

Inaugural dissertation
for
obtaining the doctoral degree
of the
Combined Faculty of Mathematics, Engineering and Natural Sciences
of the
Ruprecht - Karls - University
Heidelberg

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Oral examination: 17 September 2026

*Characterising HNSCC Heterogeneity: From
HPV Status to Spatial Tumour Organisation*

Referees

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I dedicate my thesis to the children of Gaza who are no longer with us. A lot of them would have a PhD, a lot of them would have become great scientists. I am just lucky to be born into a different geographical location. May you rest in peace in heaven, a far better place.

Abstract

Head and neck squamous cell carcinoma (HNSCC) is a heterogeneous malignancy with variable clinical outcomes. In this dissertation, resolving this heterogeneity is essential for improving patient stratification and treatment-response prediction. I address two forms of that heterogeneity: patient-level outcome variability in HPV-positive disease, and the spatial organisation of transcriptional programmes within HNSCC tumours.

In the first part I investigate the molecular basis of clinical response heterogeneity in HPV-positive HNSCC. Using differential gene expression and DNA methylation analyses in a preclinical xenograft cohort ($n = 18$), intersected with TCGA-HNSCC patient data ($n = 487$), I identified a set of HPV-associated genes from which I derived a cancer-testis (CT) gene panel. In an independent cohort treated with postoperative radiochemotherapy ($n = 195$; HPV-positive subset $n = 87$), I found that low CT-panel expression identified a subgroup with inferior overall survival. This low-CT group was enriched for TP53 alterations and aggressive hallmark pathways, and I show that combining CT expression with TP53 status stratified survival better than either marker alone. These results define an aggressive subset within HPV-positive HNSCC and establish the CT panel as a tool for treatment de-escalation decisions.

In the second part I characterise the spatial transcriptional architecture of HNSCC using Visium FFPE spatial transcriptomics. Profiling twenty sections from seven HNSCC xenograft cell lines with co-registered histology and pimonidazole hypoxia labelling, I applied a non-negative matrix factorisation pipeline and identified five reproducible metaprograms (MPs): epithelial-mesenchymal transition (MP1), hypoxia response (MP2), squamous differentiation (MP3), ECM/stromal-like (MP4), and keratinocyte/squamous-epithelial (MP5). These MPs correspond to conserved cancer cell states and map onto established HNSCC bulk molecular subtypes.

These findings show that HNSCC heterogeneity acts at multiple scales. The CT panel resolves clinically relevant subgroups within HPV-positive disease that current biomarkers do not capture, while the spatial MPs describe transcriptional architecture at tissue resolution. Despite differing in scale, both approaches convert HNSCC heterogeneity into discrete, quantifiable features: a CT expression pattern and a

defined MP composition. Whereas the CT panel was prognostic in clinical cohorts, the spatial MP framework was established in preclinical models and remains to be validated in patient material; both nonetheless provide quantifiable features for future clinical studies.

Zusammenfassung

Das Plattenepithelkarzinom des Kopf-Hals-Bereichs (HNSCC) ist eine heterogene maligne Erkrankung mit variablen klinischen Verläufen. Die Auflösung dieser Heterogenität ist entscheidend, um die Patientenstratifizierung und die Vorhersage des Therapieansprechens zu verbessern. In dieser Dissertation untersuche ich zwei Formen dieser Heterogenität: die patientenbezogene Variabilität des Outcomes bei HPV-positiver Erkrankung und die räumliche Organisation transkriptioneller Programme innerhalb von HNSCC-Tumoren.

Im ersten Teil untersuche ich die molekulare Grundlage der Heterogenität des klinischen Ansprechens beim HPV-positiven HNSCC. Mittels differentieller Genexpressions- und DNA-Methylierungsanalysen in einer präklinischen Xenograft-Kohorte (n = 18), abgeglichen mit Patientendaten des TCGA-HNSCC (n = 487), identifizierte ich eine Reihe HPV-assoziiierter Gene, aus denen ich ein Cancer-Testis-(CT-)Genpanel ableitete. In einer unabhängigen, mit postoperativer Radiochemotherapie behandelten Kohorte (n = 195; HPV-positive Teilgruppe n = 87) zeigte sich, dass eine niedrige CT-Panel-Expression eine Untergruppe mit schlechterem Gesamtüberleben identifizierte. Diese Niedrig-CT-Gruppe war für TP53-Alterationen und aggressive Hallmark-Signalwege angereichert, und ich zeige, dass die Kombination der CT-Expression mit dem TP53-Status das Überleben besser stratifizierte als jeder Marker allein. Diese Ergebnisse definieren eine aggressive Untergruppe innerhalb des HPV-positiven HNSCC und etablieren das CT-Panel als Instrument für Entscheidungen zur Therapiede Eskalation.

Im zweiten Teil charakterisiere ich die räumliche transkriptionelle Architektur des HNSCC mittels Visium-FFPE-Raumtranskriptomik. Durch die Profilierung von zwanzig Schnitten aus sieben HNSCC-Xenograft-Zelllinien mit ko-registrierter Histologie und Pimonidazol-Hypoxiemarkierung wandte ich eine Pipeline zur nichtnegativen Matrixfaktorisierung an und identifizierte fünf reproduzierbare Metaprogramme (MPs): epithelial-mesenchymale Transition (MP1), Hypoxieantwort (MP2), squamöse Differenzierung (MP3), EZM/stromaähnlich (MP4) und Keratinozyten/Plattenepithel

(MP5). Diese MPs entsprechen konservierten Tumorzellzuständen und lassen sich den etablierten molekularen Bulk-Subtypen des HNSCC zuordnen.

Diese Ergebnisse zeigen, dass die Heterogenität des HNSCC auf mehreren Ebenen wirkt. Das CT-Panel löst klinisch relevante Untergruppen innerhalb der HPV-positiven Erkrankung auf, die von aktuellen Biomarkern nicht erfasst werden, während die räumlichen MPs die transkriptionelle Architektur auf Gewebeebene beschreiben. Trotz ihres unterschiedlichen Maßstabs überführen beide Ansätze die Heterogenität des HNSCC in diskrete, quantifizierbare Merkmale: ein CT-Expressionsmuster und eine definierte MP-Zusammensetzung. Während das CT-Panel in klinischen Kohorten prognostisch war, wurde das räumliche Metaprogramm-Konzept in präklinischen Modellen etabliert und muss noch an Patientenmaterial validiert werden; beide liefern jedoch quantifizierbare Merkmale für zukünftige klinische Studien.

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1.Introduction

1.1. Head and Neck Squamous Cell Carcinoma

1.1.1.Epidemiology, Anatomy, and Risk Factors

Head and neck squamous cell carcinoma (HNSCC) is the collective term for malignancies arising from the squamous epithelium of the upper aerodigestive tract, encompassing the oral cavity, oropharynx, hypopharynx, larynx, nasopharynx, and the paranasal sinuses (Johnson et al. 2020). Globally, HNSCC accounts for approximately 900,000 new cases and 450,000 deaths each year, ranking it among the seven most common cancers by incidence (Sung et al. 2021). Incidence varies markedly by anatomical subsite, geography, and exposure to specific risk factors. Tobacco use, both smoking and smokeless, and chronic alcohol consumption together account for the majority of HNSCC cases in most regions, with the two exposures acting synergistically on epithelial carcinogenesis (Johnson et al. 2020). Over the past three decades, the epidemiological landscape has shifted substantially: in North America and parts of Western Europe, infection with high-risk Human papillomavirus (HPV) has emerged as the principal cause of oropharyngeal squamous cell carcinoma, while the incidence of tobacco-associated cancers at other subsites has declined in line with reductions in smoking prevalence (Chaturvedi et al. 2011; Johnson et al. 2020).

