

INTRODUCTION

Accurate histological grading of thymomas is pivotal for predicting prognosis and guiding therapeutic strategies. The World Health Organization classification system categorizes thymomas into low-grade (types A, AB, and B1) and high-grade (types B2 and B3) subtypes [1,2]. However, distinguishing between B1 and B2 thymomas remains a notorious diagnostic challenge. Both subtypes are heavily infiltrated by immature lymphocytes, making the visual assessment of the sparse, neoplastic epithelial cells highly subjective and prone to inter-observer variability [3,4]. Clinically, the transition to high-grade thymoma is accompanied by distinct cytological alterations in the epithelial compartment, notably increased nuclear size and pleomorphism. To overcome the limitations of human visual estimation, digital nuclear morphometry provides a quantifiable, highly reproducible approach to capture these critical dimensional changes objectively [5].

In the era of precision medicine, transcriptomics—particularly bulk RNA sequencing (RNA-seq) via cohorts such as The Cancer Genome Atlas (TCGA)—has been widely used to identify objective prognostic biomarkers and therapeutic targets [6,7]. Among these, the immune checkpoint molecule programmed death-ligand 1 (PD-L1) and its upstream transcriptional driver, signal transducer and activator of transcription 1 (STAT1), are of paramount clinical interest for guiding immunotherapy [8,9]. Yet, the unique, lymphocyte-rich microenvironment of thymomas poses a critical bioinformatic pitfall [10]. While advanced technologies such as single-cell RNA-seq and spatial transcriptomics can, in principle, resolve cellular heterogeneity [11], their high costs and technical complexities preclude their use in routine clinical diagnostics. Consequently, bulk RNA-seq remains the dominant modality for biomarker discovery, leaving researchers susceptible to the “lymphocyte dilution effect,” where abundant immune transcripts obscure the intrinsic molecular signatures—such as the true STAT1/PD-L1 status—of the neoplastic epithelial cells [12].

METHODS

Aim

To bridge this gap, we investigated the discrepancy between morphological reality and bulk transcriptomic data. By integrating routine immunohistochemistry with digital nuclear morphometry—a cost-effective, spatial-aware, and highly reproducible diagnostic adjunct—we highlight a critical transcriptomic paradox and underscore the indispensable value of in situ spatial evaluation over bulk sequencing for accurately assessing thymoma grading and clinically actionable targets.

Design

Following Institutional Review Board approval (National Cheng Kung University Hospital, A-ER-111-319), we retrospectively enrolled surgically resected thymomas (1988–2023) that were classified according to the current WHO criteria and managed in accordance with institutional guidelines. Given the retrospective nature of this study, the sample size was not determined by a formal power calculation but was based on the availability of all eligible tissues in our archive.

Tissue microarrays (TMAs) were constructed using duplicate 2-mm formalin-fixed, paraffin-embedded tumor cores per patient. Immunohistochemistry was performed for PD-L1 (clone E1L3N; Cell Signaling Technology) and STAT1 (clone M249; ArigoBio) using standard automated protocols. Expression was evaluated semi-quantitatively by experienced, blinded pathologists. Cases exhibiting strong staining intensity at any percentage, or moderate intensity in >50% of neoplastic cells, were categorized as having high expression.

To specifically isolate neoplastic epithelial cells from abundant admixed lymphocytes, the TMA sections were immunohistochemically stained for p63 (clone Z2003L; ZETA) in addition to routine hematoxylin and eosin staining. The well-stained sections were digitized into high-resolution whole-slide images at 20x magnification using a PANNORAMIC 250 Flash III scanner (3D HISTECH Ltd.). Digital morphometric analysis was performed utilizing the NuclearQuant module within the QuantCenter® software (3D HISTECH Ltd.). By applying a targeted segmentation algorithm, the software automatically identified and analyzed all p63-positive nuclei across the sections, effectively excluding the surrounding non-neoplastic lymphocytes. For each case, the mean nuclear area (μm^2) and nuclear perimeter (μm) of all captured neoplastic cells were calculated and extracted for subsequent statistical comparison. Bulk RNA-seq data (RNA Seq V2 RSEM) from the TCGA-THYM cohort ($n = 119$) were accessed via cBioPortal. Spearman's rank correlations were utilized to assess relationships between STAT1 and targeted immune or proliferation genes (CXCL9, CXCL10, CD274, UBTF, MKI67, FBL, and TOP2A), with Benjamini–Hochberg false discovery rate (FDR) correction. For unbiased pathway evaluation, genes were ranked by their Pearson correlation with STAT1 and subjected to Gene Set Enrichment Analysis (GSEA) using the clusterProfiler R package against the MSigDB Hallmark collection (FDR < 0.25) [13,14]. Clinical and morphological data were analyzed using SPSS v25.0. Continuous variables were compared using Student's t-test, and categorical variables via the χ^2 or Fisher's exact test. A two-sided $P < 0.05$ was considered statistically significant.



