



Niche-resolved transcriptional programs and Δ Np63/NRF2-associated enrichment in post-chemoradiation esophageal squamous cell carcinoma



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Spatial organization in esophageal squamous cell carcinoma resection specimens after chemoradiation remains poorly characterized. We profiled 56 patients using Visium spatial transcriptomics and identified eight niches. All 37 evaluated transcriptional signatures showed marked variation across the five niches with adequate patient representation, revealing structured biological compartmentalization. Δ Np63/NRF2-associated enrichment was highest at the Tumor Leading Edge and lowest at the Stromal Boundary; within Fibro-Immune Niche spots, it was also higher in perineural-invasion-positive tumors. In exploratory analyses of patients with pathological regression Grades 1–2, higher inflammation-, Δ Np63/NRF2-, SPP1-, multigene radiotherapy-resistance-, and oncogenic-transcription-factor-associated enrichment in the Fibro-Immune Niche was associated with shorter failure-free survival. Related Fibro-Immune programs also showed concordant adverse trends in the full cohort, although independent prognostic value was not established in either analysis. Post-treatment Fibro-Immune programs provide biologically coherent candidates for independent validation.

Esophageal squamous cell carcinoma (ESCC) is the predominant histologic subtype of esophageal cancer, accounting for approximately 90% of cases worldwide, with the highest incidence in East Asia¹. Preoperative concurrent chemoradiation therapy (CCRT) followed by surgery is used in selected patients for locally advanced ESCC², yet pathologic responses to CCRT vary enormously: a recent meta-analysis reported pathologic complete response rates of 32% (range: 8–66%) for squamous cell carcinomas³, indicating that the majority of patients harbor residual viable tumor after treatment. Notably, patients in the “gray zone” between near-complete and partial response (Grades 1 or 2) present the greatest clinical challenge, as their long-term outcomes remain highly unpredictable under current grading systems.

Single-cell RNA sequencing has advanced understanding of the ESCC tumor microenvironment by defining immune, stromal, and epithelial transcriptional states^{4–6}. However, tissue dissociation removes information about where those states occur. Spatial transcriptomics preserves tissue

architecture, although each Visium spot contains multiple cells and therefore measures mixed local expression rather than a cell-resolved functional state. This distinction is important when interpreting cell-associated gene sets in spatial data.

Spatial transcriptomic studies have begun to map ESCC carcinogenesis and treatment response^{7–9}. CCRT can remodel the tumor-stroma interface through fibrosis, immune redistribution, cell loss, and selection of residual disease. Consequently, transcriptional patterns measured in surgical specimens after CCRT may reflect both treatment-induced remodeling and residual tumor burden; they cannot by themselves identify pre-existing resistance or predict treatment response before therapy.

Here, we analyzed Visium profiles from 56 ESCC resection specimens obtained after CCRT to define spatial domains and determine whether post-treatment multicellular environments harbor distinct transcriptional programs. We focused biological interpretation on the dominant Fibro-

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Immune Niche and on literature-derived Δ Np63/NRF2-, SPP1-, inflammatory-, and radiotherapy-response-associated signatures, while evaluating patient-level associations with pathology and subsequent outcomes. The biological patient was the unit of inference, and all clinical analyses were treated as exploratory under explicit multiplicity, model-assumption, and small-subgroup stability assessments.

Results

Spatial transcriptomic landscape of post-CCRT esophageal squamous cell carcinoma

Spatial transcriptomics of 56 post-CCRT ESCC specimens identified six transcriptomic spot clusters (Fig. 1A, B) and eight hidden Markov random field (HMRF) spatial niches (Fig. 1C, D): (i) ACTA2+/DES+ Muscular Niche, (ii) IGHG1+/CD74+ Immune-Rich Stroma, (iii) LCN2+/TACSTD2+ Mucosal Transition Zone, (iv) COL1A1+/SERPINE1+ Fibro-Immune Niche, (v) MYH11+/TAGLN+ Stromal Boundary, (vi) S100A8+/KRT13+ Stress-Response Niche, (vii) KRT5+/FTH1+ Metabolically Active Core, and (viii) TP63+/WNT5A+ Tumor Leading Edge. Patient-level niche composition was heterogeneous; the Fibro-Immune Niche was most abundant on average (53.0%), followed by the Stromal Boundary (26.9%) and Tumor Leading Edge (8.4%) (Fig. 1E). Leiden spot clusters were distributed non-uniformly across HMRF niches (Fig. 2A), showing that transcriptomic spot identity and spatial-domain assignment were related but not equivalent. Within the evaluated HMRF framework, $k = 8$ and $\beta = 9$ were retained as a stable, parsimonious working segmentation without reference to clinical outcomes; no single k was uniquely optimal, and no alternative spatial-domain algorithm was evaluated. Detailed stability, domain-support, and image-histology comparisons are provided in Supplementary Fig. 1 and Supplementary Data.

Spot-level signature enrichment differs across spatial niches after accounting for repeated observations within patients

Parametric Analysis of Gene Set Enrichment (PAGE) scores for 37 curated gene sets were calculated at each Visium spot. Because spots were nested within patients, scores were averaged for each observed patient-niche combination and analyzed using slide-adjusted mixed models with a patient random intercept. Niches represented in fewer than 10 patients were descriptive only; the five niches represented in at least 10 patients were included in inference. All 37 omnibus niche tests passed Benjamini–Hochberg (BH) correction within this characterization family and remained significant without the slide term (Fig. 2B; Supplementary Data). The adjusted max-min difference for the Δ Np63/NRF2-associated signature was 7.16 (95% confidence interval [CI], 6.08–8.24; $q < 0.001$), with the highest mean in the Tumor Leading Edge (HMRF 8) and the lowest in the Stromal Boundary (HMRF 5). SPP1-associated spot-signature enrichment also differed across niches (adjusted range: 1.76, 95% CI: 0.59–2.93; $q < 0.001$). Together, these results demonstrate structured variation in local transcriptional programs across post-CCRT spatial niches. Because Visium spots are multicellular, the patterns do not establish cell-specific abundance, polarization, or function.