The anatomical heterogeneity of the head and neck region is clinically consequential. Tumours of the oral cavity, oropharynx, larynx, and hypopharynx differ in their natural histories, patterns of lymphatic spread, and treatment options. Surgical access varies considerably across subsites; functional impacts on swallowing, breathing, and speech are profound; and the proximity of critical neural and vascular structures constrains the curative use of high-dose radiotherapy (RT) (Chow 2020). These

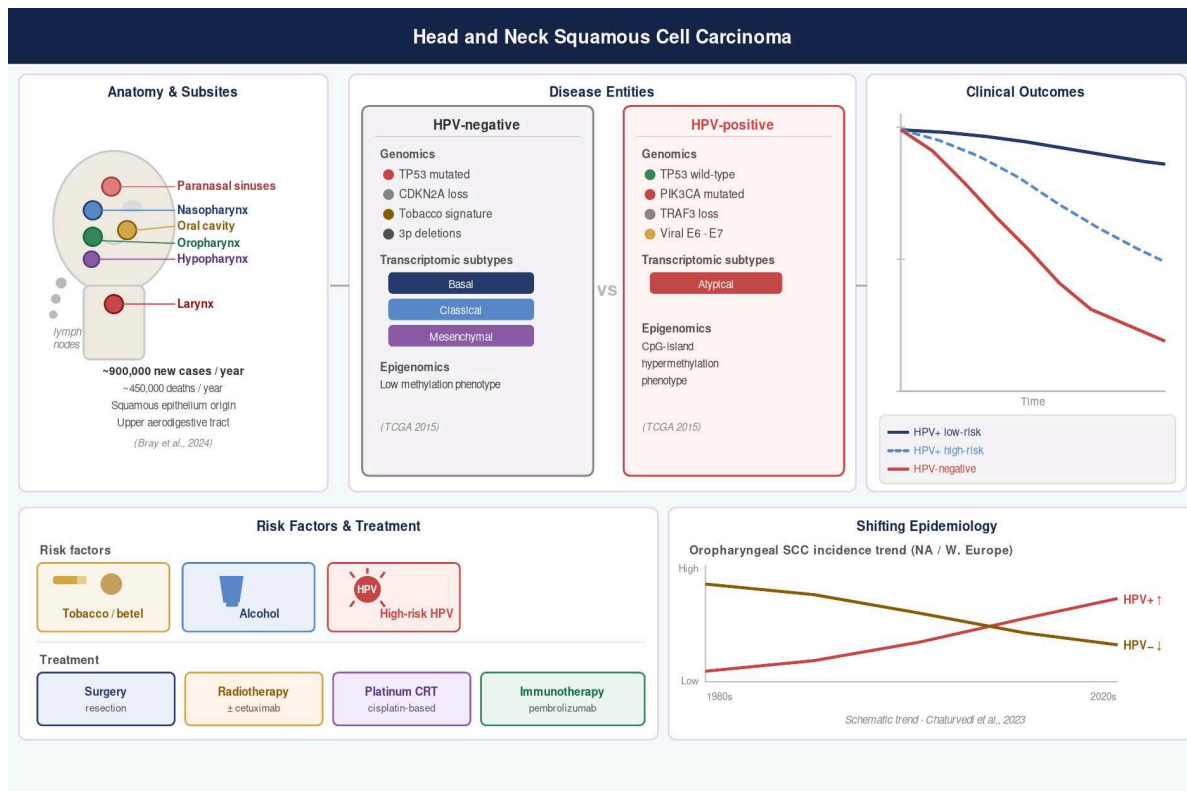


Figure 1. Overview of head and neck squamous cell carcinoma. Depicted are the major anatomical subsites, molecular disease entities (HPV-negative vs. HPV-positive), clinical outcomes, risk factors, treatment approaches, and the shifting epidemiology of oropharyngeal SCC. Figure created with assistance from Claude (Anthropic). Adapted from Bray et al. (2024), Chaturvedi et al. (2023), and Cancer Genome Atlas Research Network (2015).

anatomical considerations shape both clinical decision-making and the design of clinical trials, in which subsite is now routinely used as a stratification variable.

1.1.2. Standard Treatment Types

Treatment of locally advanced HNSCC combines surgery, RT, and platinum-based chemotherapy in subsite- and stage-specific protocols (Chow 2020; Pignon et al. 2009). Cisplatin-containing concurrent RCT remains the standard of care for patients with unresectable locally advanced disease, with the meta-analysis of chemotherapy in head and neck cancer (MACH-NC) demonstrating an absolute five-year survival benefit of approximately 6.5% over RT alone (Pignon et al. 2009). Targeted therapy with the anti-EGFR antibody cetuximab provides an alternative for patients unsuitable for cisplatin (Bonner et al. 2006), although its activity in the HPV-positive subset has

more recently been called into question (Mehanna et al. 2019). In case of recurrence and metastasis, immune checkpoint blockade with pembrolizumab, either as monotherapy or combined with chemotherapy, has become the first-line standard following the KEYNOTE-048 trial (Burtneess et al. 2019; Cohen et al. 2019)

Despite these advances, outcomes in HNSCC remain heterogeneous. Five-year overall survival (OS) ranges from below 40% in locally advanced HPV-negative disease to above 80% in low-risk HPV-positive oropharyngeal carcinoma (Ang et al. 2010). A substantial fraction of patients with similar clinical stage and histology nonetheless experience early recurrence, treatment-refractory disease, or severe long-term toxicity, while others achieve durable cure. Conventional staging systems and pathology assessment do not predict this variability with sufficient resolution to support individualised therapy decisions, motivating intensive efforts in molecular characterisation.

1.1.3. Molecular Characterisation

The Cancer Genome Atlas Network (TCGA) reported a comprehensive characterisation of 279 HNSCCs in 2015, defining the somatic mutational, copy-number, methylation, and transcriptomic landscape of the disease (“Comprehensive Genomic Characterization of Head and Neck Squamous Cell Carcinomas” 2015). The work confirmed that HPV-positive and HPV-negative HNSCCs are genomically distinct: HPV-positive cancers carry few somatic mutations, retain wild-type TP53, and frequently harbour activating PIK3CA mutations and loss of TRAF3, whereas HPV-negative cancers are characterised by TP53 inactivation, CDKN2A loss, 3p deletions, and a strongly tobacco-associated mutational signature. These findings reinforced the view that HPV-positive HNSCC constitutes a biologically separate disease entity within the same histological category (Leemans, Snijders, and Brakenhoff 2018).

Transcriptomic subtyping has provided a complementary aspect on inter-tumour variation. Walter et al. defined four bulk RNA-based subtypes, Atypical, Basal, Classical, and Mesenchymal, that recur across multiple HNSCC datasets and capture distinct biological entities ranging from squamous differentiation programmes through to mesenchymal and immune-infiltrated states (Walter et al. 2013). Keck et al. further subdivided HPV-positive cancers into two transcriptomic distinct subgroups with differing immune infiltrate, and partitioned HPV-negative tumours into three classes

that recapitulate the Walter subtypes (Keck et al. 2015). Epigenomic profiling has added a third axis: HPV-positive HNSCCs exhibit a characteristic hypermethylation phenotype affecting promoters of differentiation and tumour-suppressor genes, distinguishing them from their HPV-negative counterparts (Degli Esposti et al. 2017; Y. Zhang et al. 2016). Integrative multi-omics approaches that combine transcriptomic, methylation, and copy-number data offer a more complete view of these molecular axes than any single modality and have become a standard analytical basis in HNSCC research (Subramanian et al. 2020).

While bulk multi-omics has refined the classification of HNSCC, it does so at the level of the average signal across millions of cells from a tumour fragment. There are two limitations to this. First, subtypes defined on bulk profiles can be unstable in tumours of mixed transcriptional composition. Second, bulk profiles obscure the spatial organisation of distinct transcriptional states within a single tumour. Both limitations motivated me to design the dual-modality adopted in this thesis: bulk multi-omics for the high-throughput identification of robust molecular signals across patient cohorts, and spatial transcriptomics for the resolution of within-tumour architecture.

1.2. HPV-Positive HNSCC & Radiosensitivity

1.2.1. Viral Oncogenesis and Cell-Cycle Dysregulation

Human papillomaviruses are small, non-enveloped, double-stranded DNA viruses with a tropism for stratified squamous epithelium (Pal and Kundu 2019). More than 200 HPV genotypes are recognised; a subset of approximately twelve mucosal types, including HPV16, 18, 31, 33 and 45, are classified as high-risk on the basis of their causal role in cervical, anogenital, and oropharyngeal cancers (zur Hausen 2002). HPV16 accounts for the great majority of HPV-positive HNSCC cases globally (Leemans, Snijders, and Brakenhoff 2018), and HPV16 E6 and E7 expression is functionally

required for the maintenance of the transformed phenotype in vitro and in vivo (Moody and Laimins 2010).

