⁷ | John Allendorf⁸ | Yao-Zhong Liu²

¹Department of Pathology, New York University Grossman Long Island School of Medicine, Long Island, New York, USA | ²Department of Biostatistics and Data Science, Tulane University School of Public Health and Tropical Medicine, New Orleans, Louisiana, USA | ³Department of Laboratory Medicine and Pathology, University of Washington School of Medicine, Seattle, Washington, USA | ⁴Department of Chemistry, University of Washington School of Medicine, Seattle, Washington, USA | ⁵Skyline High School, Sammamish, Washington, USA | ⁶Department of Oncology, New York University Grossman Long Island School of Medicine, Long Island, New York, USA | ⁷Department of Gastroenterology, New York University Grossman Long Island School of Medicine, Long Island, New York, USA | ⁸Department of Surgery, New York University Grossman Long Island School of Medicine, Long Island, New York, USA

Correspondence: Xiaofei Zhang (xiaofei.zhang@nyulangone.org)

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ABSTRACT

Neoadjuvant therapy (NAT) is increasingly used for pancreatic ductal adenocarcinoma (PDAC); yet most patients only achieve partial response. Pathological treatment response grading focuses on assessing residual tumor burden, often overlooking changes in tumor microenvironment (TME). To address this gap, we compared tumor cells and TME of 13 NAT-naïve and 23 post-NAT PDACs using integrated spatial pathomics and transcriptomics, with validation in an independent single-cell spatial dataset. NAT significantly reduced tumor burden (14.7%–6.2%, $p=0.004$), but systemic comparison of 13 cytomorphometric features of tumor cells alone did not reliably distinguish between naïve and NAT cases. In contrast, NAT profoundly remodeled TME by increasing cancer-associated fibroblast (CAF) and CD8⁺ T cell densities, promoting CD8⁺ T cell-tumor cell proximity and fibrosis, reducing tumor-associated neutrophils, and redistributing tertiary lymphoid structures (TLSs). Spatial transcriptomics shows NAT induced apoptosis, DNA-damage response, and AGC-kinase (S_TK_X) signaling in tumor cells, and upregulated complement pathway, p53 signaling, and cellular senescence program in TME. Cross-platform single-cell spatial analysis revealed decreased regulatory T cells (Treg) and a shift from myofibroblastic (mCAF) to inflammatory CAF (iCAF). Importantly, post-NAT patients with more fibrosis had longer overall survival ($p=0.02$), and higher B-cell density showed a favorable trend ($p=0.06$). Together, these results suggest that beyond tumor debulking, NAT induces a coordinated TME remodeling characterized by fibroblast reprogramming, matrix fibrosis, and immune spatial reorganization. Incorporating assessment of NAT-induced stromal and immune changes into TRG may improve prognostication and guide more precise therapy in post-NAT PDAC.

Abbreviations: CAF, cancer-associated fibroblast; CPA, collagen proportion area; DDR, DNA damage response; EC, enrichment coefficient; FDR, false discovery rate; HR, hazard ratio; NAT, neoadjuvant therapy; PDAC, pancreatic ductal adenocarcinoma; TAN, tumor associated neutrophil; TLS, tertiary lymphoid structure; TME, tumor microenvironment; TPA, tumor proportion area; Treg, regulatory T cell; TRG, tumor regression grading.

Xiaofei Zhang and Ruoxin Lan contributed equally to this study.

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1 | Introduction

Pancreatic ductal adenocarcinoma (PDAC) has a 5-year survival rate of only 13% [1, 2]. This poor prognosis is mostly driven by its late diagnosis, early metastasis, aggressive biology, therapeutic resistance, and immunosuppressive tumor microenvironment (TME) [3–6]. While surgical resection remains the only potentially curative option, fewer than 20% of patients are eligible at diagnosis. Therefore, neoadjuvant therapy (NAT), including chemotherapy or chemoradiation administered prior to surgery, is increasingly becoming the standard of care for borderline resectable or high-risk resectable PDAC [7, 8]. It is also increasingly being used in some locally advanced cases. Studies have shown that NAT can improve rates of margin-negative (R0) resections, eradicate early micrometastases, and enable a more informed patient selection for surgery. Furthermore, recent evidence has also shown that NAT may exert an immune priming effect on TME [9–11]. For example, NAT can increase stromal fibrosis [12]; decrease stromal CD4⁺ T cells, CD20⁺ B cells, and FoxP3⁺ T cells [13]; increase CD8⁺ T cells and decrease T regulatory cells (Tregs) as well as M2 macrophages [14]. Furthermore, NAT can also up-regulate genes involved in antigen presentation [15]. Our recent spatial transcriptomics study showed that NAT can induce orchestrated upregulation of complement cascade genes (C3, C1R, C1S, C4B, C7) within CAFs, which is associated with prolonged overall survival [16, 17].

As NAT becomes more commonly used in PDAC treatment, pathologists play an increasingly important role in evaluating treatment response in post-NAT specimens [18]. Pathologic tumor regression grading (TRG) provides essential prognostic information that complements traditional TNM staging [19]. It also guides postoperative clinical decision making by identifying poorly responded patients who may benefit from alternative adjuvant therapy, and those with excellent responses who may avoid overtreatment. Multiple pathologic TRG systems have been proposed, including the AJCC/CAP [20], MD Anderson [21], Evans [22], ART (Area of Residual Tumor) [23], JPS (Japanese Pancreas Society) [24], Hartman [25], Royal North Shore [26], and Integrated Pathology Score [27]. However, these systems largely rely on semiquantitative microscopic assessments that are prone to inter-observer variability [28–30]. Moreover, they do not evaluate changes in the TME, which constitute up to 70%–80% of the PDAC tumor mass and contain many components playing pivotal roles in tumor progression, therapeutic resistance, and immunomodulation, such as cancer-associated fibroblasts (CAFs), extracellular matrix, and immune cells, etc. [31]. To address this gap, we aim to comprehensively quantitate how NAT alters both tumor and the TME compartments using a multimodal approach, and to examine how these spatial and molecular changes correlate with prognosis. Our eventual goal is to develop a more holistic treatment response evaluation framework that integrates traditional TRG with spatial, stromal and immunological features to improve prognostic accuracy and clinical utility.

2 | Materials and Methods

2.1 | Patient Cohort and Data Collection

This study was approved by the Institutional Review Boards at University of Wisconsin—Madison and New York University

Grossman Long Island School of Medicine. Our study cohort contains 13 upfront resections without NAT and 23 resections after NAT, including 17 cases that received FOLFIRINOX (six of which were with radiation) and six cases that received Gemcitabine/Abraxane (three of which were with radiation) (Table 1).

2.2 | Histology and Immunohistochemistry (IHC)

Serial 4μm-sections were cut from the most representative Formalin-Fixed Paraffin Embedded (FFPE) block for each case and used for H&E, immunohistochemical (IHC), and spatial transcriptomics. Primary antibodies used include rabbit monoclonal antibodies against CD8 (SP57), CD20 (L26), and CD21 (EP3093) (Ventana). Detection was performed using HRP-conjugated secondary antibodies with Teal HRP Kit, Purple Kit, and ChromoMap DAB Kit. Slides were scanned at 40× using the Aperio Scanner (Leica Biosystems).