Patient-level associations between spatial features and clinicopathologic characteristics

In an exploratory analysis of 48 evaluable patients, perineural-invasion (PNI)-positive tumors ($n = 10$) showed higher patient-level Δ Np63/NRF2-associated enrichment and lower plasma-cell-associated enrichment within the Fibro-Immune Niche than PNI-negative tumors ($n = 38$; PNI-family BH $q = 0.015$ and 0.029 , respectively). Neither association remained significant after correction across all clinical comparisons. Across all 1543 valid clinicopathologic comparisons, the sole result with clinical-wide $q < 0.05$ was the Immune-Rich Stroma (HMRF 2) niche proportion association with post-treatment pathological nodal stage (ypN) ($P < 0.001$; ypN-family $q = 0.002$; clinical-wide $q = 0.012$). This feature was nonzero in only 2 of 56 patients; the smallest ypN group contained four patients, and the result failed the sensitivity analysis addressing sparse presence (ypN-family

$q = 0.794$; clinical-wide $q = 0.401$); it was therefore not interpreted biologically. None of the 10 regression-grade candidate associations in Table 1 passed BH correction across the 231 regression-grade tests (smallest $q = 0.051$) or the 1543-test clinical-wide family (smallest $q = 0.136$). Tumor regression grade (TRG) was evaluated only as an alternative same-cohort pathological classification. Full effect estimates and confidence intervals are provided in Supplementary Data.

Whole-cohort associations between spatial features and survival outcomes

Failure-free survival (FFS) was measured from radiotherapy (RT) start. An event was the first documented progression/failure or death from any cause; patients without an event were censored at last follow-up. Event counts among 56 patients were 26 for overall survival (OS), 33 for FFS, 31 for locoregional recurrence-free survival (LRFS), and 32 for distant metastasis-free survival (DMFS). Across 194 eligible spatial features and four endpoints (776 continuous Cox models reporting hazard ratios (HRs) per one-standard-deviation (SD) increase), two previously highlighted Fibro-Immune Niche signatures showed consistent adverse nominal associations across all four endpoints. Among 52 patients with evaluable Fibro-Immune Niche scores, higher SPP1-associated enrichment was associated with HRs per SD of 1.51–1.62 (nominal $P = 0.007$ – 0.020), while Δ Np63/NRF2-associated enrichment had HRs of 1.36–1.42 (nominal $P = 0.017$ – 0.035). For FFS (31 events), the corresponding HRs were 1.57 (95% CI: 1.11–2.21; $P = 0.010$) and 1.38 (95% CI: 1.04–1.84; $P = 0.024$), respectively.

Within the broad screen, neither signature met BH $q < 0.05$ across the 194 features evaluated for an endpoint, and no model met BH $q < 0.05$ across all 776 analyses. As a restricted sensitivity analysis, we applied the same 12-feature set used for the within-group analyses to the whole cohort. Seventeen of the 48 feature-endpoint associations passed endpoint-specific BH correction (OS 7, FFS 0, LRFS 8, and DMFS 2), including SPP1-associated enrichment for OS, LRFS, and DMFS and Δ Np63/NRF2-associated enrichment for OS and LRFS. None passed BH correction across all 48 models (minimum $q = 0.054$). None of the corresponding 48 models adjusted for pathological regression grade and ypN passed endpoint-specific or 48-model BH correction. These results support selected exploratory associations, but not robustness to the broader, global, or adjusted analyses. Complete model-level N, event counts, HRs, 95% CIs, nominal P values, BH q values, and proportional-hazards checks are provided in Supplementary Data.

Grades 1–2 subgroup survival associations

The Grades 1–2 subgroup comprised 29 patients. Two patients had no spots assigned to the Fibro-Immune Niche; consequently, niche-specific signature means were structurally undefined and were not imputed, leaving 27 evaluable patients with 22 FFS events for Fibro-Immune Niche signature models. Continuous Cox models reporting HRs per 1-SD increase were used for inference. An exploratory 12-feature family comprised three niche proportions and nine Fibro-Immune Niche spot-signature enrichment scores. The 12-feature set was defined independently of the continuous Cox results. Median-split Kaplan–Meier curves were used only as secondary visualizations.

Among the targeted 12 FFS models, five continuous associations passed BH correction: inflammation-associated enrichment (HR per SD 2.53, 95% CI: 1.50–4.28; $P < 0.001$; BH $q = 0.006$), Δ Np63/NRF2-associated enrichment (HR: 1.90, 95% CI: 1.28–2.83; $P = 0.002$; BH $q = 0.009$), SPP1-associated spot-signature enrichment (HR: 1.99, 95% CI: 1.27–3.12; $P = 0.003$; BH $q = 0.011$), multigene RT-resistance-associated enrichment (HR: 2.00, 95% CI: 1.24–3.23; $P = 0.005$; BH $q = 0.014$), and oncogenic-transcription-factor-associated enrichment (HR: 1.76, 95% CI: 1.12–2.75; $P = 0.014$; BH $q = 0.034$). All five also passed correction across the corresponding 48 feature-endpoint models (largest BH $q = 0.040$) (Fig. 3A; Table 2; Supplementary Data). The risk direction was retained in 99.2–100% of 5000 patient bootstraps and in every leave-one-out fit. Within the

