From tissue architecture to precision oncology: spatial transcriptomics of bladder cancer

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Urothelial carcinoma of the urinary bladder is a biologically heterogeneous malignancy characterized by substantial variability in its morphological and molecular features. The aim of this study was to evaluate the potential of spatial transcriptomics for identifying biologically distinct tumour populations within invasive urothelial carcinoma. Spatial transcriptomic profiling was performed on a formalin-fixed paraffin-embedded (FFPE) specimen of invasive bladder carcinoma using the 10x Genomics Visium Spatial Gene Expression platform. Transcriptomic data were analysed using cluster analysis and differential gene expression analysis. Six transcriptionally distinct clusters with a clear spatial organization were identified. Cluster 1 corresponded to superficial papillary non-invasive tumour formations, whereas Clusters 2 and 4 were localized within deeper papillary structures. Cluster 6 was localized at the invasive tumour front. Clusters 3 and 5 represented transitional populations containing stromal and inflammatory cells. Expression of EPCAM was detected in all tumour-associated clusters, confirming their epithelial origin. The superficial cluster was characterized by genes associated with epithelial differentiation, mucosal defence, and maintenance of epithelial polarity. The deeper Cluster 2 exhibited increased expression of cell-cycle and proliferation-related genes, including PCLAF, PLK1, STMN1, and E2F3. Cluster 4 was associated with metabolic and signalling pathways. The invasive Cluster 6 demonstrated increased expression of genes related to mitotic activity as well as genes involved in immune processes. This pilot analysis confirmed marked transcriptional heterogeneity among individual tumour compartments. The findings demonstrate the potential of spatial transcriptomics for investigating the biological diversity of urothelial carcinoma and for identifying molecular mechanisms associated with tumour progression and invasion.

Keywords: bladder cancer, spatial transcriptomics, diagnostic markers, prognostic markers

Od tkanivovej architektúry k precíznej onkológii: priestorová transkriptomika karcinómu močového mechúra

Abstrakt

Urotelový karcinóm močového mechúra patrí medzi biologicky heterogénne nádory s výraznou variabilitou morfologických a molekulových vlastností. Cieľom práce bolo zhodnotiť možnosti priestorovej transkriptomiky pri identifikácii biologicky odlišných nádorových populácií v invazívnom urotelovom karcinóme. Analýza bola realizovaná pomocou platformy 10X Genomics Visium Spatial Gene Expression na formalínom fixovanej v parafíne zaliatej (FFPE) vzorke invazívneho karcinómu močového mechúra. Transkriptomické dáta boli analyzované prostredníctvom klastrovej analýzy a diferenciálnej expresnej analýzy génov. Identifikovaných bolo šesť transkripčne odlišných klastrov s jasnou priestorovou organizáciou. Klaster 1 zodpovedal povrchovým papilárnym neinvazívnym nádorovým formáciám, zatiaľ čo klastre 2 a 4 boli lokalizované v hlbších papilárnych štruktúrach. Klaster 6 bol lokalizovaný v oblasti invazívneho infiltračného frontu. Klastre 3 a 5 predstavovali prechodné populácie so zastúpením stromálnych a zápalových buniek. Vo všetkých nádorových klastroch bola prítomná expresia génu EPCAM, potvrdzujúca ich epitelový pôvod. Povrchový klaster bol charakterizovaný génmi spojenými s epitelovou diferenciáciou, slizničnou ochranou a bunkovou polaritou. Hlbší klaster 2 vykazoval zvýšenú expresiu génov bunkového cyklu a proliferácie vrátane PCLAF, PLK1, STMN1 a E2F3. Klaster 4 bol asociovaný s metabolickými a signalizačnými procesmi. Invazívny klaster 6 vykazoval zvýšenú expresiu génov súvisiacich s mitotickou aktivitou a zároveň génov zapojených do imunitných procesov. Realizovaná pilotná analýza potvrdila výraznú transkripčnú heterogenitu medzi jednotlivými nádorovými kompartmentmi. Výsledky dokumentujú potenciál priestorovej transkriptomiky pri štúdiu biologickej diverzity urotelového karcinómu a pri identifikácii molekulových mechanizmov spojených s nádorovou progresiou a inváziou

Kľučové slová: karcinóm močového mechúra, priestorová transkriptomika, diagnostické markery, prognostické markery

Newslab, 2025, roč. 15 (2): 66-77

Introduction

Urinary bladder carcinoma is a relatively common malignancy with an increasing incidence worldwide. According to GLOBOCAN 2022 data, it is the 9th most frequently diagnosed cancer, with over 610.000 new cases and over 220.000 deaths annually. Approximately 75% of cases are observed in males, who are affected 3-4x more frequently than females (1, 2).