DISCUSSION AND CONCLUSION

Accurate histopathological grading of thymomas is essential for clinical management but remains challenging due to the heavy admixture of reactive lymphocytes. In this study, we demonstrated that digital nuclear morphometry provides a highly objective and reproducible metric for distinguishing high-grade from low-grade thymomas, effectively bypassing the visual interference from the immune microenvironment. By specifically targeting p63-positive neoplastic cells, we confirmed that high-grade thymomas intrinsically overexpress STAT1 and PD-L1.

Crucially, our study exposes a significant observational paradox inherent in bulk transcriptomic analyses. In the TCGA cohort, STAT1 expression exhibited a deceptive negative correlation with critical proliferation markers (MKI67, TOP2A) and MYC/E2F targets. A purely bioinformatic interpretation might erroneously conclude that STAT1 is a marker of low proliferative potential. However, our spatially resolved IHC results contradict this, revealing that high-grade neoplastic cells strongly express STAT1. This discrepancy can be attributed to the “lymphocyte dilution effect” [15]. In bulk RNA-seq, low-grade thymomas with massive lymphocyte infiltration contribute overwhelmingly to the total STAT1 pool while diluting the scarce signals of epithelial proliferation [7,12], thereby generating a mathematically inverse yet biologically misleading correlation.

To overcome the bioinformatic pitfalls of bulk tissue analysis, spatial transcriptomics and single-cell RNA-seq are emerging as definitive solutions [11]. However, their high costs and technical complexities currently limit their routine clinical application. In this context, our approach—combining targeted immunohistochemistry with digital morphometry—serves as a highly accessible and cost-effective spatial profiling tool. This spatial resolution is critically important for ongoing and future clinical trials investigating immune checkpoint inhibitors in thymic epithelial tumors [16]. If patient selection relies solely on bulk transcriptomic signatures, high-grade thymomas might be erroneously dismissed as “immunologically cold” due to the lymphocyte dilution effect. Our findings strongly advocate for tissue-based, spatially resolved evaluation of PD-L1 and intrinsic immune signatures, ensuring that patients with high-grade thymomas are not inappropriately excluded from potentially life-saving immunotherapies.

We acknowledge certain limitations in our study. First, the retrospective, single-institution design of our TMA cohort necessitates future multi-center prospective validation. Second, although duplicate cores were used, TMAs may not capture the full intratumoral heterogeneity of thymomas as comprehensively as whole-slide evaluations. Finally, although our p63-directed approach clarifies the epithelial expression of STAT1, the precise transcriptomic input of the lymphoid stroma remains unmeasured in our cohort.

Conclusions

Bulk RNA-seq in thymomas is heavily confounded by the immune microenvironment, creating a paradox where inflamed, high-grade tumors appear transcriptionally quiescent. Therefore, bulk transcriptomics should be interpreted with extreme caution for biomarker discovery in thymomas. Instead, integrating digital nuclear morphometry with spatial protein evaluation (IHC) provides a necessary, objective adjunct to overcome inter-observer variability, ensuring accurate grading and reliable assessment of therapeutic targets like PD-L1. In conclusion, digital nuclear morphometry provides an objective framework for thymoma grading, highlighting the necessity of spatial context in the era of precision oncology.