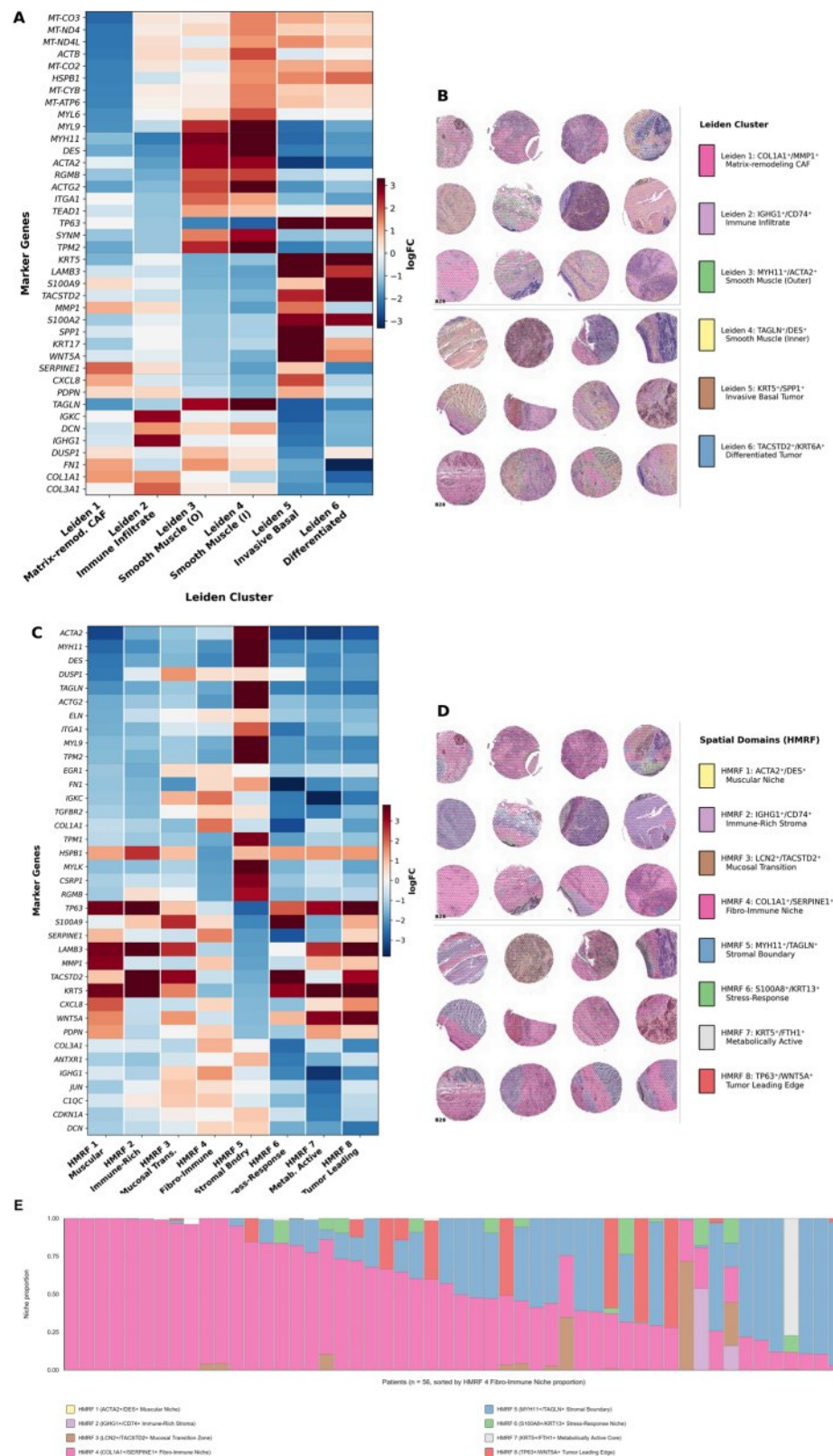


Fig. 1 | Spatial transcriptomic atlas of ESCC resection specimens after CCRT. **A** Marker-gene heatmap across six Leiden transcriptomic spot clusters. **B** Representative TMA cores showing Leiden clusters. **C** Marker-gene heatmap across eight HMRF spatial niches. **D** Representative cores showing HMRF domains. **E** Patient-level niche composition across 56 specimens. **E** Is calculated from the 56 patient-level composition profiles. HMRF identifiers and colors follow the locked

dictionary; abbreviated panel labels correspond to the same canonical niche labels. The eight displayed niche proportions retain their patient-level denominators; the unassigned residual (9/56 patients; maximum 3.8%) is shown as white residual height rather than renormalized. CCRT concurrent chemoradiation therapy, ESCC esophageal squamous cell carcinoma, HMRF hidden Markov random field, TMA tissue microarray.