The highest incidence is reported in high-income countries, and the strongest demographic risk factor is increasing age, with 90% of patients diagnosed after the age of 55. The overall prognosis is variable. The 5-year survival ranges from 96% for non-invasive and lamina propria-invasive tumours to 5% in cases with distant metastases (3, 4). Tobacco smoking is an important environmental and modifiable risk factor. Tobacco smoke contains polycyclic aromatic hydrocarbons and aromatic amines, which, when excreted in urine, are potent urothelial carcinogens. Additional risk factors include chronic bladder inflammation and chronic irritation from long-term catheterisation. In some countries, the crucial risk factor is chronic Schistosoma infection. (5).

Genetic predisposition is less prominent, but hereditary syndromes, such as Lynch syndrome, may also predispose to urothelial carcinoma, and germline DNA mutations can be identified in approximately 14% of bladder cancer patients. (6, 7). Bladder carcinogenesis follows at least partially distinct molecular pathways that differ from early dysplasia through papillary precursor lesions to invasive carcinoma. Early events in low-grade papillary tumours frequently include loss of chromosome 9 and activating mutations in the FGFR3 gene. TP53 mutations and additional chromosomal aberrations characterise progression to high-grade dysplasia. The critical point in carcinogenesis is a progression from non-invasive disease to lamina propria-invasive (pT1) and subsequently muscle-invasive (pT2) disease (8, 9). At this stage, urothelial tumours exhibit pronounced intratumoral molecular heterogeneity.

Gene	Full name	Main biological function	Functional category
C10orf99 (GPR15L)	G protein-coupled receptor 15 ligand	Antimicrobial peptide expressed in epithelial tissues	Mucosal defence / epithelial protection
KIAA1324 (EIG121)	Estrogen-induced gene 121	Regulates autophagy and epithelial cell survival	Epithelial homeostasis
CLIC3	Chloride intracellular channel 3	Endosomal recycling and extracellular matrix remodelling	Membrane trafficking / ECM remodelling
FBLN1	Fibulin-1	Extracellular matrix glycoprotein involved in tissue integrity and epithelial differentiation	Extracellular matrix / epithelial differentiation
P2RY2	Purinergic receptor P2Y2	Regulates proliferation, differentiation and ion secretion in epithelial cells	Cell signalling
FDX1	Ferredoxin 1	Mitochondrial protein involved in steroid biosynthesis and copper metabolism	Cellular metabolism
PIFO	Pitchfork	Participates in ciliogenesis and ciliary formation	Ciliogenesis
SLPI	Secretory leukocyte protease inhibitor	Antimicrobial and anti-inflammatory protein of mucosal surfaces	Mucosal defence / inflammation control
MAL	Myelin and lymphocyte protein	Lipid raft-associated protein involved in apical transport in polarized epithelial cells	Epithelial polarity / membrane transport
MREG	Melanoregulin	Regulates vesicular trafficking and exocytosis	Vesicular transport
MALL	MAL-like protein	Involved in apical membrane transport, functionally related to MAL	Epithelial polarity / membrane transport

Table 1. Representative marker genes identified in Cluster 1 and their principal biological functions. The cluster was predominantly localised within superficial papillary non-invasive tumour structures and was characterised by genes associated with epithelial differentiation, mucosal defence, membrane trafficking, epithelial polarity, and tissue homeostasis (21–23, 38).