2.3 | Digital Pathology and Spatial Analysis

Digital pathology analysis was performed using QuPath (version 0.5.1). A 2.5×2.5 mm² region of interest (ROI) was selected

TABLE 1 | Clinicopathologic comparison of naïve and NAT groups.

	Naïve (13)	NAT (23)	<i>p</i>
Median age (mean, range)	65 (49–86)	67 (50–79)	0.44
Age group (years), <i>n</i> (%)			
≤ 65	7 (53.8)	10 (43.5)	0.73
> 65	6 (46.2)	13 (56.5)	
Gender, <i>n</i> (%)			
Male	8 (61.5)	8 (34.8)	0.17
Female	5 (38.5)	15 (65.2)	
Cancer grade, <i>n</i> (%)			
G1	3 (23.1)	4 (17.4)	0.46
G2	8 (61.5)	11 (47.8)	
G3	2 (15.4)	8 (34.8)	
Tumor size (range)			
≤ 2.0 (T1)	2 (15.4)	5 (21.8)	0.73
2.1–4.0 (T2)	8 (61.5)	11 (47.8)	
> 4.0 (T4)	3 (23.1)	7 (30.4)	
(y) pN, <i>n</i> (%)			
0 (N0)	3 (23.1)	11 (47.8)	0.19
1–3 (N1)	6 (46.1)	10 (43.5)	
≥ 4 (N2)	4 (30.8)	2 (8.7)	
(y) TNM stage, <i>n</i> (%)			
I and II	10 (76.9)	13 (56.5)	0.30
III and IV	3 (23.1)	10 (43.5)	

from the most representative area inside each tumor, avoiding necrosis, crush artifact, or tissue edges. Cell detection was performed using QuPath's built-in algorithm. A minimum of 500 cells containing all cell types were annotated based on the consensus of two pancreaticobiliary pathologists (X.Z. and Y.L.) to train an Artificial Neural Network—Multilayer Perceptron (ANN_MLP) based cell classifier for each case, which was iteratively refined through multiple rounds of post-training to reach at least >90% PPV and NPV. Then the classification output was exported for spatial analyses. Trichrome-stained images were analyzed using QuPath's pixel classifier module. A customized pixel classifier was trained and validated for each image. After reaching at least >90% PPV and NPV, it was used to quantify collagen proportionate area (CPA), the percentage of areas occupied by collagen fibers. All spatial analyses were performed in R (v.4.3.1) using the spatstat package (v.2.3-4).

2.4 | Spatial Transcriptomics Using Nanostring GeoMx DSP

The 4- μ m unstained sections were hybridized to UV-photocleavable barcode-conjugated RNA in situ hybridization probe set (Nanostring Cancer Transcriptome Atlas) and stained with immunofluorescence morphology markers set (1:10 SYTO13, 1:20 anti-panCK-Alexa Fluor 532, and 1:100 anti- α SMA-Alexa Fluor 647). For each slide, 2–5 circle ROIs were selected and segmented into the tumor AOI (Area Of Illumination, pan-CK+, SMA–) and the TME AOI (pan-CK–,

SMA+). Photocleavage of spatial barcode and sample collection were done on GeoMx Digital Spatial Profiler (DSP) to prepare RNA Libraries. Next Generation Sequencing (NGS) read-out was done on Illumina NextSeq 550AR sequencer, exported as digital count conversion (DCC) files and analyzed using R and Bioconductor packages. Linear modeling analysis was conducted in R to detect differentially expressed genes (DEGs) using Benjamini–Hochberg FDR controlled at 0.05, which were used for DAVID (The Database for Annotation, Visualization, and Integrated Discovery) gene set enrichment analysis (<https://david.ncifcrf.gov/home.jsp>) [32, 33].

2.5 | Single-Cell Spatial Transcriptomics

The Spatial Molecular Imaging (SMI) data of an independent single-cell spatial transcriptomics dataset [34] were obtained from Mendeley Data (<https://doi.org/10.17632/kx6b69n3cb.1>) and Zenodo (<https://doi.org/10.5281/zenodo.7963531>), which included 7 NAT-naïve and 6 post-NAT patients. A median of 20 fields of view (FOVs, each measuring 984.96 $\times$ 662.04 μ m²) was analyzed for each case. The cell-type calling made by this published study was used for our spatial analysis as described above [34].

2.6 | Other Statistical Analyses

Overall survival was analyzed using Cox proportional hazards model and Kaplan–Meier/log-rank analyses within naïve and