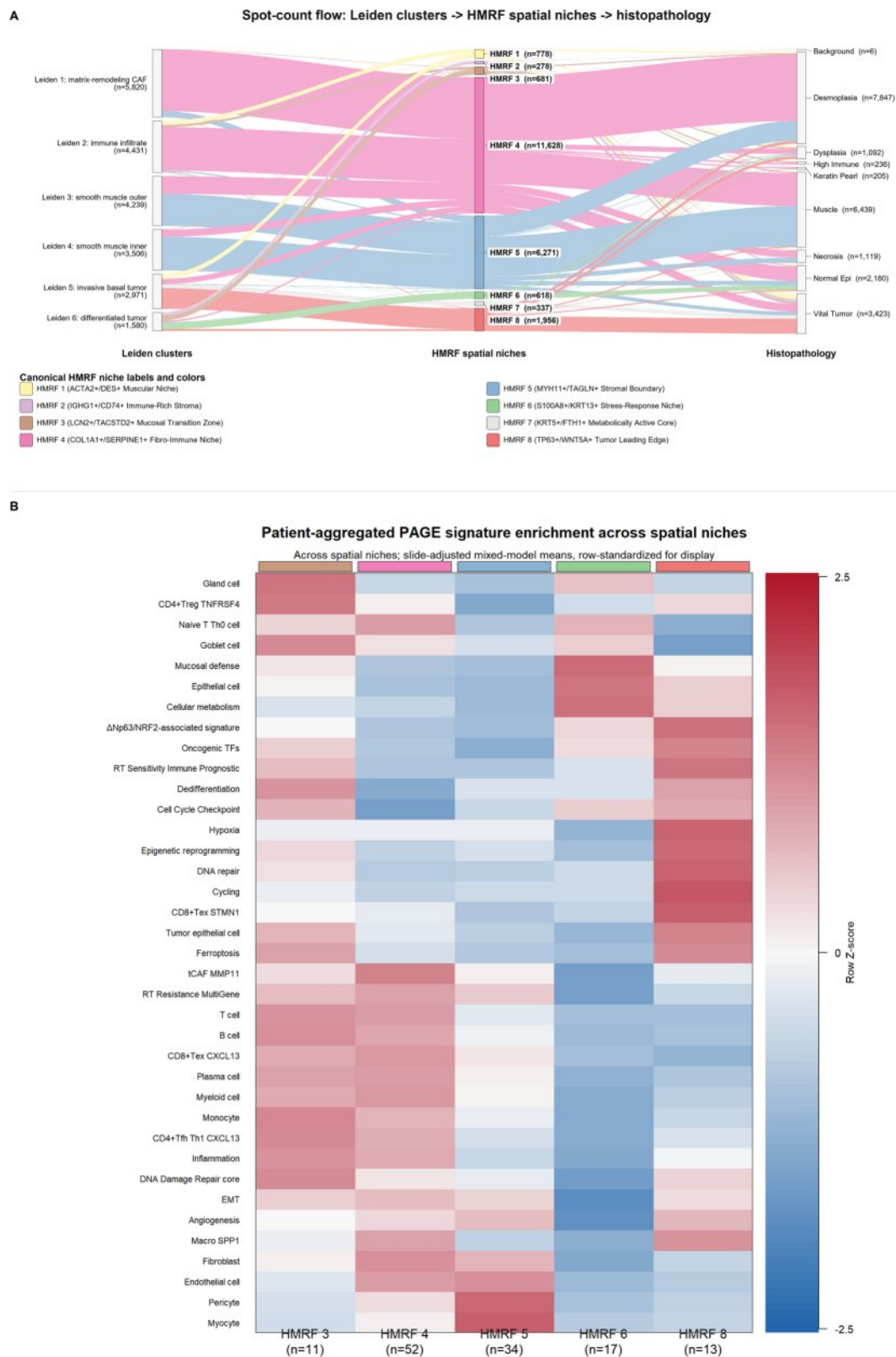


Fig. 2 | Niche composition and spot-signature enrichment accounting for repeated observations within patients. **A** Sankey diagram showing the distribution of Leiden transcriptomic spot clusters across canonical HMRF niches and image-derived histopathological categories. Ribbon widths are proportional to the 22,547 HMRF-assigned spots, and HMRF nodes and ribbons use the locked identifier-label-color dictionary. **B** Slide-adjusted mixed-model means for 37 spot-derived PAGE

signatures across the five niches represented in at least 10 patients; rows are standardized for display. HMRF 1, 2, and 7 are excluded from inference because they occur in 3, 2, and 1 patients, respectively. Signature names do not indicate measured cell abundance or cell-intrinsic function. HMRF hidden Markov random field, PAGE Parametric Analysis of Gene Set Enrichment.

Table 1 | Associations between spatial features and pathological regression grade

Spatial niche	Feature	P value	Grade-family BH <i>q</i>	Clinical-wide BH <i>q</i>	Patients, <i>n</i> (grade 0/1/2/3)
Muscular Niche	Niche proportion	<0.001	0.051	0.136	56 (23/12/17/4)
Fibro-Immune Niche	Δ Np63/NRF2 RT-resistance-associated enrichment	0.003	0.221	0.260	52 (23/12/15/2)
Fibro-Immune Niche	Epithelial-cell-associated enrichment	0.010	0.440	0.565	52 (23/12/15/2)
Fibro-Immune Niche	Oncogenic-TF-associated enrichment	0.020	0.589	0.594	52 (23/12/15/2)
Tumor Leading Edge	Niche proportion	<0.001	0.051	0.136	56 (23/12/17/4)
Metabolically Active Core	Niche proportion	0.005	0.268	0.377	56 (23/12/17/4)
Stromal Boundary	Niche proportion	0.012	0.478	0.569	56 (23/12/17/4)
Stress-Response Niche	Gland-cell-associated enrichment	0.026	0.678	0.669	17 (8/2/5/2)
Fibro-Immune Niche	SPP1/macrophage-associated enrichment	0.019	0.589	0.594	52 (23/12/15/2)
Unassigned	EMT-associated enrichment	0.047	0.746	0.769	9 (3/4/2/0)

Data are shown for 10 candidate associations between spatial features and pathological regression grade; rows are retained irrespective of statistical significance. Grade-family BH *q* values were calculated across 231 regression-grade tests, and clinical-wide BH *q* values across all 1543 valid clinical tests. No association in this table had *q* < 0.05 in either family. Grade counts are presented as grade 0/1/2/3. Rank-based effect sizes and bootstrap 95% CIs are provided in Supplementary Data. Cell-associated labels indicate gene-signature enrichment in multicellular Visium spots, not measured cell abundance or cell-intrinsic function.

BH Benjamini-Hochberg, CI confidence interval, EMT epithelial-mesenchymal transition, NRF2 nuclear factor erythroid 2-related factor 2, RT radiotherapy, SPP1 secreted phosphoprotein 1, TF transcription factor.

targeted testing scopes, these results nominate a coherent set of Fibro-Immune Niche transcriptional programs—including Δ Np63/NRF2, SPP1, inflammation, multigene RT resistance, and oncogenic transcription factors—for independent study. None passed BH correction within the broad 120-feature Grades 1–2 FFS family (strongest *q* = 0.061), and none of the 12 models adjusted for regression grade and ypN passed BH correction. A median-split Δ Np63/NRF2 Kaplan–Meier curve with censoring marks and numbers at risk is shown only as a secondary exploratory illustration (Fig. 3B). These same-cohort subgroup associations were directionally stable under bootstrap and leave-one-out resampling but remained family-dependent and unadjusted; independent prognostic value was not established.