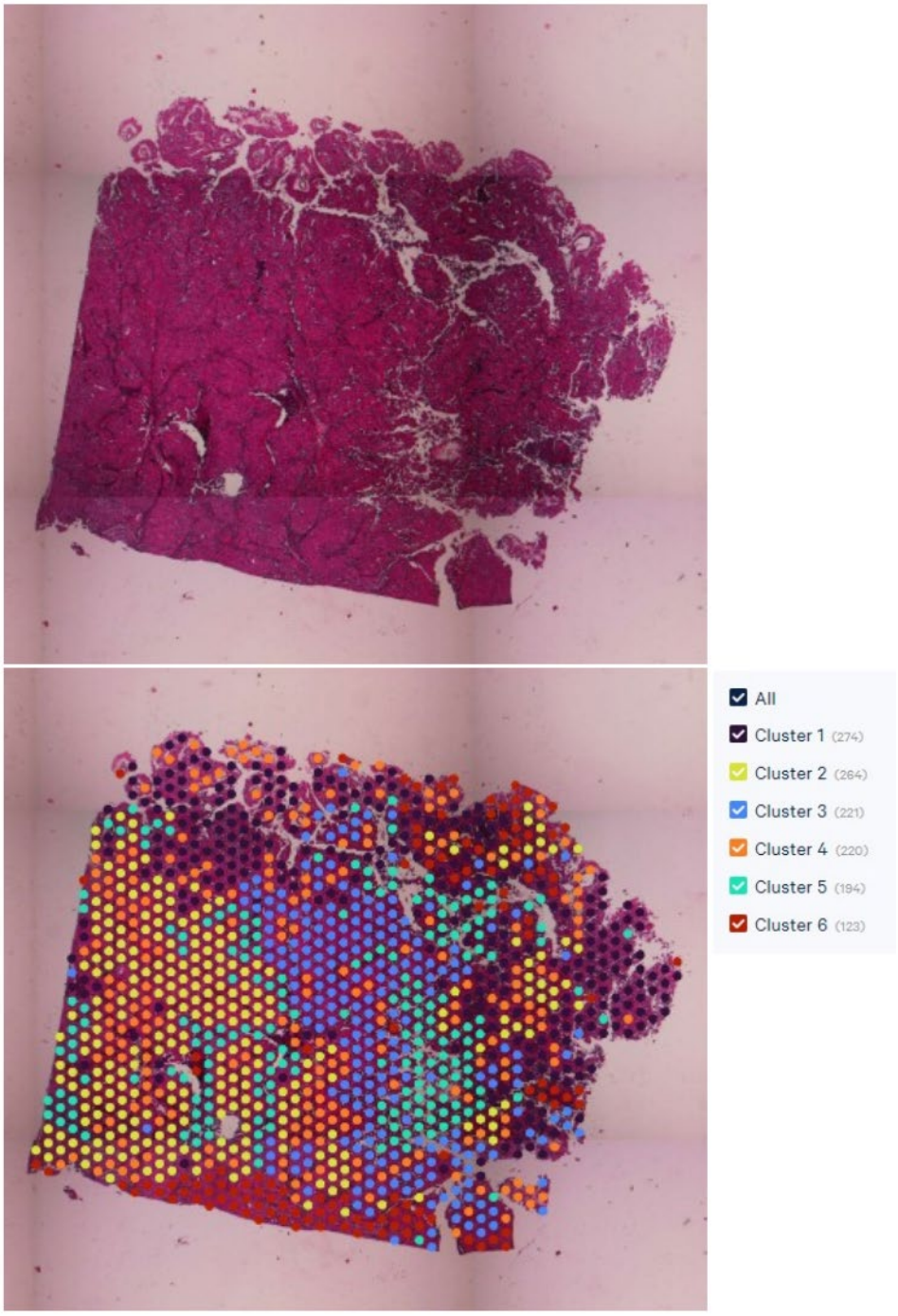

Figure 1. Spatial transcriptomic clustering of invasive urothelial carcinoma. Representative hematoxylin and eosin (H&E)-stained tissue section (top) and corresponding spatial transcriptomic map generated using the 10x Genomics Visium platform (bottom). Unsupervised graph-based clustering identified six transcriptionally distinct clusters within the analysed tumour specimen. Cluster 1 (black) was predominantly localised within superficial non-invasive papillary tumour structures. Clusters 2 (yellow-green) and 4 (orange) were mainly distributed within deeper papillary formations extending towards the bladder wall. Cluster 6 (dark red) was concentrated at the invasive tumour front and corresponded to areas of infiltrative growth. Clusters 3 (blue) and 5 (green) represented transitional populations located between the major tumour compartments and were associated with regions containing stromal elements, inflammatory infiltrates, and mixed cellular populations. The spatial distribution of clusters demonstrates marked intratumoral transcriptional heterogeneity and reveals distinct molecular profiles associated with superficial, deep, and invasive tumour compartments.

Functional group	Genes
Epithelial differentiation and homeostasis	KIAA1324, FBLN1, MAL, MALL
Mucosal defence and antimicrobial activity	C10orf99, SLPI
Membrane trafficking and epithelial polarity	CLIC3, MAL, MALL, MREG
Cell signalling and regulation	P2RY2
Metabolism	FDX1
Ciliogenesis	PIFO

Table 2. Summary of the major functional groups represented by genes in Cluster 1. The identified genes were categorized according to their predominant biological functions. The transcriptional profile of Cluster 1 is consistent with a differentiated epithelial phenotype characterized by preserved epithelial polarity, active membrane transport, mucosal protective mechanisms, and tissue homeostasis. These findings are in accordance with the spatial localization of Cluster 1 within superficial papillary non-invasive tumour formations.