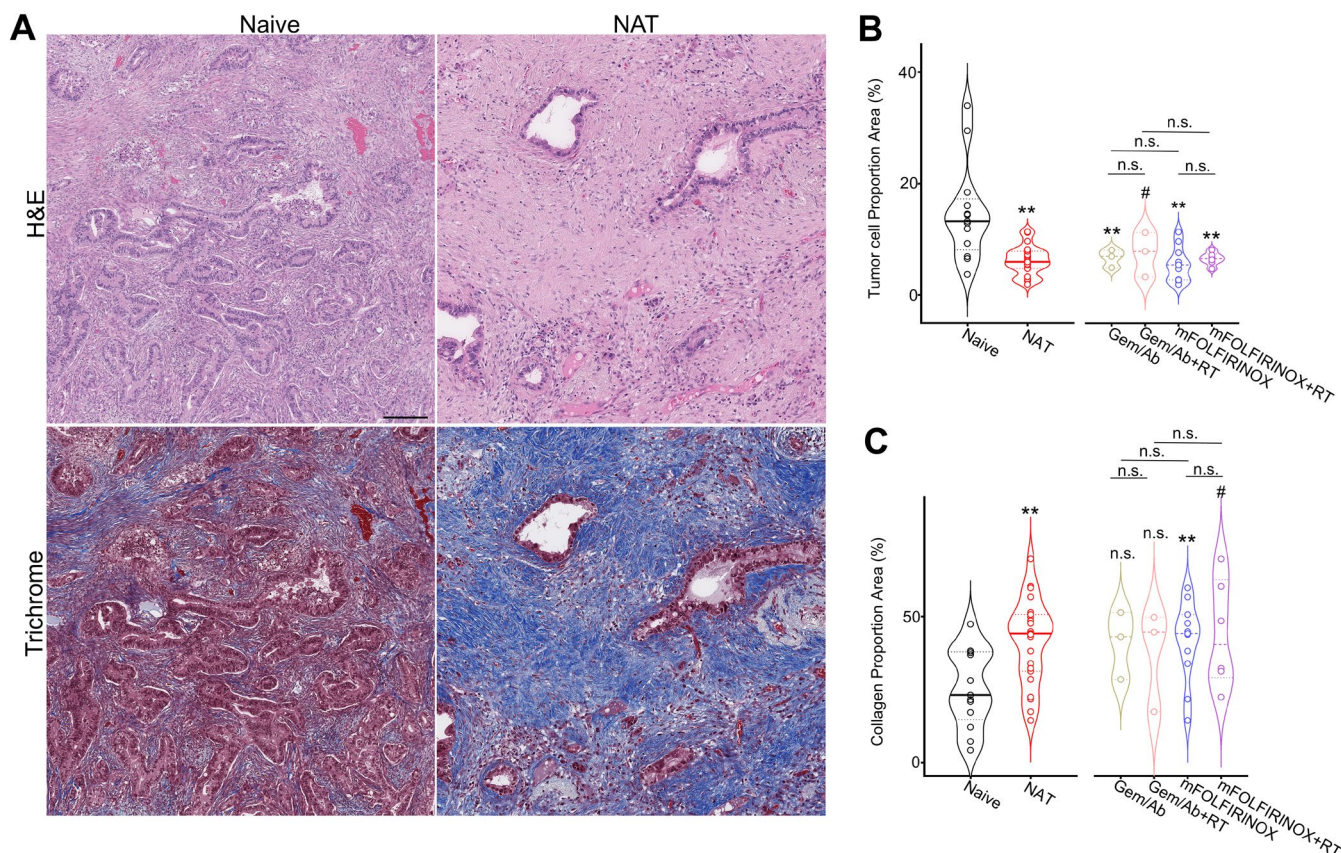


FIGURE 1 | NAT significantly reduces tumor burden and increases stromal fibrosis. (A) H&E (top) and Masson's trichrome (bottom) images from representative naïve and NAT cases. (20 $\times$ magnification, scale bar: 200 μ m). (B, C) Violin plots show tumor cell proportionate area (B) and collagen proportionate area (C) in naïve versus NAT groups (left), and among different NAT regimens (right). (**, $p < 0.01$; #, $p < 0.1$; n.s., not significant).

NAT groups. Discrimination was assessed by Harrell's C-index (95% CI) and time-dependent AUC at 365 and 730 days. Group comparisons used Wilcoxon rank-sum and Fisher's exact tests with FDR correction; analyses were conducted in R and GraphPad Prism. See Methods S1 for a detailed description of the patient cohort and the methodologies used for digital pathology, spatial transcriptomics, and survival analyses.

3 | Result

3.1 | NAT Induces Coordinated Tumor Regression and TME Remodeling in PDAC

We first measured key cellular and extracellular components in both tumor and TME. NAT led to a ~60% reduction in tumor cell density (890.6 ± 147.6 to 362.8 ± 37.1 cell/mm², $p < 0.0001$) and a corresponding decrease in tumor proportionate area (TPA)

($14.7\% \pm 2.4\%$ to $6.1\% \pm 0.6\%$, $p < 0.01$) with no significant differences among different NAT regimens (Figure 1A,B). We did not observe any significant differences in a comprehensive set of tumor-cell cytomorphometric metrics between naïve versus NAT groups (Table 2). In contrast, NAT significantly increased stromal cell density (2078.2 ± 143.5 vs. 1463.9 ± 149.1 cell/mm², $p < 0.05$) and decreased tumor-to-stroma cell ratio (Table 2). Trichrome-based fibrosis quantification revealed that NAT significantly increased collagen proportion area (CPA, $45.8\% \pm 3.3\%$ vs. $28.2\% \pm 4.1\%$, $p < 0.01$), which was also independent of NAT regimens (Figure 1A,C).

3.2 | NAT Increases Stromal CD8⁺ T-Cell Density and Spatial Proximity to Tumor Cells

NAT significantly increased the density of CD8⁺ T cells in the TME (318.6 ± 49.8 vs. 127.4 ± 30.4 cells/mm², $p < 0.01$). The B cell

TABLE 2 | Comparison of cytomorphometric metrics between naïve and NAT groups.

	Naïve (13)	NAT (23)	<i>p</i> (<i>q</i>)
Tumor segment			
Tumor cell density (/mm ²)	890.6 ± 147.6	362.8 ± 37.1***	0.0001 (0.0006)
Nuclear features			
Area	53.7 ± 2.1	60.0 ± 2.8	0.121 (0.31)
Perimeter	29.8 ± 0.6	30.9 ± 0.8	0.348 (0.57)
Aspect ratio	1.64 ± 0.02	1.60 ± 0.02	0.168 (0.37)
Circularity	0.74 ± 0.01	0.76 ± 0.01	0.053 (0.29)
Complexity ratio	1.17 ± 0.004	1.16 ± 0.006	0.073 (0.29)
Eccentricity	0.74 ± 0.006	0.73 ± 0.005	0.196 (0.37)
Hematoxylin OD mean	0.63 ± 0.03	0.55 ± 0.03	0.077 (0.29)
Hematoxylin OD SD	0.16 ± 0.009	0.13 ± 0.007	0.055 (0.29)
Cellular features			
Area	169.7 ± 5.3	179.4 ± 8.4	0.414 (0.74)
Perimeter	50.0 ± 0.8	50.9 ± 1.1	0.694 (0.79)
Aspect ratio	1.49 ± 0.01	1.48 ± 0.01	0.730 (0.79)
Complexity ratio	1.11 ± 0.002	1.11 ± 0.003	0.677 (0.79)
Eccentricity	0.69 ± 0.005	0.69 ± 0.005	0.997 (1.00)
N/C	0.32 ± 0.005	0.33 ± 0.008	0.109 (0.31)
Stroma segment			
Stroma cell density	1463.9 ± 149.1	2078.2 ± 143.5**	0.008 (0.05)
T/S ratio	0.66 ± 0.12	0.20 ± 0.03***	0.0001 (0.0006)
CD8 density	127.4 ± 30.4	318.6 ± 49.8**	0.009 (0.001)
CD20 density	10.6 ± 3.7	16.3 ± 4.6	0.391 (0.30)
Neutrophil density	22.2 ± 3.4	11.8 ± 2.1**	0.010 (0.01)
Collagen proportion area	28.2 ± 4.1	45.8 ± 3.3*	0.010 (0.03)

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$.

density in the TME was not significantly increased (16.3 ± 4.6 vs. 10.6 ± 3.7 cells/mm², $p=0.39$, Table 2). Spatial analysis revealed tumor cells in the NAT group had a ~2.3-fold higher likelihood in direct contact (connected by Delaunay edge) with at least one CD8⁺ T cell ($24.4 \pm 3.3\%$ vs. $10.6 \pm 1.9\%$, $p=0.004$, Figure 2A–C). However, functional cytotoxicity by CD8⁺ T cells on tumor cells requires their spatial proximity. Our cytomorphometry measurement showed the average radii of PDAC cancer cells and CD8⁺ T cells are ~10 and ~5 μm respectively. Therefore, 20 μm is a biologically plausible range for functional cytotoxic interaction. Within this range, tumor cells in the NAT group had ~2.5-fold higher likelihood in direct contact with at least one CD8⁺ T cell than the naïve group ($18.7 \pm 2.8\%$ vs. $7.5 \pm 1.5\%$, $p=0.005$) (Figure 2A–C).

We also studied NAT's impact on tertiary lymphoid structure (TLS). Lymphoid aggregates in TME were categorized into three stages [7, 35–37]. Lymphoid aggregates (LA) are defined

as ≥ 50 spatially organized T and B cells without CD21⁺ follicular dendritic cells. Primary follicle-like TLSs (pfl-TLS) are LAs with CD21⁺ dendritic cell networks but no germinal center (GC). Secondary follicle-like TLSs (sfl-TLS) contain GC recognizable on H&E (confirmed by CD21⁺CD23⁺ staining) (Figure 3A). In the peritumoral region, NAT led to a significant reduction of all three stages of TLS. In the intratumoral region, NAT led to a trend of increased LA (0.006 ± 0.009 vs. 0.016 ± 0.020 /mm², $p=0.077$) and sfl-TLS (0.0 ± 0.0 vs. 0.002 ± 0.006 /mm², $p=0.066$), while pfl-TLS remained not significantly changed (Figure 3B,C).