E6 and E7 are the principal viral oncoproteins. E7 binds the retinoblastoma family of pocket proteins, RB1, p107 and p130, and targets them for proteasomal degradation, releasing E2F transcription factors and forcing entry into S phase irrespective of upstream growth signals (Moody and Laimins 2010). E6 forms a complex with the cellular ubiquitin ligase E6AP and promotes the ubiquitination and degradation of p53, neutralising the principal cellular checkpoint that would otherwise initiate apoptosis or growth arrest in response to E7-driven unscheduled proliferation (Pal and Kundu 2019). Together, E6 and E7 produce a state of sustained proliferation in cells that retain a functional but repressed p53 axis. The diagnostic surrogate p16^{INK4a}, the protein product of CDKN2A, is markedly overexpressed in HPV-positive HNSCC: p16 normally serves as a feedback brake on cyclin-D-CDK4/6 activity, but with RB1 inactivated by E7 this feedback is uncoupled from the cell cycle, and p16 accumulates to immunohistochemically detectable levels (Leemans, Snijders, and Brakenhoff 2018). Having p16 staining of more than 70% of tumour cells is the standard surrogate marker used clinically to indicate HPV-driven oncogenesis in oropharyngeal cancer, although it does not distinguish transcriptionally active from inactive virus and is not equivalent to direct detection of viral DNA or E6/E7 transcripts (Lewis et al. 2018).

1.2.2. Prognostic Characteristics and Treatment De-Escalation

The prognostic distinctiveness of HPV-positive oropharyngeal carcinoma was definitively established by Ang et al. (2010), whose retrospective analysis of the RTOG 0129 trial demonstrated a three-year OS of 82.4% in HPV-positive patients versus 57.1% in HPV-negative patients receiving identical concurrent RCT. The prognostic advantage holds across multiple cohorts and is independent of stage and smoking status, although heavy smoking history attenuates it (Ang et al. 2010; Faraji et al. 2019). The consistency of these data led to the introduction of a separate staging system for HPV-positive oropharyngeal cancer in the eighth edition of the AJCC/UICC TNM classification.

The favourable outcomes of HPV-positive HNSCC under standard RCT raised the question of whether treatment intensity, and the associated long-term toxicity,

including xerostomia, dysphagia, and second malignancy, could be safely reduced. Several large randomised de-escalation trials have addressed this question. RTOG 1016 (Gillison et al. 2019) and De-ESCALaTE HPV (Mehanna et al. 2020) compared cetuximab-based RCT with cisplatin-based RCT in HPV-positive oropharyngeal cancer; both trials demonstrated inferior survival with cetuximab, contradicting the hypothesis that cetuximab could replace cisplatin without loss of efficacy. Smaller single-arm and multi-arm trials have explored reductions in radiation dose, omission of concurrent chemotherapy, or transoral surgery followed by adjuvant therapy (Chera et al. 2018), with encouraging but not yet definitive results. The collective experience is that the HPV-positive population is heterogeneous in its response to de-intensified treatment, and that current clinical biomarkers, p16 status, smoking history, and stage, are insufficient to identify patients who would benefit from de-escalation without unacceptable risk of recurrence (Faraji et al., 2017; Chera et al., 2018). The need for additional molecular biomarkers to refine risk stratification within HPV-positive HNSCC was the primary motivation for me for the first experimental part of this thesis.

1.2.3. Molecular Biomarkers for Risk Stratification

Multiple analytical approaches have been pursued to identify molecular biomarkers that could stratify HPV-positive HNSCC patients beyond clinical and pathological criteria. Transcriptomic signatures have been the most extensively developed. Hypoxia gene-expression classifiers, initially developed by Toustrup et al. (2011) and Eustace et al. (2013), capture the radioresistant phenotype of hypoxic tumours and have shown predictive value for benefit from hypoxia-modifying therapy (Eustace et al. 2013; Toustrup et al. 2011). Immune-infiltrate signatures distinguish T-cell-inflamed from immune-excluded tumours and correlate with response to checkpoint blockade in case of recurrence and metastasis (Chakravarthy et al. 2016). DNA damage response signatures have been proposed to capture the intrinsic radiosensitivity differences between HPV-positive and HPV-negative HNSCCs (Nickson et al. 2017), although these have not yet been incorporated into clinical practice.

Epigenomic biomarkers have developed in parallel. HPV-positive HNSCC exhibits a CpG-island methylator-phenotype-like pattern that distinguishes it from HPV-negative

disease and from normal mucosa (Lechner et al. 2013). Differentially methylated regions affect promoters of immune, differentiation, and developmental genes, and methylation-derived classifiers can distinguish HPV-driven from HPV-naïve tumours with high accuracy (Lechner et al. 2013; Chakravarthy et al. 2016). The intersection of transcriptomic and methylation data, multi-omics integration, provides a more constrained and biologically interpretable signal than either modality alone, by restricting candidate biomarkers to genes for which both expression and regulatory state vary coherently across patient subgroups (Hasin, Seldin, and Lusi 2017).

Despite this analytical progress, no single biomarker or composite signature has achieved the combination of predictive power, reproducibility, and clinical accessibility required to guide treatment de-escalation in HPV-positive HNSCC.

1.3. Tumour Heterogeneity and Spatial OMICS

1.3.1. Intra-Tumoral Heterogeneity in HNSCC

Cancer is not a homogeneous entity even within a single tumour. Intra-tumoural heterogeneity, variation in genotype, transcriptional state, and phenotype among cells of the same tumour, is now recognised as a fundamental feature of solid cancers and a major driver of treatment failure (Marusyk, Janiszewska, and Polyak 2020; Tirosh et al. 2016). Genetic heterogeneity arises from the clonal evolution of somatic mutations under selective pressure and gives rise to spatially distinct subclones within a tumour (Gerlinger et al. 2012). Non-genetic, transcriptional heterogeneity reflects reversible cell-state plasticity, including epithelial-mesenchymal transition, differentiation status, and stress-response programmes, and contributes to therapy resistance independently of genetic clonality (Marjanovic, Weinberg, and Chaffer 2013; Tirosh et al. 2016).

Intra-tumoral heterogeneity matters clinically because a tumour's response to treatment is often driven not by its average phenotype but by subpopulations within it. In HNSCC, hypoxic, mesenchymal, and stem-like states are recognised drivers of radioresistance that can survive radiotherapy and seed local recurrence, so cell states

present at low frequency can determine outcome (Marie-Egyptienne, Lohse, and Hill 2013; Qiao et al. 2022). Resolving this heterogeneity is therefore a prerequisite for understanding why tumours assigned the same bulk molecular subtype (Walter et al. 2013) can respond differently to the same treatment. Intra-tumoral heterogeneity has been characterised by single-cell RNA sequencing of primary tumours. Puram et al. (2017) profiled eighteen HNSCC primary tumours by single-cell RNA-sequencing and identified a partial epithelial-mesenchymal transition (pEMT) programme, characterised by VIM, AXL, and matrix-remodelling gene expression, that was confined to a leading-edge subpopulation of malignant cells and was associated with nodal metastasis (Puram et al. 2017). The pEMT programme co-existed with differentiated squamous, stress, hypoxia, and cell-cycle programmes within the same tumours, demonstrating that bulk subtype labels reflect the dominant rather than the only transcriptional state present. Subsequent pan-cancer single-cell analyses have generalised this picture, identifying recurrent transcriptional programmes, including hypoxia, EMT, cell cycle, stress, and differentiation, across many tumour types (Kinker et al. 2020; Gavish et al. 2023).