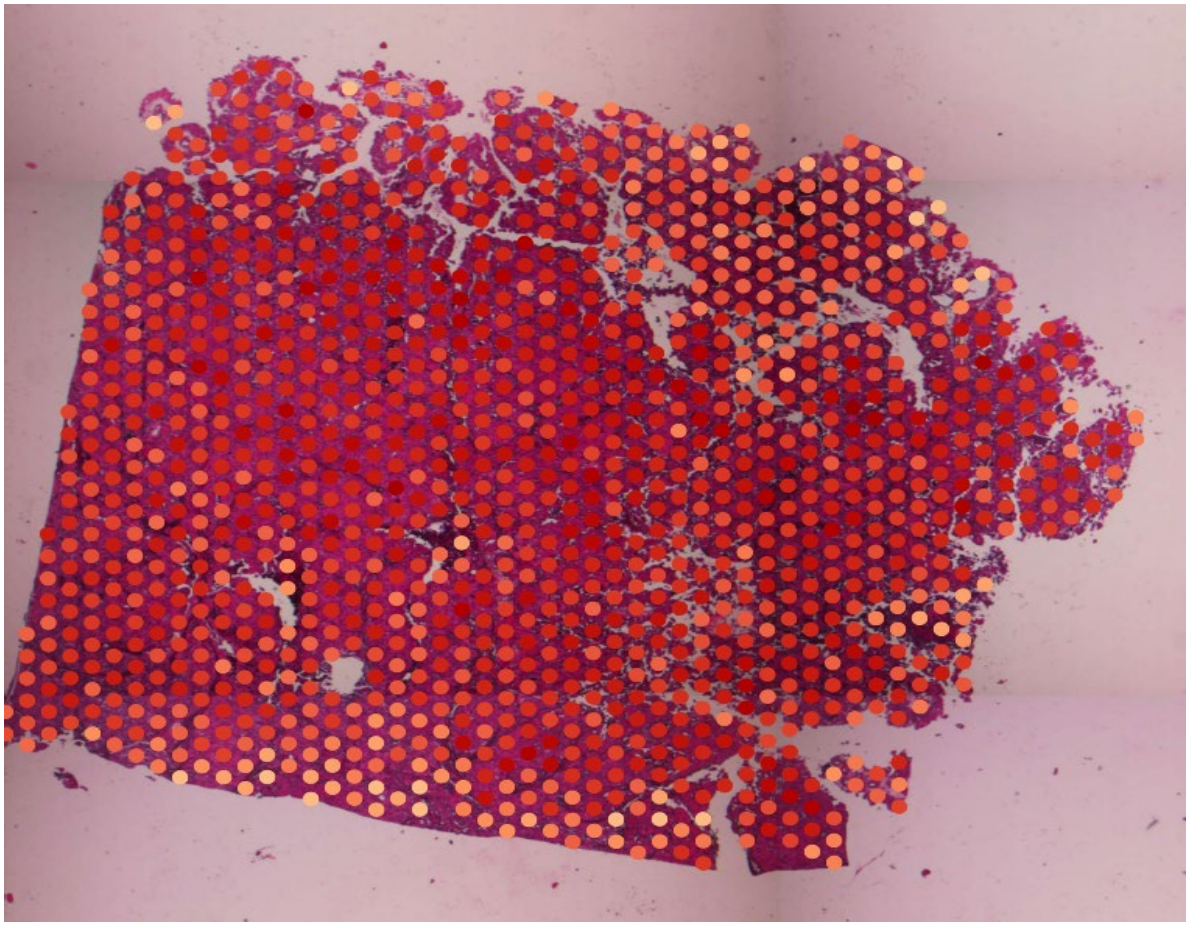

Figure 2. Spatial distribution of EPCAM expression within the analysed urothelial carcinoma specimen. Spatial transcriptomic mapping demonstrated widespread EPCAM expression across the tumour tissue. EPCAM-positive spots were detected throughout all major tumour-associated clusters, confirming the predominantly epithelial origin of the analysed cellular populations. The relatively homogeneous distribution of EPCAM expression across superficial, deep, and invasive tumour compartments indicates preservation of epithelial lineage characteristics despite the marked transcriptional heterogeneity observed between individual clusters. Colour intensity corresponds to normalised EPCAM expression levels within individual Visium spots.

Gene	Full name	Main biological function	Functional category
PCLAF	PCNA Clamp Associated Factor	Regulates DNA replication and progression through the S phase of the cell cycle	DNA replication / Cell cycle
PTTG1	Pituitary Tumor-Transforming Gene 1 (Securin)	Controls chromatid segregation during mitosis by inhibiting separase until anaphase	Mitosis / Chromosome segregation
PLK1	Polo-Like Kinase 1	Essential for mitotic entry, spindle assembly, and cytokinesis	Cell cycle / Mitosis
STMN1	Stathmin 1	Regulates microtubule dynamics and mitotic spindle formation	Mitosis / Cytoskeleton regulation
E2F3	E2F Transcription Factor 3	Controls G1/S transition and transcription of DNA replication genes	Cell cycle regulation
ASF1B	Anti-Silencing Function 1B Histone Chaperone	Histone chaperone associated with cellular proliferation and cell-cycle progression	Chromatin regulation / Proliferation
ASRGL1	Asparaginase-Like Protein 1	Catalyzes hydrolysis of L-asparagine and isoaspartyl peptides; linked to tumorigenesis	Metabolism / Tumour progression
FABP5	Fatty Acid-Binding Protein 5	Promotes tumour cell proliferation and survival through lipid signalling pathways	Lipid metabolism / Tumour proliferation

Table 3. Representative marker genes identified in Cluster 2 and their principal biological functions. The cluster was predominantly localized within deeper papillary tumour structures and was characterized by genes involved in DNA replication, cell-cycle progression, mitotic regulation, cellular proliferation, and tumour growth (23, 39–41).