REFERENCES & ACKNOWLEDGEMENTS

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RESULTS

A total of 107 thymoma patients were included, comprising 72 with low-grade (types A, AB, and B1) and 35 with high-grade (types B2 and B3) thymomas. Patients in the low-grade group were significantly older ($P = 0.009$) and presented with larger macroscopic tumor maximum diameters ($P = 0.005$). Peripheral white blood cell counts were significantly higher in the high-grade group ($P = 0.020$), indicating a more pronounced systemic inflammatory status. No significant differences were observed regarding sex, association with myasthenia gravis, Masaoka stage, hemoglobin levels, or platelet counts (Table 1).

At the cellular level, digital morphometric analysis demonstrated that high-grade thymomas had significantly larger nuclear areas and perimeters than their low-grade counterparts (both $P = 0.004$; Figure 1A, B), and a significantly higher proportion of high-grade cases exhibited elevated STAT1 ($P = 0.014$) and PD-L1 ($P < 0.001$) expression within the neoplastic compartment (Table 1). Paradoxically, in the TCGA-THYM bulk RNA-seq cohort, STAT1 expression showed strong positive correlations with key immune and inflammatory regulators (CXCL10, CXCL9, CD274) but significant negative correlations with nucleolar and proliferation markers (UBTF, MKI67, FBL, TOP2A) (Figure 1C); GSEA confirmed this dichotomy, with positive enrichment for the interferon gamma response pathway and negative enrichment for MYC and E2F targets (Figure 1D).

Table 1. Clinicopathological characteristics, nuclear morphometry, and immunohistochemical findings according to thymoma grade

Parameters	Low-Grade Group (Type A, AB, B1)	High-Grade Group (Type B2, B3)	P-value
Cases (n)	72	35	
Demographic and clinical factors			
Age (years)	58.7 ± 12.9	51.7 ± 11.7	0.009
Sex (male %)	40.3	60.0	0.055
Myasthenia gravis (%)	27.8	42.9	0.119
Masaoka stage			0.139
1 / 2a / 2b / 3 / 4a	37 / 27 / 2 / 4 / 2	14 / 11 / 5 / 4 / 1	
Pathological tumor size (cm)	5.5 ± 3.0	4.1 ± 1.8	0.005
Laboratory parameters			
White blood cells ($\times 10^3/\mu\text{L}$)	7.3 ± 2.3	8.9 ± 3.5	0.020
Hemoglobin (g/dL)	13.1 ± 1.8	13.6 ± 1.8	0.192
Platelet concentration ($\times 10^3/\mu\text{L}$)	244.8 ± 78.5	244.1 ± 71.2	0.966
Nuclear morphometry			
Mean nuclear area (μm^2)	23.2 ± 14.0	31.3 ± 17.4	0.004
Nuclear perimeter (μm)	18.6 ± 6.9	22.4 ± 7.1	0.004
Immune microenvironment			
STAT1 high-expression (%)	31.9	66	0.014

Data are mean ± standard deviation for continuous variables and percentages for categorical variables, except for Masaoka stage (case numbers). P-values: Student's t-test for continuous variables and χ^2 or Fisher's exact test for categorical variables. High STAT1 and PD-L1 expression was defined as strong staining at any percentage or moderate staining in >50% of neoplastic cells.

Table 2. Univariate and multivariable logistic regression analyses of factors associated with high-grade thymoma

Variable	Univariate OR (95% CI)	P-value	Multivariate aOR (95% CI)*	P-value
Age (years)	0.958 (0.927–0.987)	0.0073	0.972 (0.937–1.006)	0.1154
White blood cells ($\times 10^3/\mu\text{L}$)	1.147 (1.004–1.320)	0.0472	1.095 (0.948–1.270)	0.2202
Mean nuclear area (μm^2)	1.172 (1.087–1.277)	< 0.001	1.146 (1.057–1.254)	0.0015

* The multivariable model included age, white blood cell count, and mean nuclear area. Abbreviations: aOR, adjusted odds ratio; CI, confidence interval; OR, odds ratio.