1.3.2. The Tumour Microenvironment

Tumour cells exist within a complex ecosystem of stromal fibroblasts, endothelial cells, infiltrating immune cells, and extracellular matrix (ECM), collectively termed the tumour microenvironment (TME) (Hanahan and Weinberg 2011; Quail and Joyce 2013). The TME is not a passive scaffold; it actively shapes tumour growth, angiogenesis, immune evasion, and therapy response. Stromal-derived cytokines and ECM components collectively regulate the inflammatory microenvironment of the tumour stroma (Mantovani et al. 2008); endothelial cells determine the vascular density and, by extension, the oxygenation status of the tissue; and infiltrating immune cells, ranging from cytotoxic T cells and natural-killer cells to immunosuppressive regulatory T cells, myeloid-derived suppressor cells, and tumour-associated macrophages, determine the immune phenotype that conditions response to immunotherapy (Binnewies et al. 2018)

The HNSCC tumour microenvironment is particularly relevant because the disease is treated with modalities, RT and immunotherapy, whose efficacy depends critically on

microenvironmental context. RT efficacy is modulated by oxygenation status (see 1.3.3) and by immune activation downstream of radiation-induced cell death; checkpoint inhibitor activity depends on a pre-existing inflamed immune microenvironment. Spatial profiling of the TME, distinguishing immune-infiltrated from immune-excluded regions, normoxic from hypoxic regions, and stromal from epithelial compartments, is therefore central to understanding why patients with apparently similar tumours respond differently to identical treatments.

1.3.3. Tumour Hypoxia

Tumour hypoxia arises from the mismatch between the oxygen demand of proliferating cancer cells and the chaotic, often insufficient tumour vasculature (Vaupel and Mayer 2007). Regions of hypoxia are found in essentially all solid tumours above a few millimetres in diameter and adopt a characteristic spatial topology, with relatively well-oxygenated regions adjacent to functional capillaries giving way to graded hypoxia at increasing diffusion distance, and ultimately to necrosis where oxygen tensions fall below the threshold for survival (Vaupel and Mayer 2007; Wigerup, Pålman, and Bexell 2016).

At the molecular level, the cellular response to reduced oxygen availability is coordinated by the hypoxia-inducible factor (HIF) family of transcription factors (Semenza 2003). Under normoxia, HIF-1 α and HIF-2 α are hydroxylated on conserved proline residues by oxygen-dependent prolyl hydroxylases, polyubiquitinated by the von Hippel-Lindau E3 ligase complex, and degraded by the proteasome. Hypoxia inhibits prolyl hydroxylation, allowing HIF- α to stabilise, translocate to the nucleus, and dimerise with HIF-1 β to activate a transcriptional programme of more than two hundred target genes, including those encoding glycolytic enzymes, vascular endothelial growth factor (VEGF), erythropoietin, and the cell-state regulators DDIT4, NDRG1, ADM, ANGPTL4 and the prolyl-hydroxylase EGLN3 itself (Schito and Semenza 2016; Semenza 2012). These genes constitute the canonical hypoxia transcriptional signature that underlies clinical gene-expression classifiers of the hypoxic phenotype.

In HNSCC, hypoxia is associated with poorer prognosis and reduced response to RT and chemotherapy (Vaupel and Mayer, 2007; Toustrup et al., 2011; Eustace et al., 2013). The hypoxia-induced transcriptional programme upregulates pathways, including angiogenesis, EMT, and metabolic adaptation, that drive resistance and

metastasis (Schito and Semenza, 2016). Gene-expression classifiers such as the fifteen-gene Toustrup signature (Toustrup et al., 2011) and the twenty-six-gene Eustace signature (Eustace et al., 2013) have been validated as predictors of benefit from hypoxia-modifying therapies in head and neck and other tumours, illustrating both the clinical relevance of the hypoxia phenotype and the limits of a single global score in capturing its spatial complexity.

Direct measurement of hypoxia at tissue resolution has historically been challenging. Polarographic oxygen electrodes provide accurate pO_2 measurements but are invasive and impractical at scale. Endogenous markers such as HIF-1 α and carbonic anhydrase IX (CAIX) are commonly used in immunohistochemistry (IHC) but have stability and specificity limitations (Wigerup et al., 2016). Exogenous 2-nitroimidazoles, most commonly pimonidazole (PIMO) provide an alternative that reports cumulative exposure to oxygen tensions below approximately 10 mmHg during the labelling window (Raleigh et al. 1999; Kaanders et al. 2002). I used PIMO-based hypoxia annotation to label hypoxic regions in the spatial dataset analysed in this thesis.

1.3.4. Limitations of Bulk Molecular Profiling

Bulk RNA sequencing has been the traditional method of HNSCC molecular characterisation for some times. Its principal limitation is the loss of spatial information. A bulk transcriptome integrates the contributions of every cell in the input material, weighted by abundance and capture efficiency, into a single average vector (Marx 2021; Williams et al. 2022). For tumours composed of a small number of dominant cell populations, this average is informative; for tumours composed of multiple co-existing transcriptional states in distinct spatial compartments, the average is a mixture from which the underlying components cannot be uniquely recovered.

Two related consequences follow. First, subtype assignment on bulk profiles can be unstable: tumours in which two or more programmes are present in roughly equal proportion will receive intermediate or mixed subtype labels that may misrepresent the dominant biology in any specific tumour region. Second, biological relationships that depend on spatial juxtaposition, such as the position of a hypoxic compartment relative to vasculature, or of an immune-infiltrated region relative to the tumour-stroma interface, are inaccessible in bulk data, because the spatial coordinates have been

discarded. Single-cell RNA-sequencing recovers cellular resolution but, since it requires tissue dissociation, also discards spatial information (Marx, 2021; Williams et al., 2022). The methodological gap that motivated the development of spatial transcriptomics is the recovery of cellular- or near-cellular-resolution gene expression measurements while preserving the spatial coordinates of each measurement on the tissue section.

1.3.5. Spatial Transcriptomics and Multi-Modal Integration

Spatial transcriptomics encompasses a family of methods that produce gene-expression measurements indexed by position in a tissue section, allowing the joint analysis of transcriptional state and tissue architecture (Asp, Bergenstråhle, and Lundeberg 2020; Marx 2021; Williams et al. 2022). The first array-based platform, Spatial Transcriptomics, since commercialised as 10x Genomics Visium, captures messenger RNA by hybridisation to spatially barcoded oligonucleotide spots arrayed on a glass slide, with each spot covering a defined area of tissue (Ståhl et al. 2016). Subsequent methods have improved resolution to near single-cell scale, including Slide-seq (Rodriques et al. 2019), high-definition spatial transcriptomics (Vickovic et al. 2019), and a range of sequencing- and imaging-based platforms in active development. Each platform balances spatial resolution, gene coverage, and compatibility with formalin-fixed paraffin-embedded (FFPE) tissue; the Visium FFPE platform I used in this thesis provides whole-transcriptome coverage at approximately 55- μ m spot resolution from archival FFPE material, which makes it well suited to the integration of spatial transcriptomics with classical histopathology.

The analytical task in spatial transcriptomics is to extract biologically interpretable transcriptional states from a high-dimensional, spatially structured dataset. Several broad approaches have emerged. Clustering of spots in transcriptional space identifies discrete tissue states and is a standard first step (Marx 2021). Cell-type deconvolution algorithms such as RCTD (Cable et al. 2022) decompose each spot into a mixture of single-cell-derived cell-type signatures, providing a bridge between spatial and single-cell modalities. Matrix-factorisation approaches, in particular non-negative matrix factorisation (NMF), decompose the expression matrix into a small number of additive transcriptional programmes (or MPs) that can be activated to varying degrees within each spot, providing a parts-based representation of cell state that is well suited to

tissue contexts in which multiple MPs co-exist (Stein-O'Brien et al. 2018; Brunet et al. 2004; Kotliar et al. 2019).

The MP concept has been extended by Tirosh and colleagues from melanoma single-cell data (Tirosh et al., 2016) through to pan-cancer single-cell analyses (Kinker et al. 2020) and, most recently, to a thousand-tumour single-cell and spatial atlas that defined a small number of recurrent intra-tumoural transcriptional programmes, including hypoxia, EMT, stress, cell cycle, and differentiation, that appear across many tumour types (Gavish et al. 2023; Barkley et al. 2022). These recurrent programmes provide the concept within which the MPs identified in any new dataset can be interpreted. The combination of NMF-based programme discovery, validation against graph-based community detection (Traag, Waltman, and van Eck 2019), and consensus aggregation across samples is the analytical strategy that underpins the spatial chapter of this thesis.