Discussion

This study identifies eight spatial niches in ESCC resection specimens obtained after CCRT and shows that all 37 evaluated gene signatures vary across the five niches represented in at least 10 patients after accounting for repeated observations within patients and slides. Δ Np63/NRF2-associated enrichment was highest at the Tumor Leading Edge and lowest at the Stromal Boundary, illustrating the spatial organization of a literature-derived radioresistance-related program. SPP1- and Δ Np63/NRF2-associated enrichment in the Fibro-Immune Niche also showed concordant adverse nominal associations across all four endpoints in the full cohort. In the restricted whole-cohort sensitivity using the same 12-feature set, SPP1-associated enrichment passed endpoint-specific correction for OS, LRFS, and DMFS, while Δ Np63/NRF2-associated enrichment passed for OS and LRFS; neither passed correction across all 48 feature-endpoint models, and none of the 48 grade/ypN-adjusted models passed correction. Within the dominant Fibro-Immune Niche, five biologically related spot-signature enrichments—including Δ Np63/NRF2-, SPP1-, inflammation-, multigene RT-resistance-, and oncogenic-transcription-factor-associated programs—were associated with FFS in Grades 1–2 after correction within both targeted testing scopes. The spatial characterization is the strongest evidence; the subgroup and restricted whole-cohort findings identify family-dependent candidates that require independent validation.

The non-equivalence of Leiden spot clusters and HMRF niches, together with mixed-model differences across all 37 signatures, indicates that post-CCRT tissue contains local multicellular transcriptional environments not captured by spot-cluster identity alone. Gene sets associated with stromal, immune, epithelial, and stress-response programs varied across niches after accounting for patient clustering and slide. Because

Visium spots contain multiple cells and no reference-based deconvolution was performed, these patterns cannot determine the cell of origin or demonstrate that an individual cell type changed functional state. This spatial interpretation remains compatible with prior work on context-dependent stromal programs and ESCC spatial niches^{7,8,10,11}.

The TP63/PLEC/NFE2L2/KEAP1 signature was selected because preclinical ESCC studies implicate the Δ Np63 α -PLEC-KEAP1-NRF2 axis in radioresistance¹². Prior disease-specific studies also link NRF2/SLC7A11 signaling and suppression of ferroptosis to reduced ESCC radiosensitivity^{13,14}. In the present post-CCRT specimens, Δ Np63/NRF2-associated enrichment in Fibro-Immune Niche spots was higher in PNI-positive tumors in the PNI-family analysis and was associated with shorter FFS in Grades 1–2 in the targeted analyses. Although the strength of these associations depended on the multiplicity scope and covariate adjustment, the Grades 1–2 FFS association showed a consistent risk direction across bootstrap and leave-one-out analyses. Together with the preclinical evidence, these convergent observations support independent evaluation of the Δ Np63/NRF2-related program as a post-treatment spatial correlate of adverse clinical features and outcomes in ESCC.

SPP1-associated and immune-associated spot-signature gene sets also varied across niches, and SPP1- and inflammation-associated enrichment appeared among the five targeted-family FFS candidates in the Fibro-Immune Niche. This pattern provides a spatially coherent rationale for further investigation. However, the PAGE labels derive from published marker sets rather than measured cell counts or cell-resolved states. Prior reports of SPP1-positive macrophages in ESCC and irradiated tissue^{9,15,16} therefore provide external biological context but do not prove macrophage infiltration, polarization, or causal crosstalk in these Visium spots.

Several considerations temper interpretation. This was a single-institution retrospective study without independent validation. Visium profiles multicellular spots, and no deconvolution or protein/RNA co-localization assay was performed; cell-associated labels therefore represent spot-level enrichment. Because tissue was collected after CCRT, the findings describe post-treatment associations rather than pretreatment prediction. Although Harmony addressed slide-related variation, survival models were not additionally adjusted for slide, and the Grades 1–2 subgroup was small. The observed whole-cohort and subgroup signals were sensitive to broader multiplicity correction and clinicopathologic adjustment. Independent cohorts and orthogonal validation are warranted.

In conclusion, ESCC resection specimens obtained after CCRT contain spatial niches with distinct local transcriptional programs after accounting

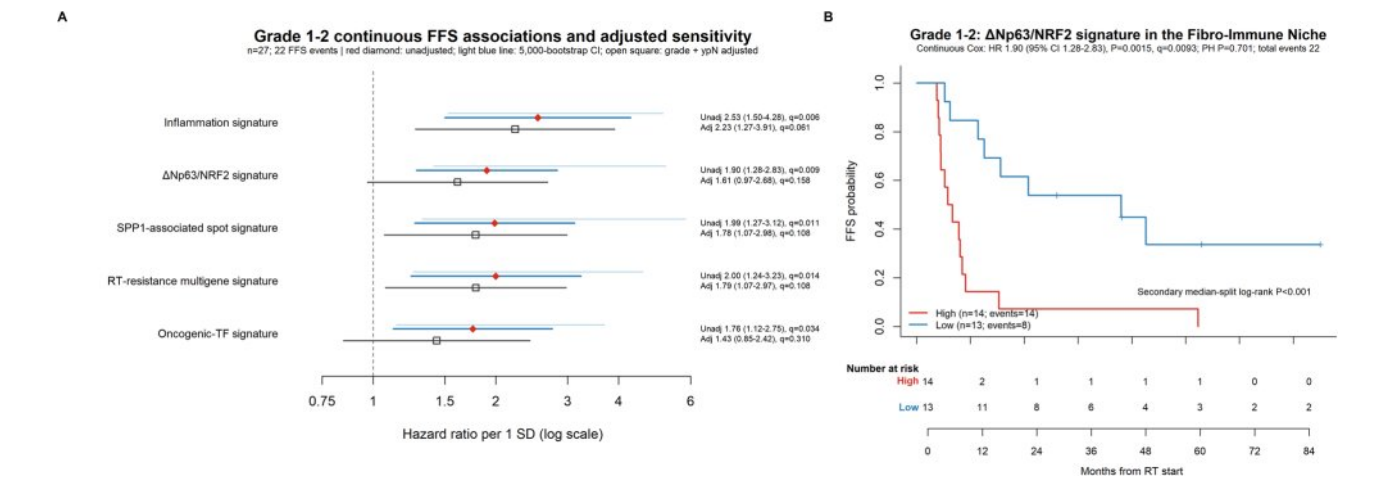


Fig. 3 | Exploratory Grades 1–2 failure-free survival analysis. **A** Continuous Cox estimates (HR per 1-SD increase) for five Fibro-Immune Niche spot-signature enrichments that passed BH correction across the targeted 12-feature FFS family described in “Methods” ($n = 27$; 22 events). Filled diamonds and dark intervals show unadjusted HRs and 95% CIs; light blue intervals show percentile 95% CIs from 5000 bootstrap resamples; open squares and gray intervals show regression-grade- and ypN-adjusted sensitivity estimates. None of 12 adjusted FFS models passed BH $q < 0.05$, and none of the five displayed associations passed BH within the broader 120-feature Grades 1–2 FFS endpoint family (strongest broad FFS $q = 0.061$).