Functional group	Genes
DNA replication and S-phase progression	PCLAF, E2F3
Mitosis and chromosome segregation	PTTG1, PLK1, STMN1
Cell-cycle regulation and proliferation	PCLAF, PLK1, E2F3, ASF1B, STMN1
Chromatin organization	ASF1B
Metabolism and tumour progression	ASRGL1, FABP5
Lipid signalling and tumour survival	FABP5

Table 4. Summary of the major functional groups represented by genes in Cluster 2. The transcriptional profile of Cluster 2 is consistent with a highly proliferative tumour cell population characterised by active DNA replication, cell-cycle progression, mitotic activity, and enhanced tumour cell survival. The predominance of genes involved in cell-cycle regulation and mitosis suggests that this cluster represents a biologically aggressive proliferative compartment located within the deeper papillary regions of the tumour.

This heterogeneity reflects the diverse histological and molecular subtypes and participates in variable clinical behaviour (10, 11).

The molecular complexity makes diagnosing and classifying urothelial carcinoma more difficult and can result in varied responses to treatment. Traditional morphological classification is often insufficient to fully describe disease biology (10, 11). Integrating mutational, transcriptomic, epigenetic, and proteomic profiles enables molecular subtyping with potential prognostic and predictive value. In this context, spatial transcriptomics has recently emerged as a promising approach to better characterise tumour biology. The spatial analysis of gene expression mapped to specific locations within a tissue section helps to better understand cellular changes in their histological context, which is especially important in malignancies characterised by marked intratumoral heterogeneity, such as urothelial carcinoma. (12, 13).

Methods

The multidisciplinary study "BLAC-GPRO" (Genetic Profile Variability in Bladder Cancer: Implications for Diagnosis and Therapy) was realised in collaboration with Comenius University in Bratislava (Faculty of Medicine and University Science Park) and the University Hospital in Bratislava. The study protocol was approved by the ethics committee, and the participants signed the informed consent. To date, 257 patients are enrolled. The inclusion criteria comprised patients with clinical pathology of the urinary bladder or suspected bladder carcinoma who were indicated for

Gene	Full name	Main biological function	Functional category
OAS1	2'-5'-Oligoadenylate Synthetase 1	Synthesizes 2'-5'-oligoadenylates and activates RNase L as part of the innate antiviral response	Antiviral immunity
MAPK3 (ERK1)	Mitogen-Activated Protein Kinase 3	Regulates signalling pathways involved in proliferation, differentiation, and innate immune responses	Signal transduction
ASS1	Argininosuccinate Synthase 1	Catalyzes argininosuccinate synthesis in the urea cycle and arginine metabolism	Amino acid metabolism
PLA2G2F	Phospholipase A2 Group IIF	Secreted phospholipase involved in lipid metabolism and inflammatory responses	Lipid metabolism / Inflammation
SCNN1G	Sodium Channel Epithelial 1 Gamma Subunit	Component of the epithelial sodium channel (ENaC), essential for sodium transport and reabsorption	Ion transport
ZNF90	Zinc Finger Protein 90	Putative transcription factor involved in the regulation of gene expression	Transcriptional regulation

Table 5. Representative marker genes identified in Cluster 4 and their principal biological functions. This cluster exhibited a heterogeneous transcriptional profile associated with signal transduction, metabolic processes, interferon-related responses, ion transport, and transcriptional regulation (26, 42, 43).

Functional group	Genes
Antiviral and innate immunity	OAS1, MAPK3
Signal transduction	MAPK3
Amino acid metabolism	ASS1
Lipid metabolism and inflammatory response	PLA2G2F
Ion transport and epithelial homeostasis	SCNN1G
Transcriptional regulation	ZNF90

Table 6. The transcriptional profile of Cluster 4 represents the most functionally heterogeneous cluster identified in the analysis. The enriched genes are involved in diverse biological processes, including innate antiviral immunity, signal transduction, amino acid metabolism, lipid metabolism, ion transport, and transcriptional regulation. Unlike Clusters 1, 2, and 6, no single dominant biological pathway is evident. These findings suggest that Cluster 4 may represent a metabolically and functionally adaptive tumour population reflecting local microenvironmental influences within deeper tumour compartments.

diagnostic biopsy. Patients with concurrent multiple malignancies and patients who had previously received neoadjuvant chemotherapy were excluded from the study.

In this report, we present the results of a pilot spatial transcriptomic analysis of invasive urothelial carcinoma, comparing superficial and deep tumour compartments. This approach identified spatially distinct gene expression patterns associated with tumour invasion and interactions with the tumour microenvironment.

Spatial transcriptomic profiling was performed on a selected formalin-fixed, paraffin-embedded (FFPE) bladder tumour specimen using the 10x Genomics Visium Spatial Gene Expression platform (10x Genomics, Pleasanton, CA, USA). Whole-transcriptome sequencing libraries were generated from tissue sections captured on Visium slides, enabling alignment and visualisation of mRNA expression profiles directly within their histological context. Raw sequencing data were processed using Cell Ranger (10x Genomics), and gene expression profiles were visualised in Loupe Browser v 9.1.0 (10x Genomics) according to the manufacturer's recommendations.