3.3 | Single-Cell Spatial Transcriptomics Characterization of NAT-Induced TME Remodeling

We then analyzed an independent single-cell spatial transcriptomics dataset [34] to validate our findings. NAT

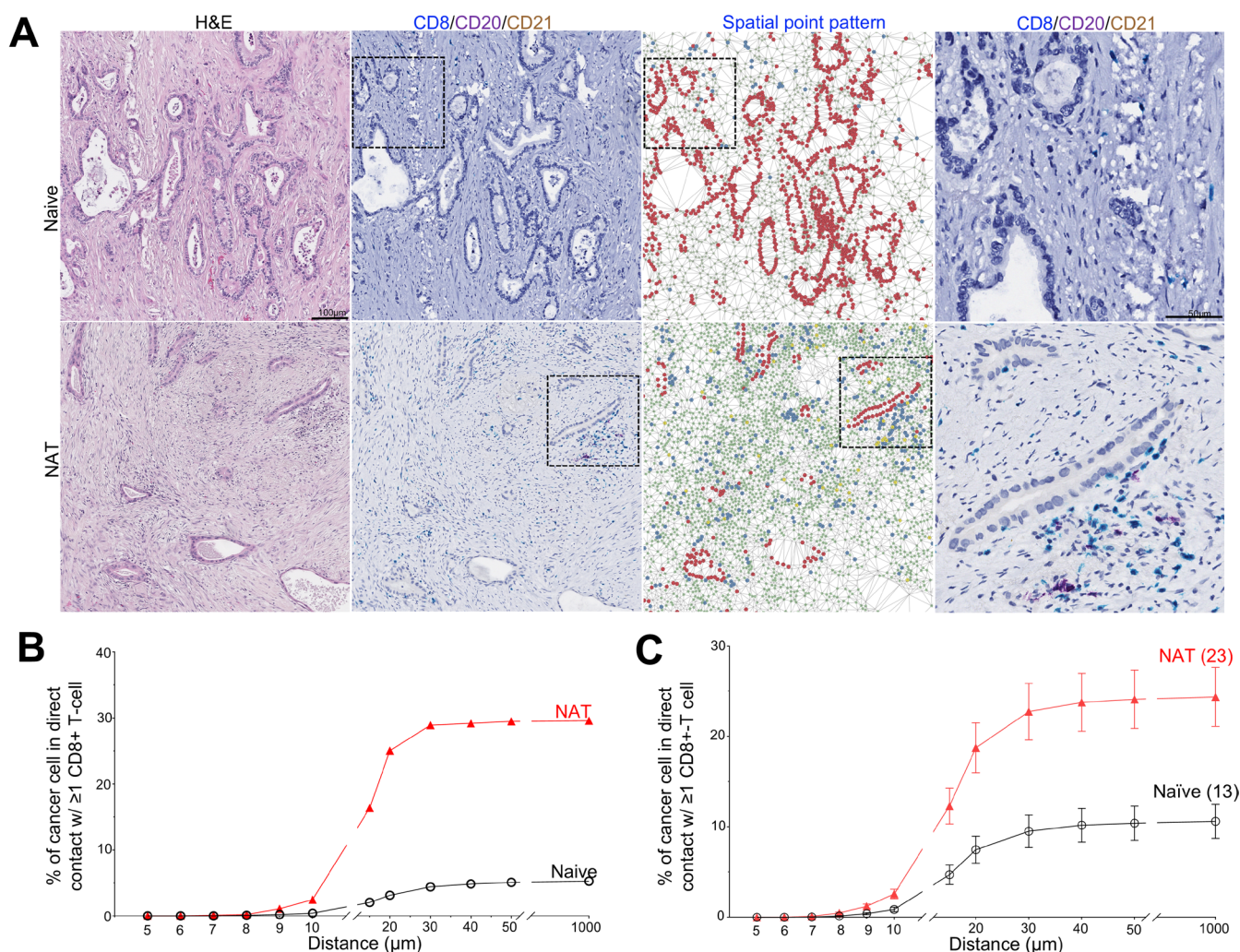


FIGURE 2 | NAT increases stromal CD8⁺ T-cell density and proximity to tumor cells. (A) Representative H&E, CD8/CD20/CD21 multiplex IHC, and spatial point pattern (tumor cells = red, CD8⁺ T cells = teal, CD20⁺ B cells = yellow, stromal cells = green) from a naïve case (top) and a NAT case (bottom). The NAT case shows reduced tumor cell density, increased stromal CD8⁺ T cell density and more CD8⁺ T cells and CD20⁺ cells clustering around tumor glands (dashed box magnified in 4th column). (20× in columns 1–3, 63× in column 4). (B) Spatial analysis of the representative naïve (black) and NAT (red) cases shown in (A), demonstrating a higher percentage of tumor cells in the NAT case that are in direct contact with at least one CD8⁺ T cell across increasing distance. (C) Group average of spatial analysis comparing the naïve and NAT groups. The NAT group shows significantly higher percentage of tumor cells in direct contact with at least one CD8⁺ T cell across distances.

significantly reduced tumor cell density by ~70% in this cohort (538.5 ± 42.9 vs. 1871.0 ± 77.7 , $p < 0.0001$). There were no differences in the degree of tumor reduction among different molecular subtypes of cancer cells (e.g., classical, basal-like, etc.). Like in our study cohort, total CAF density was significantly increased (1384.0 ± 58.5 vs. 1161.0 ± 38.1 , $p < 0.002$). Subtype analysis revealed a NAT-induced increase of iCAFs (772.9 ± 45.8 vs. 271.1 ± 15.4 , $p < 0.0001$) and decrease of mCAFs (607.1 ± 28.6 vs. 885.4 ± 32.1 , $p < 0.0001$), suggesting that NAT reprograms CAFs toward an iCAF-enriched state (Figure 4A,B).

In this dataset, NAT also significantly increased stromal CD8⁺ T cell density (52.9 ± 7.3 vs. 25.7 ± 2.7 , $p < 0.001$) without significantly changing B cell density (2.6 ± 0.4 vs. 4.2 ± 0.9 , $p = 0.12$), aligning with results from our cohort. NAT also significantly decreased the densities of regulatory T cells (Treg, 8.1 ± 1.0 vs. 15.6 ± 1.2 , $p < 0.0001$) and tumor-associated neutrophils (TAN, 1.2 ± 0.2 vs. 20.1 ± 3.5 , $p < 0.0001$). Spatial analysis demonstrated that tumor cells in post-NAT cases also exhibited significantly higher likelihood in direct contact with at least one CD8⁺ T cell than NAT-naïve cases. These results provide a cross-platform validation of the findings in our cohort.