The interpretation of spatial transcriptomic data is enriched by integration with non-transcriptomic modalities co-registered on the same tissue. First, haematoxylin and eosin (H&E) histology provides morphological annotation by an expert pathologist, identifying tumour, stroma, necrosis, keratinisation, immune infiltrate, and other classical tissue elements that can be projected onto the spot grid (Moncada et al. 2020; Ji et al. 2020). Second, immunofluorescence or IHC of biomarkers, including hypoxia markers such as PIMO, provides molecular labels for tissue regions that can be co-registered with the transcriptomic spots (Kaanders et al., 2002; Raleigh et al., 1999). Third, bulk multi-omics of the same or matched tumours provides a population-level context in which spatial findings can be situated, including bulk subtype assignment and methylation-based classification ("Comprehensive Genomic Characterization of Head and Neck Squamous Cell Carcinomas" 2015; Leemans, Snijders, and Brakenhoff 2018). Therefore, I used the combination of spatial transcriptomics with pathologist annotation, PIMO hypoxia labelling, and bulk RNA-sequencing subtype data as a multi-modal design to relate transcriptional MPs to tissue architecture and the hypoxic microenvironment.

1.4. Preclinical Models for Functional Validation

To address the unresolved questions raised above; the lack of validated stratification within HPV-positive disease, the instability of bulk subtype labels and the spatial inaccessibility of the hypoxic microenvironment, I based both experimental parts of this dissertation on a set of well-annotated preclinical models. Established HNSCC cell lines, including FaDu (hypopharyngeal), SAS and Cal33 (oral/tongue), UT-SCC-5, UT-SCC-8 and UT-SCC-45 (laryngeal and pharyngeal), SAT provide a controlled system in which to probe individual transcriptional programmes. Cell-line-derived xenograft models recapitulate three-dimensional tumour tissue architecture and permit study of both bulk molecular profiles and the spatial organisation of tumour-cell transcriptional programmes. Their microenvironment is, however, murine and immunodeficient, and does not reproduce the full tumour microenvironment of patient tumours, and their use in this dissertation reflects two distinct but complementary applications of preclinical material.

In the first experimental part, formalin-fixed, paraffin-embedded tissue sections from eighteen treatment-naïve xenograft samples, derived from three HPV-positive (UDSCC2, UMSCC47, and UPCISCC154) and three HPV-negative (FaDu, Cal33, and SAS) cell lines sourced from a previously published preclinical trial (Valentini et al. 2023), provided me with the discovery cohort for differential gene expression and methylation analysis. The controlled experimental conditions of xenograft material, in which HPV status is unambiguous and treatment history is defined, allowed me to identify molecular candidates that I subsequently intersected with TCGA clinical data to derive a cancer-testis gene panel for HPV-positive patient risk stratification.

In the second experimental part, the role of preclinical xenografts is distinct. I profiled tumours from seven HNSCC cell lines by Visium FFPE spatial transcriptomics with co-registered H&E histology and pimonidazole (PIMO) hypoxia labelling, with the aim of decomposing the spatial transcriptional landscape into recurrent metaprograms (MP) and characterising their distribution across tumour tissue compartments. While hypoxia is one of the central biological axes under investigation, given its established role in radioresistance and its amenability to spatial annotation via PIMO, I designed the spatial dataset to capture a broader range of transcriptional states, including

programmes related to squamous differentiation, cell cycle, stromal identity, and epithelial-mesenchymal transition. PIMO, a 2-nitroimidazole that undergoes selective reductive binding in cells at oxygen tensions below approximately 10 mmHg, provides a tissue-resolved readout of hypoxia that is not accessible in monolayer culture (Raleigh et al. 1999; Kaanders et al. 2002). The use of cell-line-derived xenografts, rather than patient-derived material, reflected both the practical challenges of obtaining well-annotated clinical specimens at the scale required for spatial profiling and the value of working within an established preclinical model system that permits controlled characterisation of tumour biology.

1.5. Aims and Scope of the Thesis

This dissertation comprises two experimental parts that address complementary aspects of HNSCC heterogeneity and that share an emphasis on the integration of multiple data modalities.

In the first part, my aim was to identify a molecular stratification within HPV-positive HNSCC that resolves part of the clinical heterogeneity observed in current de-escalation trials, and to characterise its underlying biology in relation to TP53 alterations and known molecular subtype structure. I address the molecular basis of clinical response heterogeneity in HPV-positive HNSCC. Starting from integrated bulk transcriptomic and methylation data in a preclinical cohort, I filter candidate genes by a multi-omics workflow and assemble a gene panel that I test for prognostic value in the independent DKTK-ROG patient cohort.

In the second part, my aim was to define the recurrent transcriptional programmes that structure HNSCC tumour tissue, to determine how hypoxia relates to this broader landscape, and to situate the identified MPs within canonical HIF-driven biology, known HNSCC molecular subtypes, and the wider pan-cancer MP framework. I address the spatial transcriptional architecture of HNSCC and its relationship with the tumour microenvironment. Using Visium FFPE spatial transcriptomics of cell-line-derived xenografts from seven HNSCC cell lines, with co-registered H&E pathology and PIMO hypoxia labelling, I decompose the spatial dataset by a combination of per-

sample NMF and inter-sample Jaccard-based consensus into a set of consensus MPs. Then I characterise the biological identity, spatial distribution, and inter-sample reproducibility of these MPs across programmes spanning hypoxia, squamous differentiation, cell cycle, stromal, and epithelial-mesenchymal transition states.

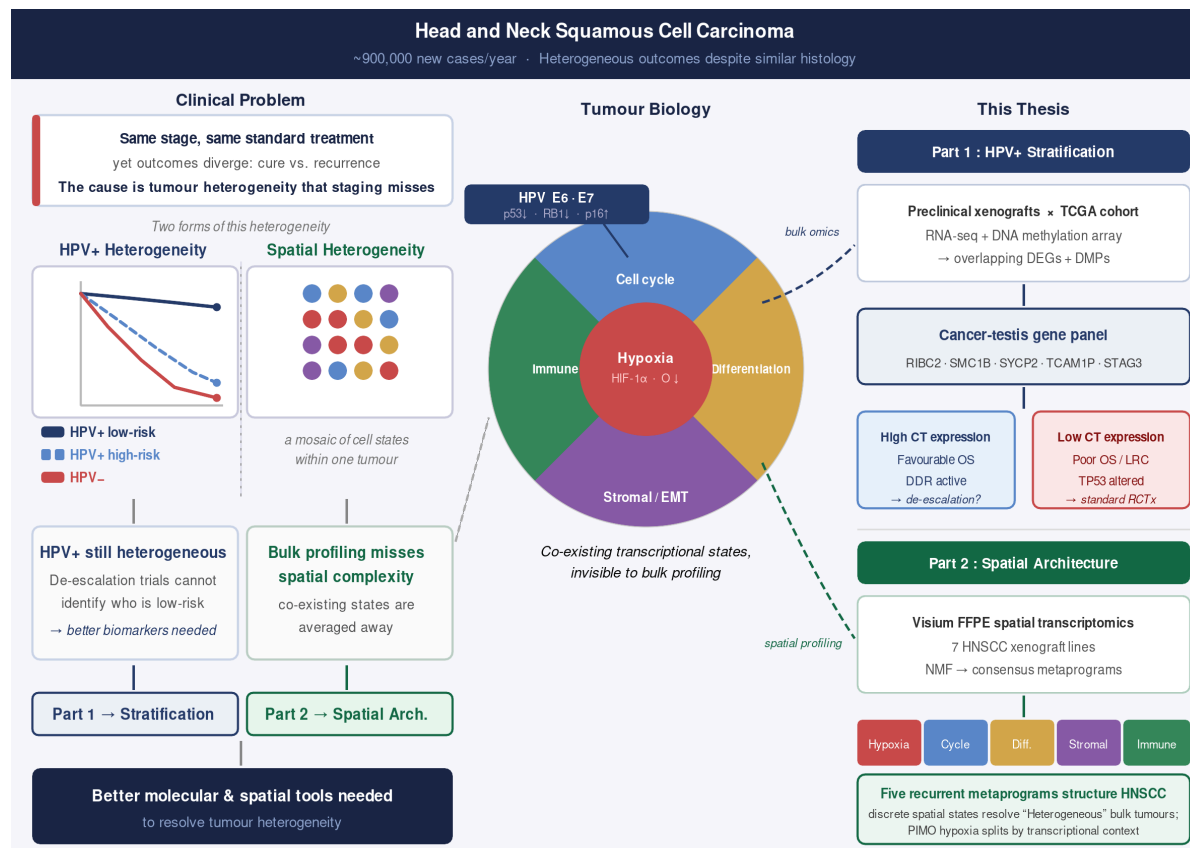


Figure 2. Overview of the dissertation. Left: the clinical problem of heterogeneous outcomes in HNSCC and the inadequacy of current biomarkers. Centre: tumour biology showing co-existing transcriptional states invisible to bulk profiling. Right: the two experimental parts of this thesis and their key outputs. Figure created with Claude (Anthropic).