To further investigate intratumoral transcriptional heterogeneity, the dataset was reanalysed. Secondary analysis and reclustering were performed directly within the software's reanalysis module. Dimensionality reduction via UMAP was executed with the parameters optimised to min_dist = 0.05 and neighbors number = 10, using the top 30 principal components. Graph-based clustering was subsequently performed using the integrated Louvain algorithm at a resolution of 0.05. Differential expression analysis was performed for each cluster, and genes with the highest positive log2 fold-change (L2FC) values were selected as representative cluster-associated markers.

The resulting L2FC values were visualised as heatmaps in GraphPad Prism version 11.0.2 (GraphPad Software,

Gene	Full name	Main biological function	Functional category
CENPF	Centromere Protein F	Localizes to kinetochores during mitosis and is essential for accurate chromosome segregation	Kinetochore / Mitosis
CENPBD1	CENP-B DNA-Binding Domain Containing 1	Centromere-associated protein involved in chromosome organization	Kinetochore / Centromere function
MIS12	MIS12 Kinetochore Complex Component	Component of the MIS12 complex linking inner and outer kinetochores and enabling chromosome attachment to spindle microtubules	Kinetochore / Chromosome segregation
BLNK	B-Cell Linker Protein	Adaptor protein required for B-cell receptor signalling and B-cell development	Adaptive immunity / B-cell signalling
IGHG1	Immunoglobulin Heavy Constant Gamma 1	Encodes the constant region of the IgG1 heavy chain and participates in humoral immune responses	Humoral immunity
NMNAT1	Nicotinamide Nucleotide Adenylyltransferase 1	Catalyzes NAD+ biosynthesis and contributes to cellular homeostasis and DNA repair	Cellular metabolism
GGPS1	Geranylgeranyl Diphosphate Synthase 1	Synthesizes geranylgeranyl diphosphate required for prenylation of small GTPases and intracellular signalling	Cellular metabolism / Signal transduction

Table 7. Representative marker genes identified in Cluster 6 and their principal biological functions. The cluster corresponded to the invasive tumour front and was characterized by genes involved in chromosome segregation, kinetochore assembly, mitotic progression, immune-related processes, and cellular metabolism (26, 28).

Functional group	Genes
Kinetochore assembly, mitosis and chromosome segregation	CENPF, CENPBD1, MIS12
B-cell signalling and humoral immunity	BLNK, IGHG1
Cellular metabolism and signalling	NMNAT1, GGPS1

Table 8. The transcriptional profile of Cluster 6 comprises two major biological modules. The first are involved in kinetochore assembly, chromosome segregation, and mitotic progression, indicating active cellular proliferation. The second consists of genes associated with B-cell receptor signalling and humoral immune responses. The coexistence of mitotic and immune-related genes suggests that Cluster 6 may represent a biologically distinct invasive compartment characterised by active proliferation and close interaction with the local immune microenvironment, rather than a single uniform biological pathway.

Boston, MA, USA), enabling direct comparison of cluster-specific transcriptional signatures across superficial, deep, and invasive tumour compartments.

The manuscript was linguistically reviewed and grammatically corrected using AI Grammarly (Grammarly Inc., San Francisco, CA, USA).

Results

Spatial transcriptomic analysis identified six transcriptionally distinct clusters within the analysed urothelial carcinoma specimen. Correlation of the spatial transcriptomic map with the corresponding histological structure revealed a clear spatial organisation of the identified clusters (Figure 1).

Cluster 1 was predominantly localised within superficial, non-invasive papillary tumour structures. Clusters 2 and 4 were mainly detected in deeper papillary formations extending into the bladder wall. Cluster 6 was concentrated at the invasive tumour front and corresponded to areas of infiltrative tumour growth. Clusters 3 and 5 represented transitional populations located between the major tumour compartments and were associated with regions containing stromal elements, inflammatory infiltrates, and mixed cellular populations.

Expression of EPCAM was detected across all analysed tumour-associated clusters, supporting the epithelial origin of the cells (Figure 2). Differential expression analysis identified distinct groups of genes showing increased expression within individual clusters compared with the remaining cell populations (Figure 3).

The superficial Cluster 1 was characterised by increased expression of C10orf99, KIAA1324, CLIC3, FBLN1, P2RY2, FDX1, PIFO, SLPI, MAL, MREG and MALL. The genes identified in this cluster are predominantly associated with the functions of differentiated epithelial tissues and mucosal surfaces, including epithelial polarity, mucosal defence, and tissue homeostasis. These findings are consistent with a relatively differentiated urothelial phenotype corresponding to superficial papillary tumour components (Table 1-2).