3.4 | Spatial Transcriptomics of NAT-Induced Changes in Tumor Cells and TME in PDAC

To investigate the molecular underpinning of these NAT-induced changes, we compared spatial transcriptomics of tumor and TME in naïve versus NAT groups. Immunofluorescence was used to segment each ROI into the tumor compartment (pan-CK+/SMA-, contains predominantly tumor cells) and the TME compartment (pan-CK-/SMA+, contains various types of CAFs and immune cells), enabling a compartment-specific comparison of NAT-induced transcriptomic changes (Figure 5A). NAT induced 22 up-regulated and 22 downregulated genes in tumor cells. Pathway enrichment analysis revealed the top 3 most significantly enriched pathways were SM00133:S_TK_X (EC 51.9, FDR 0.02), which is associated with AGC kinase activity; KW-0053~Apoptosis (EC 7.7, FDR 0.01) and GO:0005654~nucleoplasm (EC 2.7, FDR 0.06), which is associated with DNA Damage Response (DDR). In contrast, NAT induced 28 upregulated and 24 downregulated genes in TME, among which the top 3 most enriched pathways were KW-0180~Complement pathway (EC 77.3, FDR 1.0×10^{-5}), hsa04115:p53 signaling pathway (EC 28.1, FDR 1.7×10^{-4}), and hsa04218:Cellular senescence (EC 8.8, FDR 0.04). These

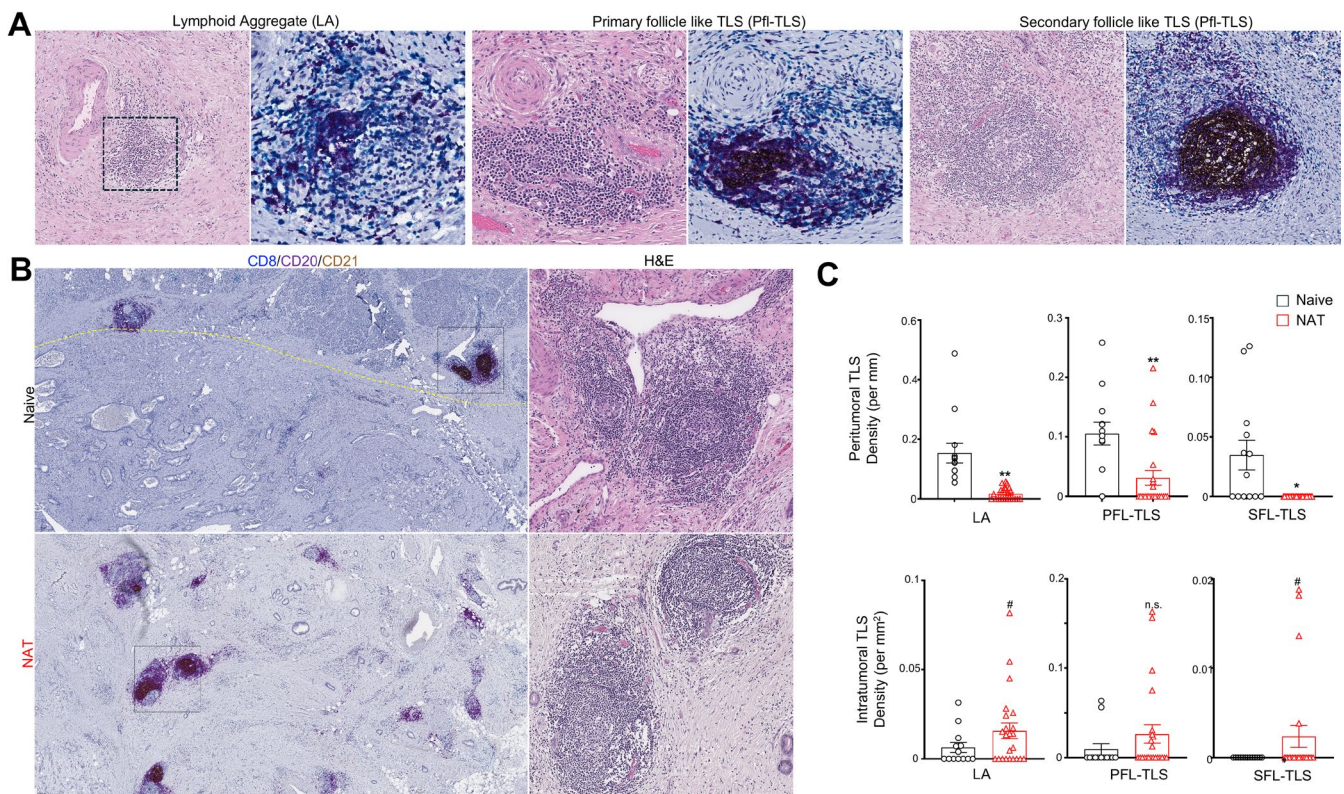


FIGURE 3 | NAT's effect on tertiary lymphoid structure. (A) Histologic and immunohistochemical classification of TLS. A lymphoid aggregate (LA) is a non-follicular cluster of ≥ 50 T and B cells with compartmentalization but lacking follicular dendritic cell (FDC) networks. Primary follicle-like TLS (pfl-TLS) exhibits distinct B-cell and T-cell zones and CD21⁺ FDC networks but lack a germinal center (GC). Secondary follicle-like TLS (sfl-TLS) is the mature form of TLS containing a GC. (B) Representative CD8/CD20/CD21 IHC (5x) and H&E (20x) images from a naïve (top) and a NAT (bottom) cases. In the naïve cases, TLSs are mostly peritumoral. High magnification H&E image (20x) highlights GCs in peritumoral sfl-TLSs in the dashed square. In contrast, the NAT case exhibits more intratumoral TLS. High-magnification H&E images highlight two intratumoral TLS (dashed box): A GC-positive sfl-TLS (CD21⁺CD23⁺, lower left) and a GC-negative pfl-TLS (CD21⁺CD23⁻, upper right). (C) Comparison of TLS subtype densities by location between naïve and NAT groups. NAT is associated with significantly reduced densities of peritumoral LA, pfl-TLS, and sfl-TLS. In contrast, intratumoral LA and sfl-TLS densities show an increasing trend, while intratumoral pfl-TLS density remains unchanged. (** $p < 0.01$, * $p < 0.05$, # $p < 0.1$; n.s., not significant).

findings suggest that NAT induces a complement-rich, p53-high, senescence-skewed TME.

3.5 | Integrating AJCC/CAP TRG With Stromal-Immune Remodeling Metrics Improves Prognostication in Post-NAT PDAC

Next, we performed univariate survival analyses to evaluate the prognostic relevance of NAT-induced changes. In the naïve group, overall survival (OS) was largely driven by standard clinicopathologic factors, whereas TME features show limited prognostic utility. High-grade (G3) tumor was associated with significantly increased mortality risk (HR 20.7; 95%

CI: 1.6–270.1; $p=0.02$) and nodal metastasis demonstrated a trend toward worse OS (HR 7.9; 95% CI: 0.9–69.9; $p=0.06$, Figure 6A, left). In contrast, in the NAT group, both clinicopathological variables and TME features hold prognostication value. High-grade tumor lost statistical significance, nodal metastasis became a strong adverse predictor (HR 20.0; 95% CI: 1.6–256.3; $p=0.02$). Patients with AJCC/CAP TRG3 had a nearly fivefold higher mortality risk than TRG2 (HR 4.8; 95% CI: 1.6–14.9; $p=0.006$). Higher CPA was associated with improved OS (HR 0.96; 95% CI: 0.94–0.99; $p=0.04$), and higher B-cell density showed a favorable trend (HR 0.97; 95% CI: 0.94–1.00; $p=0.06$). CD8⁺ T-cell density showed no significant association (Figure 6A, right). Kaplan–Meier analysis confirmed that post-NAT patients with above median CPA ($\geq 49\%$) had significantly