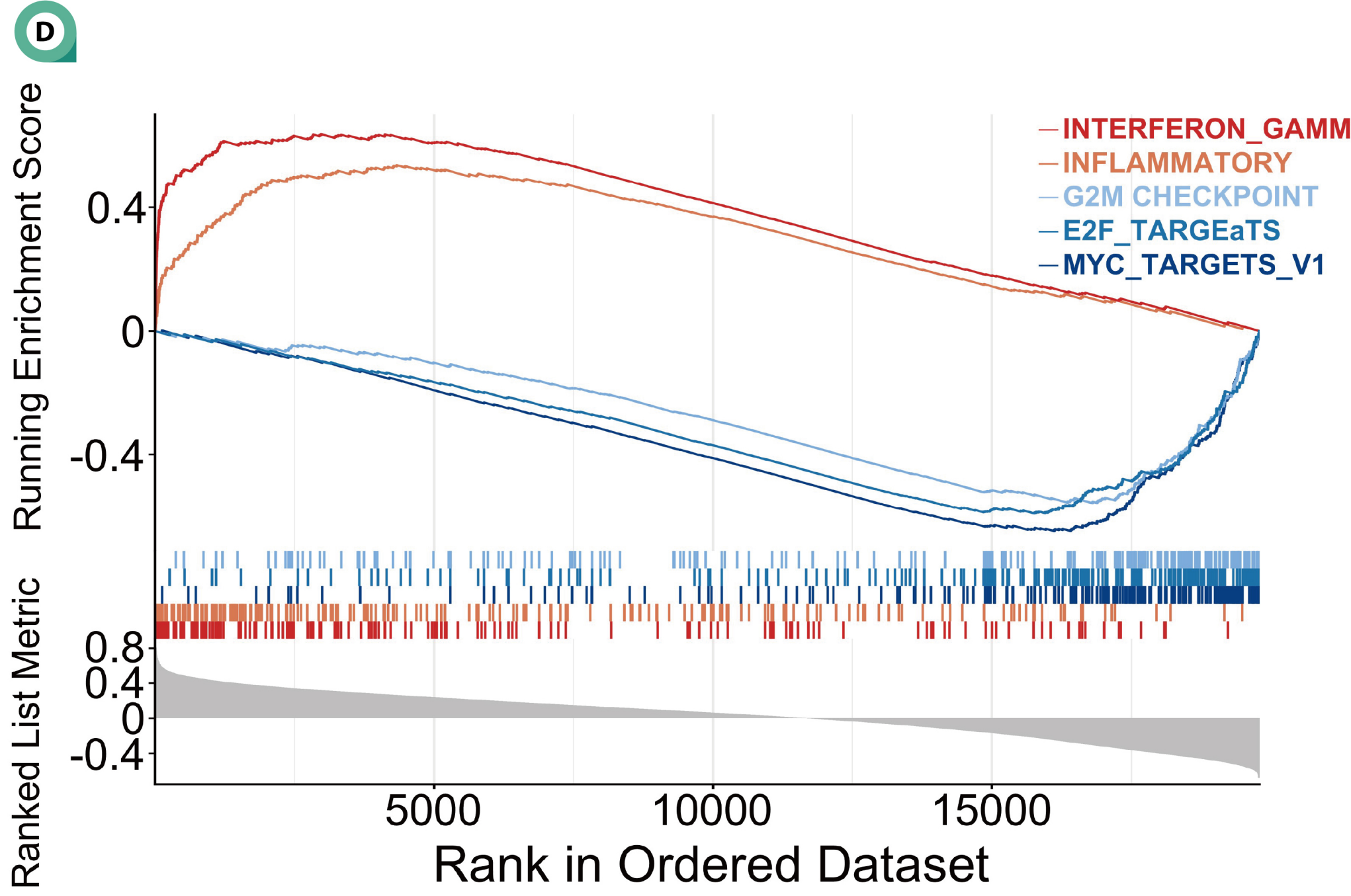
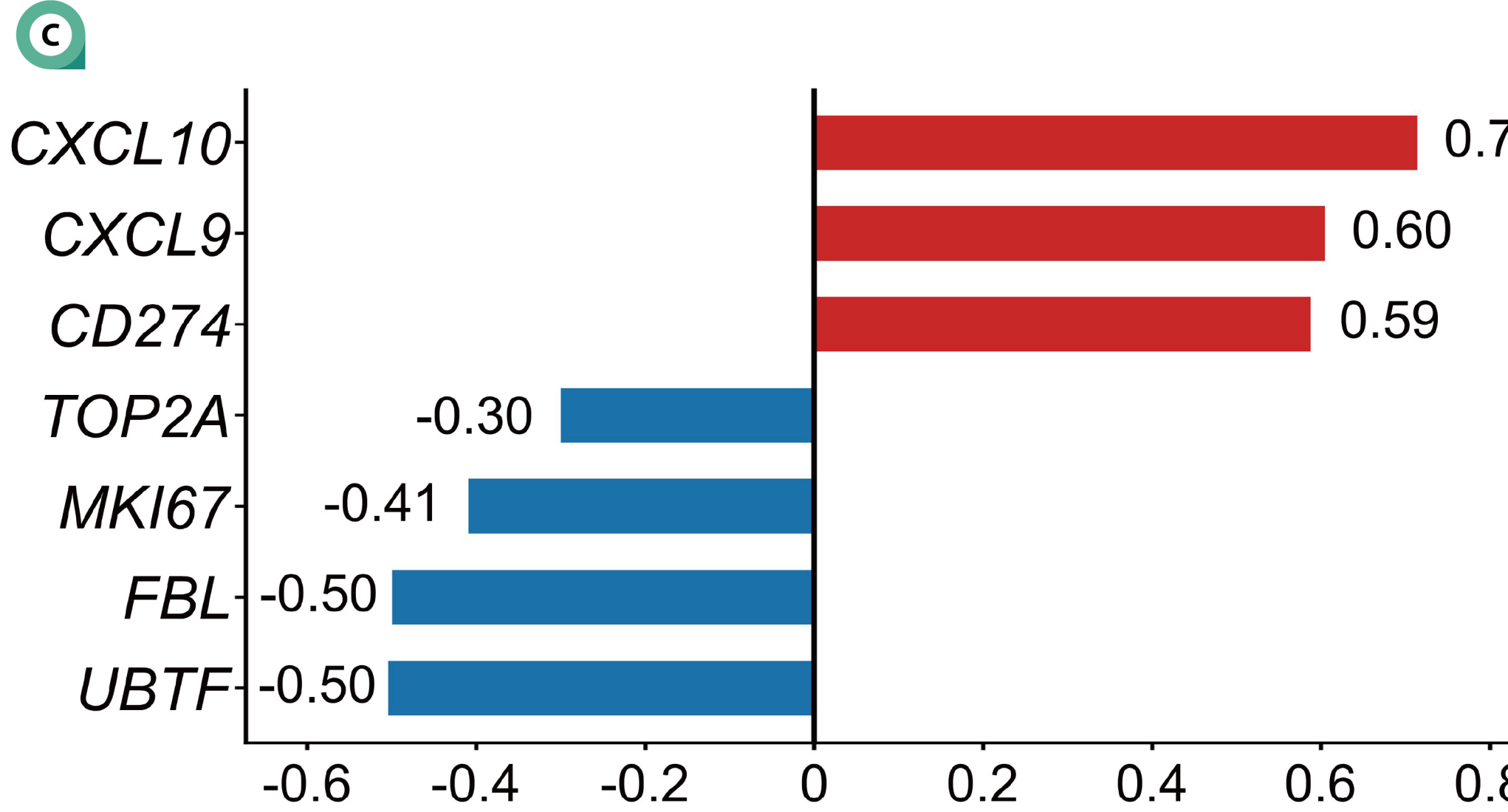
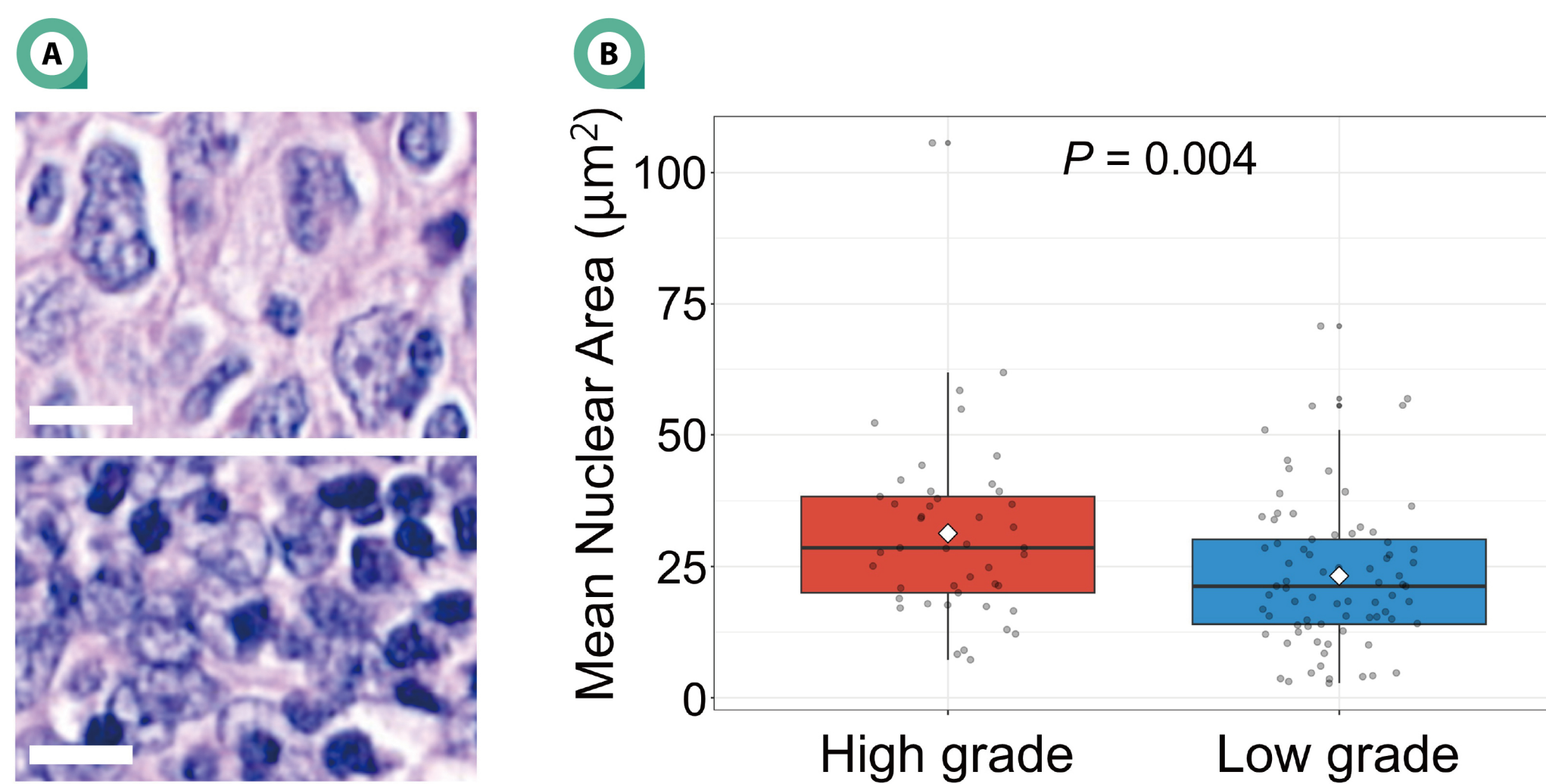


Figure 1. Divergence between morphological nuclear enlargement and bulk transcriptomic signatures in thymomas. (A) Representative hematoxylin-eosin staining of thymoma type B1 (left) and B3 (right). Scale bars: 10 μm . (B) Quantitative digital morphometry demonstrates a significantly larger mean nuclear area in high-grade thymomas ($P = 0.004$). (C) Spearman correlation coefficients between STAT1 expression and selected genes in TCGA-THYM. (D) GSEA of genes ranked by their correlation with STAT1.