Cluster 2 demonstrated increased expression of ZMAT4, PCDH10, FABP5, ASRGL1, STEAP1, PCLAF, PTTG1, PLK1, STMN1, E2F3 and ASF1B. Several of these genes are directly involved in DNA replication, cell-cycle progression, mitotic regulation and cellular proliferation. This cluster is strongly associated with cellular proliferation and cell-cycle progression. The observed transcriptional profile suggests enhanced proliferative activity within deeper tumour compartments (Table 3-4).

Cluster 4 was characterised by increased expression of ZNF90, ASS1, PLA2G2F, SCNN1G, MAPK3 and OAS1. In

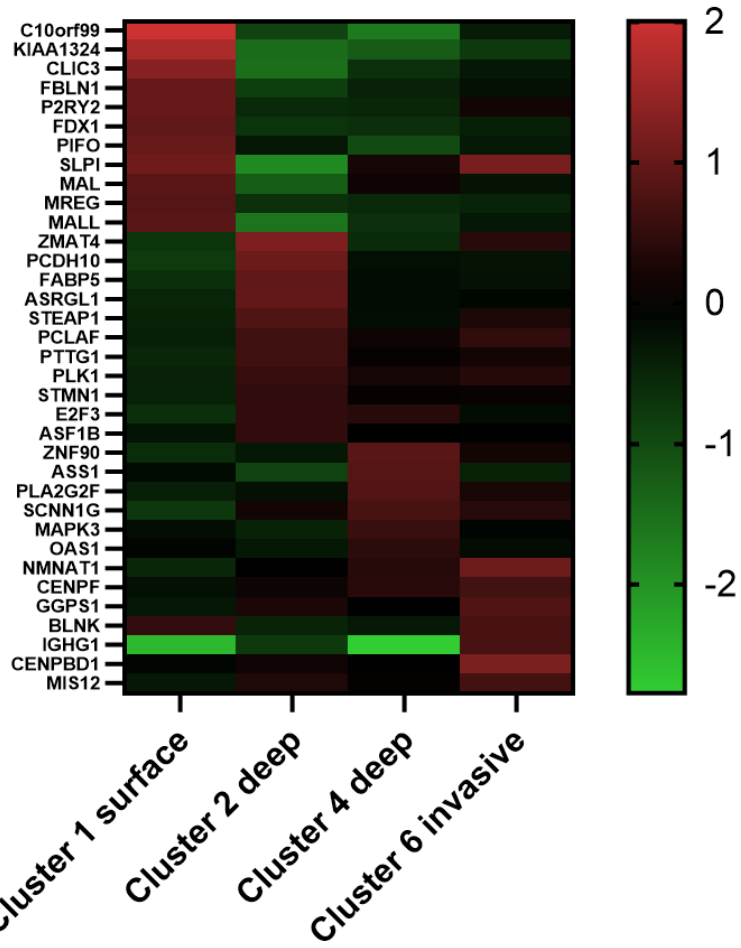

Figure 3. Heatmap of representative cluster-associated genes identified by spatial transcriptomic analysis of urothelial carcinoma. The heatmap demonstrates distinct transcriptional signatures corresponding to superficial papillary (Cluster 1), deep papillary (Clusters 2 and 4), and invasive (Cluster 6) tumour compartments. Cluster 1 was enriched for genes associated with epithelial differentiation and mucosal homeostasis, Cluster 2 for genes involved in cell-cycle progression and proliferation, Cluster 4 for genes related to signalling, metabolism, and innate immune responses, and Cluster 6 for genes associated with mitotic activity and immune-related functions. The observed expression patterns highlight substantial intratumoral transcriptional heterogeneity and clear molecular segregation of superficial, deep, and invasive tumour regions.

contrast to Cluster 2, the genes identified in this cluster did not form a single dominant biological pathway. Instead, they are associated with a broad range of cellular processes, including antiviral immunity, signal transduction, amino acid metabolism, lipid metabolism, ion transport, and transcriptional regulation (Table 5-6).

Cluster 6, corresponding to the invasive tumour front, demonstrated increased expression of NMNAT1, CENPF, GGPS1, BLNK, IGHG1, CENPBD1 and MIS12. Functional analysis revealed enrichment for genes involved in chromosome segregation, kinetochore assembly, and mitotic progression (CENPF, CENPBD1, MIS12), as well as genes associated with immune-related functions (BLNK, IGHG1). These findings indicate a biologically distinct invasive compartment characterised by active cellular proliferation and interactions with the local immune microenvironment (Table 7-8).

To compare cluster-specific transcriptional signatures, genes from Clusters 1, 2, 4, and 6 were visualised on a heatmap of log2 fold-change values (Figure 3). The heatmap showed clear separation of superficial, deep, and invasive tumour compartments and revealed transcriptional heterogeneity within the urothelial carcinoma specimen.