B Secondary median-split Kaplan–Meier display for the Δ Np63/NRF2-associated signature, with censoring marks and numbers at risk. The continuous Cox estimates provide the inference; the dichotomized curve is illustrative only. FFS begins at radiotherapy start. These same-cohort subgroup results are exploratory and require independent validation; the figure does not establish independent prognostic value. BH Benjamini–Hochberg, CI confidence interval, FFS failure-free survival, HR hazard ratio, NRF2 nuclear factor erythroid 2-related factor 2, SD standard deviation, ypN post-treatment pathological nodal stage.

Table 2 | Cox regression analysis of Fibro-Immune Niche signatures and failure-free survival in patients with pathological regression Grades 1–2

Signature	Patients/events, <i>n</i>	Unadjusted model HR per SD (95% CI)	Adjusted model <i>P</i> value	BH <i>q</i>	HR per SD (95% CI)	<i>P</i> value	BH <i>q</i>
Inflammation-associated	27/22	2.53 (1.50–4.28)	<0.001	0.006	2.23 (1.27–3.91)	0.005	0.061
ΔNp63/NRF2-associated	27/22	1.90 (1.28–2.83)	0.002	0.009	1.61 (0.97–2.68)	0.066	0.158
SPP1-associated spot signature	27/22	1.99 (1.27–3.12)	0.003	0.011	1.78 (1.07–2.98)	0.027	0.108
RT-resistance multigene	27/22	2.00 (1.24–3.23)	0.005	0.014	1.79 (1.07–2.97)	0.026	0.108
Oncogenic-TF-associated	27/22	1.76 (1.12–2.75)	0.014	0.034	1.43 (0.85–2.42)	0.181	0.310

Values are shown for the five signatures with BH $q < 0.05$ in the unadjusted targeted 12-model FFS family. All analyses included 27 patients and 22 FFS events. HRs are reported per 1-SD increase. Unadjusted BH q values were calculated across the 12 targeted FFS models. Adjusted models included regression grade and ypN; adjusted BH q values were calculated across the 12 adjusted models. None of the adjusted models had $q < 0.05$. Bootstrap and leave-one-out results are provided in Supplementary Data.

BH Benjamini–Hochberg, CI confidence interval, FFS failure-free survival, HR hazard ratio, NRF2 nuclear factor erythroid 2-related factor 2, RT radiotherapy, SD standard deviation, SPP1 secreted phosphoprotein 1, TF transcription factor, ypN post-treatment pathological nodal stage.

for repeated observations within patients. Within Fibro-Immune Niche spots, Δ Np63/NRF2-, SPP1-, inflammation-, multigene RT-resistance-, and oncogenic-transcription-factor-associated enrichments emerged as targeted-family candidates linked to FFS in Grades 1–2 and showed consistent risk directions under bootstrap and leave-one-out resampling. These findings justify independent spatial and protein/RNA validation, but they do not establish pretreatment prediction, a cell-specific resistance mechanism, independent prognostic value, or a validated therapeutic target.

Methods

Study design and patient cohort

This retrospective observational cohort study was approved by the Institutional Review Board (IRB) of Seoul National University Hospital (approval number: J-2105-156-1221; approval date: 2022-06-20) and was conducted in accordance with the Declaration of Helsinki. Because archival formalin-fixed, paraffin-embedded (FFPE) tissue and retrospective clinical data were used, the requirement for written informed consent was waived by the Institutional Review Board. We analyzed resection specimens from 56

patients with ESCC who received CCRT before esophagectomy. The source clinical database classified treatment intent as preoperative/neoadjuvant in 55 patients and as converted from definitive to neoadjuvant intent in one patient. Treatment occurred between 2005 and 2022; median age was 62.2 years (interquartile range, 57.3–66.9); 50 patients (89.3%) were male, and Eastern Cooperative Oncology Group performance status was 0, 1, and 2 in 10, 45, and 1 patients, respectively. Tissue microarrays (TMAs) were constructed from FFPE surgical specimens, with representative tumor and peritumoral regions sampled from each patient. Five TMA slides were prepared, each containing multiple cores encompassing the full spectrum of pathologic treatment responses from complete regression to minimal response. Clinical and pathological data—including pathologic regression grade (Grades 0–3, modified Ryan scheme/College of American Pathologists criteria), tumor regression grade (TRG 0–3), tumor-node-metastasis staging (ypT, ypN), lymphovascular invasion (LVI), PNI, and survival outcomes (LRFs, DMFS, OS, and FFS)—were abstracted from institutional medical records.

Spatial transcriptomics and data processing

Spatial gene expression profiling used the 10x Genomics Visium CytAssist platform with the Human Transcriptome Probe Set v2.0. Space Ranger outputs from five TMA slides were imported into Giotto Suite (v4.2.2)¹⁷. Per-spot metadata included TMA/core coordinates, slide, tma_id, a unique biological-patient identifier, clinical variables, and image-derived histopathological labels. Each of the 56 patients contributed exactly one physical TMA core. A technical field named patient_id was repeated across slides and was not used for patient-level inference. Background spots without patient assignment were excluded; spots with fewer than 100 detected genes and genes detected in fewer than 5 spots were filtered. Counts were scaled to 10,000 and log-transformed. Principal component analysis retained 100 components; Harmony¹⁸ used the first 10 principal components with slide as batch ($\theta = 2$), and uniform manifold approximation and projection used the first 10 Harmony dimensions ($n_neighbors = 40$, $min_dist = 0.01$, $n_epochs = 400$, $seed = 1234$). Individual Giotto objects were merged before a global Delaunay candidate graph was generated. That unfiltered graph contained 2449 cross-core edges among 67,681 edges, but all were removed by the analysis distance filter; the stored 62,223-edge analysis graph had zero cross-core and zero cross-patient edges. As a boundary-safe verification, Delaunay graphs were reconstructed within each of 56 physical cores and filtered with the same distance threshold; the edge set reproduced the stored graph exactly (Jaccard = 1.0).