The methodological link between the two parts is the integration of orthogonal data modalities, transcriptomics, methylation, IHC, and pathology, to produce molecular explanations of HNSCC that are not reducible to any single modality. The clinical motivation for both parts is the persistent gap between current biomarker-based risk stratification and the observed heterogeneity in treatment outcome in HNSCC.

Part I: HPV-Positive HNSCC & Radiosensitivity

HPV-positive HNSCC is generally associated with a favourable prognosis, which has motivated ongoing efforts to de-escalate treatment and reduce long-term toxicity. However, a subset of these patients still relapses, and HPV status alone does not identify them. There is therefore a clinical need for molecular markers that stratify outcome within HPV-positive disease, so that de-escalation can be offered safely to those who will benefit while sparing high-risk patients.

In this first part I ask whether transcriptional and epigenetic differences among HPV-positive tumours can define such a subgroup. Starting from a preclinical xenograft cohort and intersecting the findings with TCGA-HNSCC patient data, I derive a CT gene panel, test its prognostic value in an independent RCT-treated cohort, and integrate it with TP53 status. The aim is to identify the aggressive fraction hidden within HPV-positive HNSCC and to provide a marker that could inform treatment de-escalation.

2. Methods: HPV-Positive HNSCC & Radiosensitivity

To investigate the molecular differences associated with HPV status in HNSCC, I analysed six preclinical xenograft models comprising three HPV-positive (UDSCC2, UPCI154, and UMSCC47; HPV16-positive) and three HPV-negative (FaDu, CAL33, and SAS) cell lines. These models were derived from a previous preclinical study (Valentini et al. 2023), and I included a total of 18 treatment-naïve samples (three per cell line) in the analysis (Table 1).

Table 1. Characteristics of the preclinical HNSCC xenograft models. HPV status, cell line identity, patient sex, primary tumour location, ethnicity, and patient age are listed for the six cell lines included in the analysis.

HPV Status	Cell Lines	Gender	Location of Tumour	Ethnicity & Location	Age
HPV-positive	UDSCC2	M	Hypopharynx	European	58
	UMSCC47	M	Tongue	Unknown	53
	UPCISCC154	M	Tongue	Caucasian	54
HPV-negative	FaDu	M	Hypopharynx	Indian (79.5% South Asian Genome)	56
	SAS	F	Tongue	Japanese	69
	Cal33	M	Tongue	Caucasian	69

I included two clinical cohorts for comparative and validation analyses. The Cancer Genome Atlas HNSCC dataset (TCGA-HNSC) provided 487 treatment-naïve primary tumour samples for differential multi-omics analysis. From this cohort, I selected 293 patients who had received RT for clustering analysis, and I further included 50 HPV-positive patients who had received RT for survival stratification.

The DKTK-ROG cohort comprised 221 patients diagnosed with locally advanced HNSCC who were treated with postoperative cisplatin-based RCT (PORT-C) between 2004 and 2012 across all eight German DKTK partner sites (Tinhofer et al. 2016). RT consisted of standard fractionation to 50 Gy covering the primary tumour and regional lymph nodes, followed by a boost of 10-16 Gy. For this study, I selected 195 patients with available microarray-based gene expression data for clustering analysis. I included a subset of 87 HPV-positive patients (identified as positive by at least one of three detection methods: p16 IHC, HPV16 DNA, or HPV16 RNA testing) for survival analysis. To comprehensively assess detection concordance, I further stratified patients by individual and combined HPV positivity: any-positive (HPV16 DNA+/HPV16 RNA+/p16+), single positives (HPV16 DNA+, HPV16 RNA+, or p16+ individually), double positives (HPV16 DNA+ & p16+, HPV16 RNA+ & HPV16 DNA+, or p16+ & HPV16 RNA+), and triple positives. HPV detection and p53 immunostaining procedures have been described in detail in a previous publication on this cohort (Lohaus et al. 2014). Briefly, p53 staining was scored by percentage of positive nuclei and staining intensity (0, +, ++, +++); tumours with $\geq 70\%$ positive nuclei and moderate (++) or strong (+++) staining intensity were classified as p53-positive.

2.1. Study Design

I structured this study into two complementary analytical phases: (i) differential molecular analysis based on HPV status, and (ii) stratification of HPV-positive patient cohorts.

In the first phase, I analysed treatment-naïve preclinical and clinical (TCGA) datasets to identify differentially expressed genes (DEGs) between HPV-positive and HPV-negative samples. I performed differential methylation analysis (DMA) in parallel to identify differentially methylated positions (DMPs). Significant DEGs and DMPs identified in the preclinical cohort ($n = 18$) were intersected with results from the TCGA cohort ($n = 487$) to define a shared set of HPV-associated molecular candidates. This overlapping set was combined with literature-curated genes associated with HPV

biology, transcriptomics, and epigenomics to generate a multi-omics gene panel, from which a cancer-testis (CT) gene panel was subsequently derived.

In the second phase, I applied the CT panel to stratify patients via K-means clustering in two cohorts: a subset of TCGA-HNSCC patients treated with RT ($n = 293$) and the