Discussion

Bladder urothelial carcinoma is characterised by significant intratumoral heterogeneity in both morphology and biological behaviour. This diversity reflects distinct transcriptional programs among cells within the same tumour and strongly influences progression, invasiveness, and treatment response (14–16). The present pilot spatial transcriptomic analysis demonstrates the ability of the 10X spatial transcriptomic method to identify biologically distinct tumour cell populations within urothelial carcinoma while preserving precise histological localisation. Unlike conventional transcriptomic approaches, spatial transcriptomics enables direct correlation between gene expression profiles and morphological structures, revealing intratumoral heterogeneity.

Recent studies combining single-cell and spatial transcriptomic approaches have demonstrated the presence of multiple epithelial subpopulations within bladder cancer, each characterised by distinct biological functions, mutational burdens, and spatial localisation (17–19). Our pilot study identified six transcriptionally distinct clusters in the analysed tumour, corresponding to superficial papillary tumour formations, deeper tumour compartments, transitional stromal regions, and the invasive tumour front. These findings highlight the considerable molecular complexity of urothelial carcinoma and illustrate how morphologically related tumour areas may harbour markedly different transcriptional programs. Spatial transcriptomic analyses of recurrent bladder tumours reveal more pronounced interactions among epithelial cells, immune cells, and the extracellular matrix than in primary tumours, despite comparable or lower abundances of malignant and immune cell populations (20). Despite molecular differences among individual tumour compartments, EPCAM expression remained detectable across all major tumour-associated clusters, confirming their epithelial origin.

The identified clusters appeared to represent different biological states of tumour progression. The superficial papillary compartment was enriched in genes associated with epithelial differentiation, mucosal defence, membrane trafficking, and maintenance of epithelial polarity, consistent with a relatively differentiated urothelial phenotype (21–23). In contrast, deeper clusters demonstrated strong enrichment of genes involved in DNA replication, mitosis, and cell-cycle progression, suggesting the presence of a highly proliferative tumour population (24, 25). In deeper parts of the tumour, the expression profile was more heterogeneous, involving metabolic pathways, interferon-related responses, and signal transduction, potentially reflecting tumour adaptation to local microenvironmental conditions. The invasive tumour front exhibited increased expression of genes associated with chromosome segregation and mitotic activity, as well as immune-related genes, indicating biologically distinct features characterised by active proliferation and interactions with the surrounding immune microenvironment (26–28).

The identification of a deeper highly proliferative compartment, dominated by genes involved in DNA replication, mitosis, and cell-cycle progression, is a reproducible finding across molecular classification systems. The International Society of Urological Pathology consensus recognises that bladder cancer has three major molecular subtypes—luminal, basal-squamous, and neuroendocrine, which are associated with considerable biological diversity (29). The luminal unstable subtype is characterised by higher cell-cycle activity and genomic instability compared with other luminal subtypes. The genes identified in Cluster 2 are well-established markers of proliferative activity in urothelial carcinoma. E2F3 amplification has been reported in approximately 10% of urothelial carcinomas and is associated with tumour progression (30). The spatial localisation of this proliferative signature in deeper tumour regions suggests a gradient of increasing proliferative activity from the tumour surface toward the invasive front, a pattern observed in other epithelial malignancies.

The heterogeneous expression profile observed in Cluster 4, involving metabolic pathways, interferon-related responses, and signal transduction, may reflect the

complex metabolic and immunological adaptations that occur within the tumour microenvironment. Metabolic reprogramming is a hallmark of bladder cancer, with tumour cells exhibiting altered glycolysis and amino acid metabolism that directly influence the surrounding immune landscape (31). The presence of interferon-stimulated genes in this compartment is particularly noteworthy, as interferon signalling plays a dual role in the biology of urothelial carcinoma. On the one hand, it has been shown to drive the transition from luminal to basal-squamous transcriptional states; on the other hand, interferon signalling activates cancer-associated fibroblast subpopulations that promote cancer stemness, associated with poor outcomes and resistance to chemotherapy and immunotherapy (32–34).

The invasive tumour front exhibited a distinctive combination of mitotic activity and immune-related gene expression. This can have significant biological and clinical implications. The invasive front is recognised as a biologically special region in urothelial carcinoma. The pattern of tumour growth at the invasion front is an independent predictor of survival and progression (35–37). The increased expression of chromosome segregation genes at the invasive front indicates active cell division, consistent with active tumour expansion.

Our findings suggest that tumour invasion is accompanied by morphological alterations that reflect the activation of distinct transcriptional programmes. The spatial transcriptomic analysis enabled clear delineation of superficial, deep, and invasive tumour regions and provided insight into the biological processes associated with tumour progression. Although the present study is based on a single representative case, the results highlight the potential of spatial transcriptomics to identify biologically meaningful tumour populations, improve our understanding of tumour architecture, and facilitate the discovery of biomarkers associated with invasion and disease progression in urothelial carcinoma.

Acknowledgment

Funded by the EU NextGenerationEU through the Recovery and Resilience Plan for Slovakia under the project No. 09I03-03-V03-00046 „Genetic profile variability in urinary bladder carcinoma; implications for diagnostics and therapy“

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