Transcriptomic clustering and spatial domain identification

Leiden clustering¹⁹ of the shared nearest-neighbor graph used resolution 0.2 and seed 999, yielding six transcriptomic spot clusters. Marker genes were identified with *scraper*²⁰. Image-derived histopathological labels were assigned to spot-level hematoxylin and eosin patches with BiomedCLIP²¹ as a morphological reference, not an independent validation assay. The top 500 binSpect spatially variable genes were used for HMRF modeling²². Parameter sensitivity comprised 40 fitted HMRF solutions across $k = 6, 8, 9$, and 12 and $\beta = 0, 3, 6, 9, 12, 15, 18, 21, 24$, and 27. All settings used a common fixed initialization, so these comparisons assessed sensitivity to k and β rather than variability across independent random starts. No alternative spatial-domain algorithm was evaluated; these analyses assess internal HMRF parameter and graph-boundary sensitivity rather than cross-method validation. The retained $k = 8$, $\beta = 9$ solution used k -means $nstart = 1000$ and $maxit = 50$ and lay within a stable range of nonzero β values, across which assignments changed little. Selection considered assignment stability, spatial coherence, fragmentation, minimum domain and patient support, morphology/marker interpretability, and parsimony; spatial coherence alone did not define a unique k . Assignment consistency was verified for all 22,547 spots (adjusted Rand index [ARI] = 1.0), and a per-core threshold sensitivity changed 0 assignments. HMRF ID, canonical label, and color were joined through one locked dictionary for all figures and tables. Detailed β , cross- k , domain-support, and image-derived histology comparisons are reported in Supplementary Fig. 1 and Supplementary Data.

Gene signature curation for functional enrichment

A matrix of 37 curated gene sets was assembled from cell-marker databases, published ESCC transcriptomic studies, and literature on pathways relevant to radiotherapy response. Cell-associated labels describe the source or intended biological context of a gene set and do not imply a measured cell type within a Visium spot.

(i) *CellMarker 2.0 database*. Cell-type marker genes were retrieved from the CellMarker 2.0 database²³, filtering for human species and ESCC-related cancer types (ESCC, esophageal cancer, esophageal adenocarcinoma). This yielded marker sets for major immune populations (T cells, B cells, plasma cells, macrophages, dendritic cells, natural killer cells, mast cells), stromal cells (fibroblasts, endothelial cells, pericytes), and epithelial cells.

(ii) *Published single-cell RNA sequencing data*. Cell subtype-specific marker genes were extracted from two published ESCC single-cell transcriptomic studies. From a study characterizing immune features in ESCC⁵,

we obtained markers for SPP1⁺ macrophages, MMP11+ tumor-associated fibroblasts, CD4+ T follicular helper/T helper 1 (Tfh/Th1)-CXCL13, CD4+ regulatory T-cell (Treg)-TNFRSF4, CD8+ exhausted T-cell (Tex)-STMN1, and CD8+ Tex-CXCL13 populations. From a published ESCC single-cell transcriptomic study⁴, we extracted functional gene sets for biological processes including Angiogenesis, Epithelial-Mesenchymal Transition (EMT), Hypoxia, and additional pathway signatures.

(iii) *Literature-based radiotherapy response signatures*. Evaluated signatures included the $\Delta Np63/NRF2$ -associated set (TP63, PLEC, NFE2L2, KEAP1)¹², multigene radiotherapy-resistance sets^{24,25}, and a radiosensitivity-associated immune network²⁶. An investigator-curated eight-gene DNA-damage-repair-related set (ATM, XRCC6, TP53, RAD9, DDB2, MDM2, OGG1, and RPA3) was evaluated; ESCC-specific studies of DNA-damage-repair programs and radioresistance provide biological background^{27,28} but are not presented as the direct source of this exact set. Ferroptosis-related biological context was informed by disease-specific ESCC studies^{13,14}. Complete signature membership and source annotations are provided in Supplementary Data.

The final PAGE output contained 37 retained signatures; the source membership table and exact pathway-name mapping are included in the reproducibility package. PAGE²⁹ was computed on normalized expression at each Visium spot with a minimum two-gene overlap and returned z-scores. For corrected niche comparisons, spot scores were averaged within each observed patient-by-canonical-HMRF-niche combination; structurally absent niches were not imputed. Niche support was 3, 2, 11, 52, 34, 17, 1, and 13 patients for HMRF 1–8, respectively. HMRF 3, 4, 5, 6, and 8 (at least 10 patients) entered inferential models, whereas HMRF 1, 2, and 7 were descriptive. For each signature, a mixed model with niche and slide fixed effects and a patient random intercept was compared with a slide-plus-patient null model by likelihood-ratio test. BH correction was applied across 37 omnibus tests; a model without slide was fitted as a sensitivity analysis. Heatmaps display slide-adjusted means, row-standardized for visualization only.

Spatial interaction and clinical association analysis

Pooled Giotto proximity-permutation P values were excluded from inference because exchangeability blocks within physical core/patient were not implemented. Clinical associations used patient-level niche proportions or patient-aggregated niche-specific PAGE scores. Seven clinical-variable families were analyzed: regression grade, TRG, ypT, and ypN by Kruskal–Wallis tests; and local failure, LVI, and PNI by Wilcoxon rank-sum tests. Effect sizes were epsilon-squared for Kruskal–Wallis tests and Cliff's delta for binary comparisons, with deterministic 1000-bootstrap percentile 95% CIs. Spearman correlations between spatial features and censored OS, LRFS, or DMFS follow-up time were excluded from revised inference; time-to-event associations were evaluated with Cox models. The niche mixed models included slide as a fixed effect and patient as a random intercept. Clinical and survival analyses used one row per patient and were not additionally adjusted for slide: each patient contributed one core, and event-limited models did not support adding the four-degree-of-freedom slide factor alongside clinicopathologic covariates. These associations remain exploratory, and residual slide confounding is acknowledged. Feature-specific complete-case N and applicable group counts are provided in Supplementary Data.