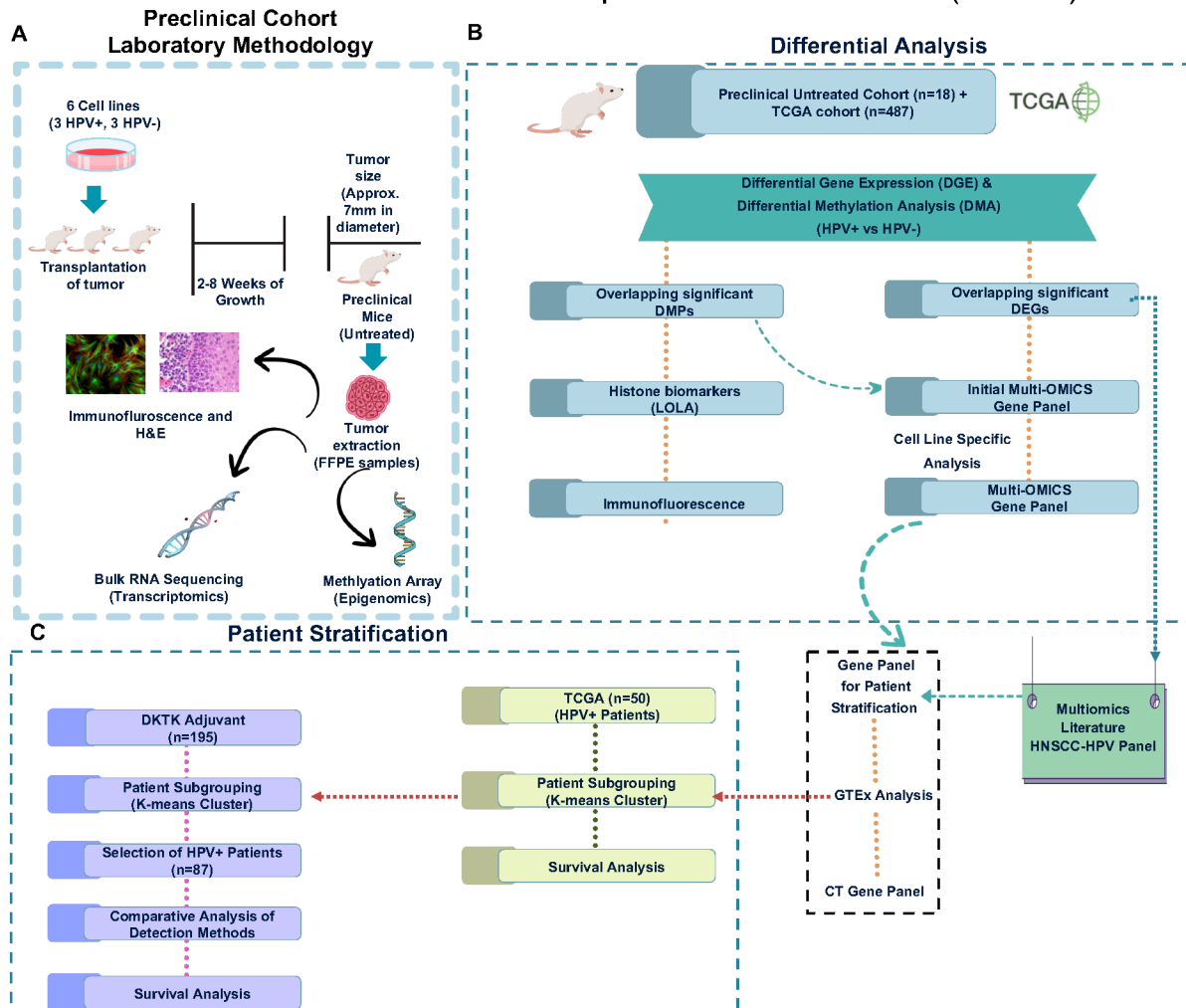


Figure 3. Schematic overview of the experimental and analytical workflow. (A) Formalin-fixed, paraffin-embedded (FFPE) samples from HPV-positive and HPV-negative HNSCC cell lines from a preclinical study used from Valentini et al. (B) Differential gene expression and methylation analyses performed in preclinical and TCGA cohorts, followed by identification of overlapping features and generation of a multi-omics gene panel. (C) Patient stratification and survival analysis in TCGA and DKTK-ROG cohorts based on CT panel expression groups.

DKTK-ROG cohort treated with postoperative RCT ($n = 195$). I then performed survival analysis in HPV-positive patients within each cohort ($n = 50$ and $n = 87$ for TCGA-HNSCC and DKTK-ROG, respectively) based on the resulting cluster assignments (Figure 3).

2.2. Experimental Methods

2.2.1. RNA Extraction and Library Preparation

I isolated total RNA from 18 formalin-fixed, paraffin-embedded (FFPE) preclinical samples using the AllPrep DNA/RNA FFPE Kit (Qiagen, Cat# 80234), following the manufacturer's protocol. I modified several steps of the initial protocol due to poor quality of FFPE samples and created a SOP. On-column DNase I digestion was performed to eliminate genomic DNA contamination. RNA purity and concentration were assessed using Qubit (Thermo Fisher Scientific), and RNA integrity was determined by TapeStation (Agilent Technologies).

Sequencing libraries were prepared at the DKFZ Genomics and Proteomics Core Facility (GPCF) using the Illumina RiboZero Stranded Kit. Briefly, 500 ng of total RNA was depleted of ribosomal RNA, fragmented to 150 bp, and reverse-transcribed into cDNA. After end repair, A-tailing, and adapter ligation, cDNA fragments were PCR-amplified for 10 cycles. Libraries were quality-checked with Qubit and TapeStation prior to sequencing on the Illumina NovaSeq 6000 platform (paired-end, 2×100 bp), yielding a minimum of 63 million reads per sample.

2.2.2. RNA-seq Alignment and Processing

RNA-seq data from xenograft samples were processed using the Roddy RNA-seq Workflow (version 1.3.0), maintained by the Omics IT and Data Management Core Facility (ODCF) at the German Cancer Research Centre (DKFZ). The process was done by ODCF and they generated me the count files from where I did the rest of analysis. Sequencing reads were aligned to a hybrid reference genome comprising human (hs37d5) and mouse (GRCm38mm) assemblies, including a PhiX spike-in control, using the STAR aligner (version 2.5.3a) with two-pass mapping enabled (-twopassMode Basic). Aligned reads were stored as sorted and indexed BAM files. Post-alignment, BAM files were merged and duplicate reads were marked using Sambamba (version 0.6.5), followed by indexing with SAMtools (version 1.6). Gene-level expression quantification was performed using FeatureCounts, targeting

exclusively human genes; mouse-derived reads were excluded from all downstream count tables.

2.2.3. DNA Extraction and Methylation Array

I extracted genomic DNA from FFPE sections of the 18 preclinical samples using the AllPrep DNA/RNA FFPE Kit and quantified the yield via Qubit. DNA methylation profiling was performed using the Illumina Infinium MethylationEPIC v2.0 BeadChip at the DKFZ Genomics and Proteomics Core Facility (GPCF) (Heidelberg, Germany), following the manufacturer's guidelines.

2.2.4. Immunofluorescence Staining and Quantification

I performed this part of the experiment with the help of our technical staff Rosemarie Euler-Lange (Bioengineer, E220, DKFZ). We performed the whole experiment together. We cut FFPE tissue sections into 3- μ m slices using a microtome and mounted onto glass slides. We deparaffinized sections in three 10-minute xylene washes and rehydrated them through a graded ethanol series (100%, 96%, 70%). We performed antigen retrieval in sodium citrate buffer (pH 6) at 99°C for 30 minutes in a water bath. Following cooling, we blocked non-specific binding with 3% bovine serum albumin (BSA) in PBS. Primary antibodies against Histone H3 (#D1H2 rabbit mAb), Tri-Methyl-Histone H3 (Lys27) (#C36B11 rabbit mAb), and Tri-Methyl-Histone H3 (Lys4) (#C42D8 rabbit mAb) (all from Cell Signalling Technology) and Histone H3 (Trimethyl Lys9) (#6F12-H4, mouse mAb, Novus Biologicals) were diluted in blocking buffer and we incubated them overnight at 4°C in a humidified chamber. We washed slides three times in PBS before applying secondary antibodies conjugated to fluorophores for 1 hour at room temperature. We counterstained samples with DAPI (100 ng/mL), mounted them with Fluoromount-G™ (Thermo Fisher Scientific), and scanned them using a Zeiss Axioscan fluorescence microscope.

The quantitative analysis was done by Cylia Ouadah, a PhD student from OncoRay, Dresden. For quantitative analysis, she excluded six samples due to suboptimal staining, image artifacts, or scanning errors (1 CAL33, 2 FaDu, 1 SAS, 1 UDSCC2, 1

UPCI154). For each valid image, she manually selected 6-8 regions of interest (ROIs) measuring $500\text{ }\mu\text{m} \times 500\text{ }\mu\text{m}$ based on staining quality, excluding necrotic areas. She carried out ROI selection in ZEN software (version 3.4, Blue Edition).

She first applied min-max normalisation to the DAPI channel prior to nuclear segmentation. She segmented nuclei using the pretrained StarDist model (2D_versatile_fluo), and she filtered the resulting nuclear masks by morphological criteria (eccentricity > 0.7 and area $> 40\text{ }\mu\text{m}^2$) to restrict analysis to tumour cells. She applied background correction for histone modification channels using the rolling ball algorithm (radius = 60). She then conducted signal intensity analysis at the single-cell level: for each selected nucleus, she calculated integrated intensity within the DAPI mask and normalised it by dividing histone integrated intensity by DAPI integrated intensity, to account for differences in DNA content. For the H3K9 modification, she performed additional normalisation to the whole histone channel, as both stainings were acquired from the same tissue sections. She performed all image analysis in Python 3.8.13 using the StarDist and scikit-image packages, following the approach described in a previous study.

2.3. Computational and Statistical Analysis

2.3.1. Preclinical Cohort Data Processing

I imported raw count matrices generated by FeatureCounts into R (v4.3.0) for downstream analysis. As an initial quality filtering step, I excluded genes with fewer than 10 total counts across all 18 samples, removing lowly expressed genes that could introduce noise in differential analysis. I then normalised the remaining count data using variance-stabilising transformation (VST) as implemented in DESeq2 (Love, Huber, and Anders 2014), which stabilises variance across the range of mean expression values and renders the data appropriate for downstream dimensionality reduction, visualisation, and clustering.