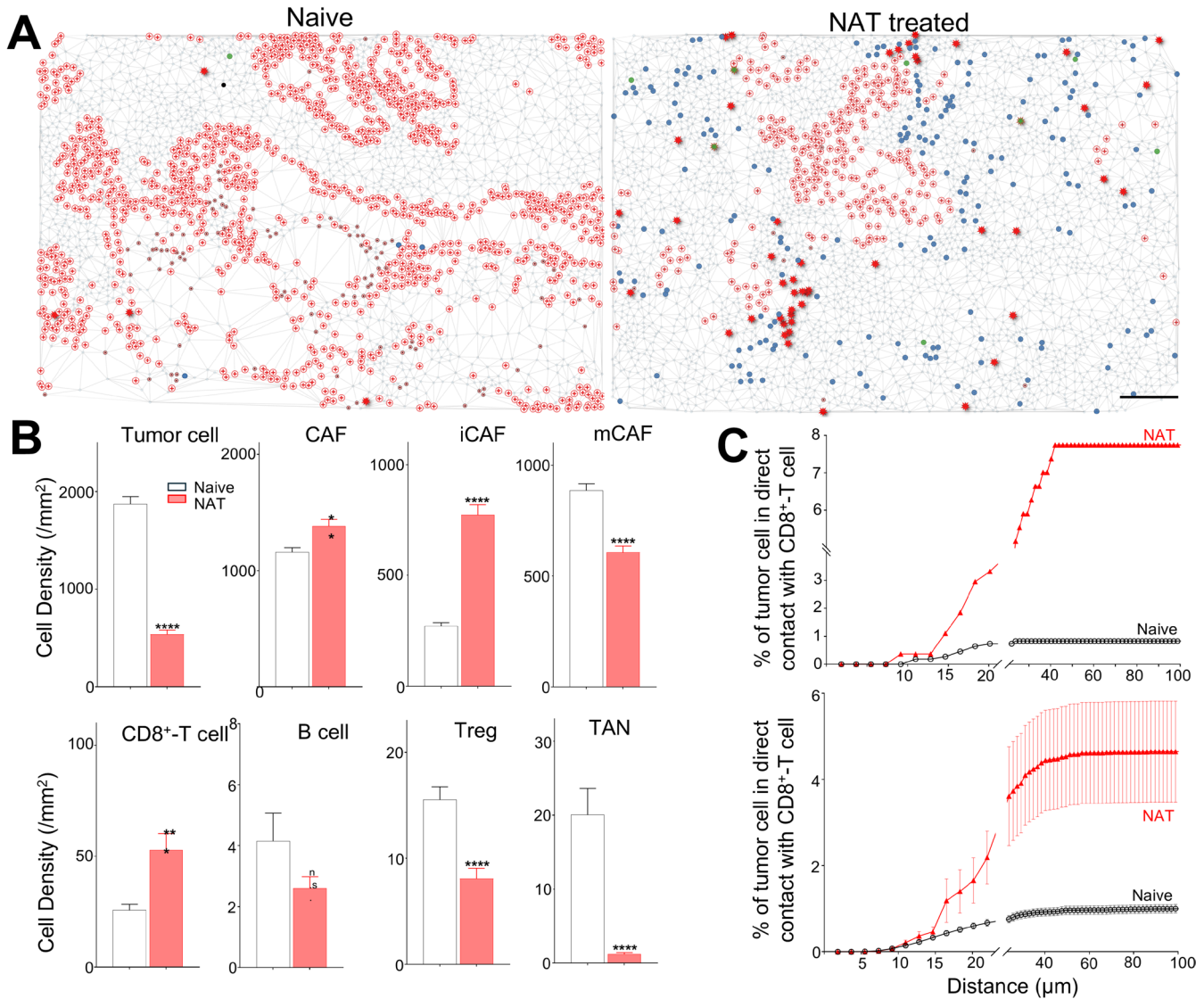


FIGURE 4 | Single-cell spatial transcriptomics reveals NAT-induced TME remodeling. (A) Representative spatial point pattern maps from independent single-cell spatial transcriptomics dataset (Nature Genetics, 2024) showing a naïve case (left) and an NAT case (right) (scale bar: 100 μm). The naïve case shows sparse CD8⁺ T cells (blue dot) located distantly from tumor cells (red circles with cross). In contrast, the NAT case (right) shows significant tumor reduction, higher CD8⁺ T cell density, reduced CD8⁺–tumor distance, and more activated dendritic cells (red star). (B) Comparison of cell-type densities between naïve and NAT groups based on single-cell spatial transcriptomics. NAT significantly reduces the densities of tumor cells, Treg, and TAN, while increasing the densities of CD8⁺ T cells and CAFs. Subtype analysis reveals a significant increase of iCAFs and a decrease of mCAFs. B-cell density does not significantly change. (C) Spatial proximity analysis of CD8⁺ T cells to tumor cells in representative cases (top) and at the group level (bottom). NAT-treated samples show a higher proportion of tumor cells in direct contact with at least one CD8⁺ T cell across increasing distances. (****, $p<0.0001$; ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$; #, $p<0.1$; n.s., not significant).

improved OS ($p=0.015$), those with above median B-cell density ($\geq 5/\text{mm}^2$) showed a non-significant trend toward improved survival ($p=0.11$, Figure 6B).

We then investigated the incremental prognostic value of the TME remodeling beyond TRG using time-dependent area-under-the-curve (AUC) analysis. TRG alone shows limited 1-year discrimination (AUC 0.60) but moderate 2-year discrimination (AUC 0.81). Adding nodal status ($N+$ vs. $N0$) improved discrimination (AUC 0.69 at 1 year; 0.94 at 2 years). Importantly, adding CPA to TRG plus nodal status yields the highest 2-year discrimination (AUC 0.97), indicating that stromal fibrosis captures long-term prognostic information not captured by TRG and nodal status. Adding B-cell density increased 1-year discrimination (AUC 0.75) but did not further improve 2-year discrimination, suggesting immune context may provide complementary early prognostic value, however, validation in larger datasets is needed.

Finally, we tested if it is clinically actionable to integrate CPA as a modifier of AJCC/CAP TRG. Although AJCC/CAP TRG alone separated TRG2 from TRG3 (log-rank $p=0.0028$; HR TRG3 vs. TRG2 = 4.84, 95% CI: 1.58–14.87, Figure 6C, left), we noticed that TRG2 cases were heterogeneous, as some TRG2 cases (6/13) with low CPA ($<49\%$) exhibited worse survival outcomes resembling the TRG3 group (Figure 6C, left, inset). Therefore, we defined a composite AJCC/CAP TRG + CPA scheme, by which the post-NAT patients were stratified into a Low-risk group containing TRG2 cases with high CPA, and High-risk group containing all TRG3 cases plus TRG2 cases with low CPA. This composite scheme produced a markedly sharper prognostic separation (median OS not reached vs. 10.1 months; log-rank $p=3.5 \times 10^{-4}$; HR high vs. low = 11.43, 95% CI: 2.41–54.22, Figure 6C, right), improved concordance index (0.72 vs. 0.66) and increased time-dependent accuracy

at both 1 year (AUC 0.62 vs. 0.60) and 2 years (AUC 0.97 vs. 0.81). In addition, the low-risk group also trended toward improved separation from naïve patients ($p=0.057$). These findings suggest that NAT induces significant TME remodeling (Figure 6D), and integrating TME metrics with AJCC/CAP TRG is clinically plausible and may refine post-NAT risk stratification.