Survival and within-group analysis

OS, FFS, LRFS, and DMFS were measured from radiotherapy start for all 56 patients. OS events were deaths. FFS was defined as the first documented progression/failure or death from any cause; when both occurred, the earlier date was used, and event-free patients were censored at last follow-up. LRFS and DMFS incorporated the corresponding failure or death. Spatial features were modeled continuously after scaling by one sample SD within the model-specific complete cases. Whole-cohort univariable Cox models covered 194 eligible features and four endpoints. A restricted whole-cohort sensitivity analysis used the same 12-feature set as the within-group

analyses; BH correction was applied within the 12 models for each endpoint and across all 48 feature-endpoint models. The same 12 features were also fitted in parsimonious whole-cohort models adjusted for pathological regression grade and ypN, with the same endpoint-specific and 48-model BH scopes. This restricted analysis was an exploratory scope extension and was not treated as a prespecified whole-cohort family. Schoenfeld residuals tested feature-level and global proportional hazards. In Grades 1–2, a broad 120-feature screen and a targeted 12-feature family were analyzed across four endpoints. The 12 features comprised three niche proportions (Fibro-Immune Niche, Stromal Boundary, and Tumor Leading Edge) and nine Fibro-Immune Niche spot-signature enrichment scores (B-cell-associated, plasma-cell-associated, cell-cycling, inflammation-associated, SPP1-associated, naive-T/Th0-associated, oncogenic-transcription-factor-associated, multigene RT-resistance-associated, and Δ Np63/NRF2-associated). The 12-feature set was defined independently of the continuous Cox results. No clinical or survival hypothesis or multiplicity hierarchy was prospectively preregistered; accordingly, this 12-feature family was treated as exploratory. For FFS, the same 12 features were fitted with regression grade and ypN adjustment; patient-level stability of the five unadjusted family- q signals was assessed by 5000 bootstrap resamples (seed 20260729) and leave-one-out refitting. Median-split Kaplan–Meier curves, censoring marks, and risk tables were used only as secondary displays. TRG was an alternative same-cohort classification sensitivity, not cross-validation or independent validation.

Statistics and reproducibility

All tests were two-sided. The unique primary/exploratory registry contained 2832 nonduplicated tests: 1543 valid patient-level clinical associations, 776 whole-cohort univariable Cox models, 476 fitted Grades 1–2 broad Cox models, and 37 niche omnibus models that accounted for repeated observations within patients. The restricted whole-cohort 12-feature set was flagged on the corresponding 48 rows of the 776-model broad screen and was not counted again; its endpoint-specific and 48-model q values are reported separately. The targeted Grades 1–2 12-feature by 4-endpoint set was similarly nested within the 476 Grades 1–2 models and flagged rather than counted again. BH q values were calculated from full-precision P values within the stated scopes. A manuscript-wide BH across all 2832 tests and a sparse-aware version replacing 28 low-presence clinical proportion P values were reported as sensitivity analyses and were not used as primary decision rules. The 97 secondary models were kept separate: 48 adjusted whole-cohort 12-feature models, 37 no-slide niche sensitivity models, and 12 adjusted Grades 1–2 FFS models. The 919-row excluded-test annex contains 693 censoring-ignorant Spearman tests, 222 spot-independent niche tests, and four technical non-fits; all are excluded from every revised false discovery rate universe. The 516 median-split subgroup rows were not used for revised inference. The integrated registry reports effect estimates, 95% CIs, model-specific N, survival event counts, P, q , model formula, support flags, and source details; applicable clinical group counts and additional diagnostics are retained in source-specific outputs. No statistical method was used to predetermine sample size. Analyses used R 4.5.1 with versioned scripts, fixed seeds where applicable, session information, and SHA-256 manifests. The integrated Supplementary Data workbook provides a reader guide, spatial-network and HMRF summaries, the clinical and survival result tables, and the primary and secondary inferential registries; the registry tiers are mutually exclusive and are not pooled as one inferential family. Excluded-test provenance is retained in the Zenodo reproducibility package. Heatmaps, alluvial plots, and other graphics were generated with ComplexHeatmap, ggalluvial, and ggplot2^{30–32}.

Data availability

De-identified processed Visium filtered count matrices, the archived normalized expression matrix, spot-level annotations and spatial coordinates, the archived exact 37-signature membership table, patient-level analysis features used for the clinical and survival models, full-precision inferential registries, and reproducibility metadata have been deposited in an

unpublished Zenodo draft under reserved digital object identifier (DOI) 10.5281/zenodo.21677711. Confidential access for editors and reviewers is provided in the point-by-point response. These resources support independent reproduction of the reported downstream analyses from the processed count-matrix stage onward. Raw sequencing files in FASTQ format, platform-specific intermediate files in binary alignment map (BAM) and CLOUPE formats, direct identifiers, the re-identification key, source pathology reports, and patient-derived source material are not included. The permitted de-identified files will be released publicly through the same Zenodo record no later than article publication.

Code availability

Version-locked scripts for graph auditing, core-restricted HMRF reproduction and sensitivity analyses, PAGE scoring, niche analyses accounting for repeated observations within patients, patient-level clinical association analyses, Cox proportional hazards and stability analyses, and figure and table generation, together with configuration files, seeds, session information, run records, and checksum manifests, have been deposited in the same Zenodo draft under reserved DOI 10.5281/zenodo.21677711. Confidential reviewer access is provided in the point-by-point response, and the permitted code and reproducibility materials will be made public no later than article publication.

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Author contributions

B.S.J. and B.H.K. conceived and designed the study. B.S.J. performed the spatial transcriptomic and statistical analyses and drafted the manuscript. K.J.N. contributed computational methodology and data interpretation. H.S.L. contributed pathological interpretation and tissue review. H.J.K. contributed clinical interpretation. B.H.K. supervised the study and acquired funding. All authors reviewed and revised the manuscript and approved the final version.

Competing interests

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Additional information

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