4 | Discussion

Multiple prior studies have shown that NAT significantly remodels the tumor immune microenvironment (TIME) of PDAC. In the stroma, NAT can induce fibrosis [12], and higher CPA has been reported as a positive prognostic marker [38]. Immunologically, multiplex immunofluorescence (mIF) studies demonstrated that NAT decreased CD4^+ T cells, CD20^+ B cells, and FoxP3^+ T cells in the stroma but not in tumor nests, and CD204^+ macrophages in tumor nests is an independent adverse prognostic factor [13]. Additionally, NAT can increase CD8^+ T cells and reduce Tregs and M2 macrophages [14], with higher CD8^+ or CD4^+ T-cell linked to improved survival [39]. Our study extended these findings by moving beyond density-focused descriptions into a spatially integrated tumor–stroma–immune framework. In addition, we benchmarked the performance of these TME features against conventional clinicopathologic variables to identify the most prognostically informative features. These results provide insight into developing a more holistic TRG paradigm.

One strength of our study is the integrated digital pathomics and spatial transcriptomics, allowing us to infer underlying molecular mechanisms of NAT-induced changes. Notably, our spatial transcriptomic demonstrated that NAT not only increases apoptosis and AGC kinase activities in tumor cells, but also

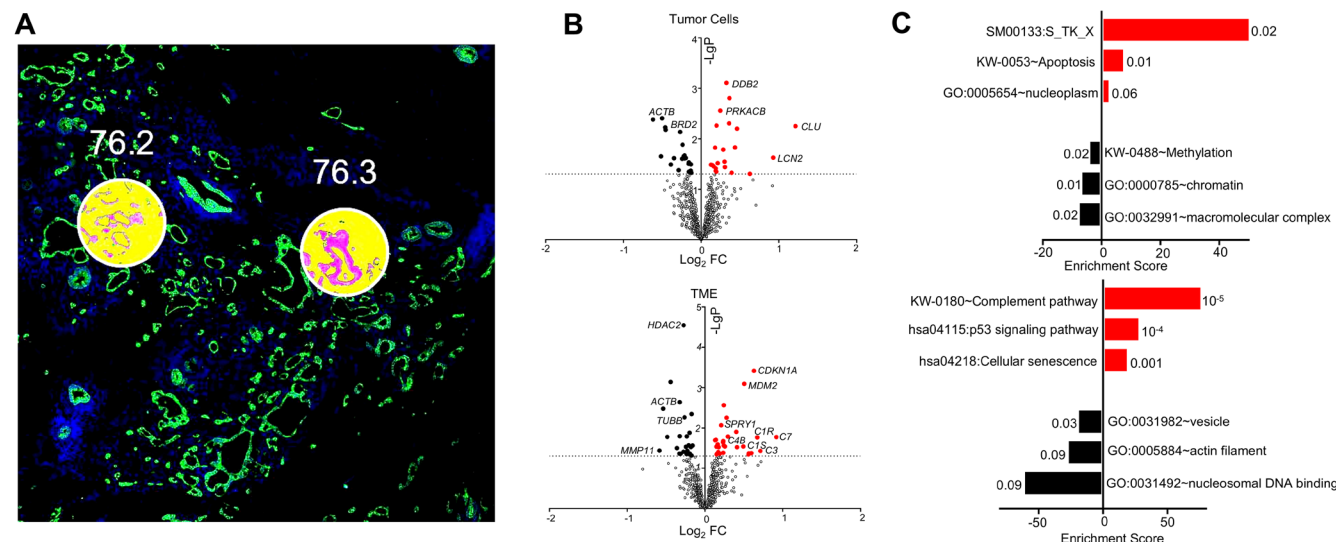


FIGURE 5 | Spatial transcriptomic reveals NAT-induced differentially enriched signaling pathways in tumor and TME. (A) Representative 20x immunofluorescence image illustrating ROI segmentation into tumor cells and TME areas of illumination (AOIs) for compartment-specific transcriptomic profiling using morphology markers: Pan-cytokeratin (green), α -SMA (yellow), and DAPI (blue). (B) Volcano plots of NAT-induced DEGs in tumor cells (top) and the TME (bottom). (C) Functional enrichment analysis (DAVID) of NAT-induced DEGs. NAT-treated tumors cells show enrichment of AGC kinase activity, apoptosis, and nucleoplasm-related signaling in tumor cells (top), and complement, p53 signaling, and cellular senescence pathways in the TME (bottom). In naïve cases, tumor cells are enriched for macromolecular complex, chromatin, and methylation-related terms, whereas the TME is enriched for nucleosomal DNA binding, Actin filament, and vesicle-related terms. Numbers on bars indicate FDR.

upregulates complement pathways, p53 signaling and cellular senescence programs in TME, suggesting that NAT not only exerts cytotoxic tumor reduction but also coordinately reprograms TME. The single-cell spatial transcriptomics analysis revealed that CD8⁺ T cells within 20 μ m to tumor cells in post-NAT cases express elevated levels of cytotoxic effector genes (e.g., *GZMB*), indicating a functionally active state (data not shown), suggesting that quantitative spatial analysis may provide a more functionally meaningful view of the immune-oncologic state following NAT.

Our study has several limitations, one of which is the lack of cases with complete or near-complete pathologic response, restricting our ability to characterize the full spectrum of treatment responses. However, studies have reported that only <5% and <10% of PDAC patients achieve complete or near complete pathologic responses, respectively, much less common than

AJCC/CAP TRG2 and TRG3 cases. Another limitation is the small study cohort size, particularly for the single-cell spatial transcriptomics dataset, limiting the statistical power. Lastly, our NAT cohort included patients treated with different regimen. Although deciphering regimen-specific attribution requires a larger cohort, prior translational/clinical data has indicated that distinct NAT regimens often converge on shared downstream effects on tumor and TME. Therefore, we feel the comparison between any NAT exposure versus NAT-naïve is the more interpretable and statistically robust. Nonetheless, these limitations require external validation in an independent larger multicenter cohort before adoption in real practice.

In summary, by doing multimodal spatial analysis, we demonstrate that NAT leads to coordinated tumor burden reduction and TME remodeling in PDAC, featuring increased densities of CAFs and CD8⁺ T cells, shorter CD8⁺ T cell-tumor distance,

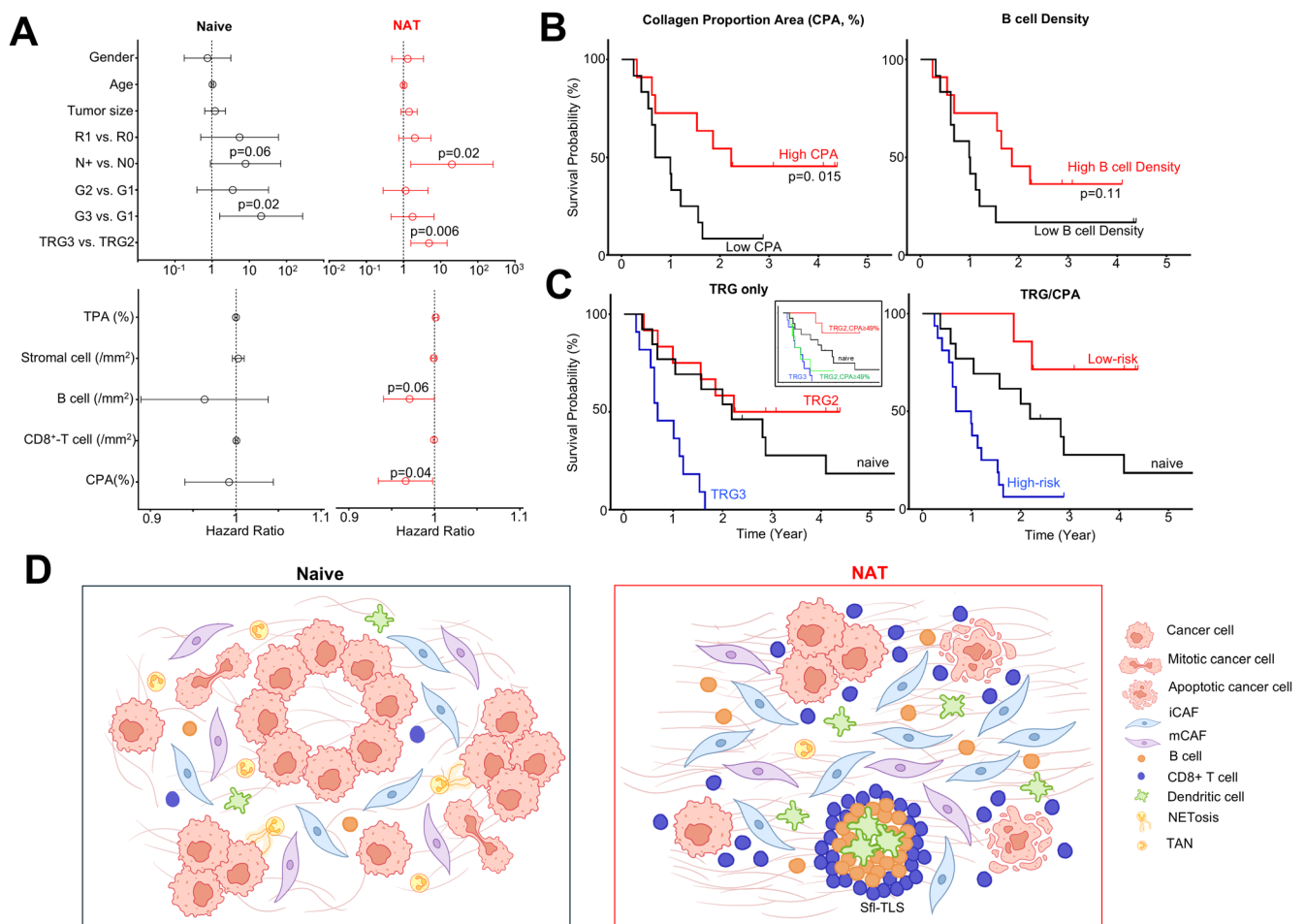


FIGURE 6 | Combining tumor regression grading with TME remodeling features improves survival stratification after NAT in PDAC. (A) Univariate Cox proportional hazards models (hazard ratio, 95% CI) evaluating prognostic variables in NAT-naïve (black) versus post-NAT (red) cases. In NAT-naïve cases, poor differentiation (G3 vs. G1) is associated with worse overall survival ($p=0.02$), and nodal metastasis shows a trend toward worse survival ($p=0.06$). After NAT, histologic grade is no longer prognostic; survival is associated with nodal metastasis ($p=0.02$), AJCC/CAP TRG3 vs. TRG2 ($p=0.006$), and TME remodeling features including collagen proportionate area (CPA; $p=0.04$) and CD20⁺ B-cell density ($p=0.06$). (B) Kaplan-Meier overall survival in post-NAT cases stratified by TME remodeling metrics. High CPA ($\geq 49\%$) is associated with improved survival ($p=0.015$), while high B-cell density shows a favorable trend ($p=0.11$). (C) Combining CPA with AJCC/CAP-TRG improves risk stratification. TRG alone separates TRG3 from TRG2; however, outcomes for TRG2 overlap with the NAT-naïve cohort. Within TRG2, low CPA ($< 49\%$; inset) identifies a poor-outcome subset with survival similar to TRG3. A composite AJCC/CAP TRG + CPA classifier stratifies post-NAT cases into low- and high-risk groups with improved separation of survival curves (right). (D) Schematic summary of NAT-associated remodeling, including increased tumor cell death, stromal fibrosis, immune infiltration, and tertiary lymphoid structure (TLS) reorganization.

fewer TANs, redistribution of TLS, and expanded fibrosis which is associated with improved prognosis. At transcriptomics level, NAT induces apoptosis, nuclear-stress responses and AGC-kinase activity in tumor cells, while enriching iCAFs and complement signaling in TME. These structural and transcriptional shifts support a modified immune-oncologic state rather than just a simple tumor burden reduction. More importantly, these TME remodeling changes carry prognostic significance. Incorporating systematic and quantitative assessment of these changes into a more holistic treatment response evaluation may improve prognostic accuracy and guide more precise therapeutic stratification of PDAC patients following NAT.

Author Contributions

Xiaofei Zhang: conceptualization, data curation, formal analysis, investigation, methodology, project administration, software, supervision, validation, visualization, writing – original draft, writing – review and editing. **Ruoxin Lan:** data curation, formal analysis, investigation, methodology, validation, visualization, writing – original draft. **Danting Li:** data curation, formal analysis, investigation, methodology, validation, visualization. **Yongjun Liu:** conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing – original draft. **Sonu Kalyan:** data curation, formal analysis, investigation, visualization. **Momin Iqbal:** data curation, formal analysis, investigation, visualization. **Nancy Liu:** formal analysis, investigation, visualization. **Jerry Zhang:** visualization. **Iman Hanna:** writing – review and editing. **Mala Gupta:** writing – review and editing. **Chaohui L. Zhao:** writing – review and editing. **Weiguo Liu:** writing – review and editing. **Jonathan Melamed:** writing – review and editing. **Michael Shusterman:** writing – review and editing. **Jessica Widmer:** writing – review and editing. **John Allendorf:** writing – review and editing. **Yao-Zhong Liu:** conceptualization, data curation, formal analysis, investigation, methodology, supervision, validation, visualization, writing – original draft, writing – review and editing.

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Ethics Statement

This is a retrospective observational study using only tissue sections cut from archived formalin-fixed, paraffin-embedded (FFPE) block without utilizing any fresh tissue from any human research participants, therefore, the requirement for informed consent was waived by the institutional ethics committees at both institutions.

Consent

The experimental design and protocols for patient selection and data collection were reviewed and approved by the Institutional Review Boards at University of Wisconsin—Madison (IRB#2018-1510, Protocol

UW18144) and New York University Grossman Long Island School of Medicine (i23-00536).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The spatial transcriptomics data used here along with the metadata were submitted to NCBI GEO (Gene Expression Omnibus) with an accession number of GSE240078.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Methods S1.** A detailed description of the patient cohort and the methodologies used for digital pathology, spatial transcriptomics and survival analysis analyses.