2.3.2. Differential Gene Expression and Methylation Analysis

I performed differential gene expression (DGE) analysis using DESeq2, with HPV-negative samples designated as the reference group. Genes with an FDR-adjusted p-value < 0.05 and an absolute fold change ≥ 1.5 were considered significantly differentially expressed. The same pipeline was applied independently to the preclinical cohort and the TCGA-HNSCC cohort.

For differential methylation analysis, I used the RnBeads package (v2.21.1) with the RnBeads.hg38 annotation package (v1.35.0) (Müller et al. 2019). Raw IDAT files from the Illumina EPICv2 arrays were imported and quality-controlled following the default RnBeads pipeline. Probes mapping to sex chromosomes were removed, background correction was applied using the methylumi.noob method, and data were normalised with wm.dasen normalisation. DMA was performed at the probe level using the limma method, with comparisons stratified by HPV status in treatment-naïve samples. Differentially methylated positions (DMPs) with a combined adjusted p-value (comb.p.adj.fdr) < 0.05 and an absolute mean methylation difference $|\Delta\beta| > 0.2$ were considered significant. For cross-cohort comparative analyses, features were ranked by combinedRank as provided by RnBeads, and the top 500 promoters and genes were selected for downstream analysis.

2.3.3. Gene Set Enrichment and Locus Overlap Analysis

I performed Gene Set Enrichment Analysis (GSEA) using the clusterProfiler (Yu 2024) package (v4.10.1) and msigdb (v10.0.2) ("MSigDB Gene Sets R Package" n.d.). Ranked gene lists derived from DESeq2 output were used as input, and enrichment analysis was conducted against hallmark (H) and curated (C1-C8) gene sets from the MSigDB database. Pathways with an FDR < 0.05 (Benjamini-Hochberg correction) were considered significant; gene sets containing fewer than 10 or more than 1000 genes were excluded. These GSEA methods were applied consistently across the preclinical, TCGA, and DKTK-ROG cohorts.

For the methylation data, I performed Locus Overlap Analysis (LOLA) to characterise the regulatory context of the differentially methylated regions. LOLA quantifies the statistical enrichment of a given set of genomic loci against publicly available region

databases, including transcription factor binding sites, histone modification marks, and chromatin state annotations; enabling functional interpretation of the identified DMPs.

2.3.4. TCGA-HNSCC Data Retrieval and Processing

I retrieved RNA-seq count data for primary HNSCC tumour samples from the TCGA-HNSC cohort using the TCGAbiolinks R package (Colaprico et al. 2016), querying the GDC Data Portal for gene expression quantification data generated by the STAR pipeline. Only samples labelled as “Primary Solid Tumour” were included. I mapped Ensembl transcript IDs to HGNC gene symbols using biomaRt (Durinck et al. 2009); for genes represented by multiple transcripts, I retained the maximum expression value. I retrieved clinical data from RadPlanBio (Skripcak 2023). I performed all data processing steps, including ID harmonization and count matrix reordering, in R (v4.3.0). I carried out DGE analysis based on HPV status using DESeq2, following the same pipeline applied to the preclinical cohort.

I processed DNA methylation data from TCGA using the RnBeads package (v2.21.1). I imported and aligned raw IDAT files from the Illumina HumanMethylation450 BeadChip to the hg19 reference genome, following the same quality control and normalisation procedure described for the preclinical cohort. I performed GSEA using the identical pipeline described above.

2.4. Multi-omics Gene Panel Design

I developed a multi-omics gene panel by integrating DEGs and DMPs from both the preclinical and TCGA cohorts. The overlapping DEG panel (ODP); comprising 172 genes consistently differentially expressed in both datasets; served as the analytical foundation. I refined the ODP by retaining only genes with concordant transcriptomic and epigenomic profiles: genes that were downregulated in HPV-positive samples and hypermethylated, or upregulated and hypomethylated. To ensure the reproducibility of gene selection, I performed a cell line-specific quality control step in which VST-normalised expression values were examined individually for each cell line. Genes

displaying inconsistent regulation trends across HPV-positive or HPV-negative cell lines, or showing substantial expression overlap between groups, were excluded. Only genes showing stable and reproducible differential expression across all analysed cell lines were retained.

The gene list was further extended by incorporating literature-curated genes with established relevance to HPV biology, transcriptomics, and epigenomics in HNSCC, provided they were present in the ODP(Pyeon et al. 2007; Slebos et al. 2006; W. Zhang et al. 2016; Schlecht et al. 2007; Lohavanichbutr et al. 2009; Koneva et al. 2018; Wood et al. 2016). I assessed tissue-specific expression of candidate genes using the Genotype-Tissue Expression (GTEx) portal(GTEx Consortium 2020), with testis-restricted expression used as a criterion for cancer-testis (CT) gene classification. I defined the final CT gene panel following this GTEx filtering step.

2.5. Survival Analysis of the TCGA cohort

For the TCGA-HNSCC cohort, I used VST-normalised count data from 293 RT-treated patients as input, and stratified patients into low and high CT panel expression groups ($k = 2$). The optimal number of clusters was validated in both cohorts using the silhouette method, implemented via the `silhouette()` function from the `cluster` package (v2.1.8.1)(“GESIS-Suche: cluster: Cluster analysis basics and extensions (R package version 2.1.1)” n.d.).

2.6. DKTK-ROG Cohort Data Processing

I preprocessed the microarray-based gene expression data from the DKTK-ROG cohort using the Robust Multichip Average (RMA) method, which combines background correction, quantile normalisation, and probe summarisation using the `oligo` package(Carvalho and Irizarry 2010). For genes represented by multiple probes on the array, I used the median expression value across probes as the representative

measure. I used the resulting log2-transformed expression matrix directly for clustering and downstream statistical analyses.

2.6.1. K-means Clustering and Patient Stratification

I performed K-means clustering using the `kmeans()` function in R, with parameters `nstart = 100` and `iter.max = 10` to ensure stable convergence. RMA-normalised expression data from all 195 patients who received postoperative RCT were used, with patients classified into three expression groups (low, intermediate, and high; $k = 3$). The optimal number of clusters was validated in both cohorts using the silhouette method same as the TCGA cohort. I performed Molecular subtyping of the DTKT-ROG cohort following the method described by Walter et al (Walter et al. 2013).

2.6.2. Survival Analysis in HPV-positive Patients

I performed Kaplan-Meier survival analysis in HPV-positive patients, using K-means expression group assignments as the stratification variable. Survival curves were estimated using the `survfit()` function from the survival package in R, and statistical significance was assessed via the log-rank test. For the DTKT-ROG cohort, survival analyses were conducted separately for each HPV detection method: any-positive (HPV16 DNA+/HPV16 RNA+/p16+), single positives (HPV16 DNA+, HPV16 RNA+, or p16+ individually), double positives (HPV16 DNA+ & p16+, HPV16 RNA+ & HPV16 DNA+, or p16+ & HPV16 RNA+), and triple positives.

2.6.3. TP53 and CT Panel Integration

I integrated TP53 mutation status and p53 immunostaining results with K-means cluster assignments in the DTKT-ROG cohort. TP53 mutation data and p53 staining scores were cross-tabulated against CT panel expression groups (low, intermediate, high) across the relevant HPV detection subgroups.

To assess whether CT panel expression carried prognostic information independent of TP53-based markers, I performed concordance index (C-Index) analysis and likelihood ratio tests (LRT), comparing models with and without TP53 mutation status or p53 staining in combination with CT panel grouping. These analyses were

conducted for OS and distant metastasis (DM) endpoints across the any-positive, HPV16 DNA+, and p16+ detection cohorts. The CT genes examined in this correlation analysis were based on those reported by Wang et al. for HNSCC (Cheng Wang et al. 2016).

2.6.4. Differential Gene Expression and Pathway Analysis

I performed DGE analysis between the high and low CT panel expression groups across different HPV detection methods in the DTKK-ROG cohort, using the limma package. I excluded Intermediate expression groups to enable focused comparison of the molecular extremes of the CT panel signature. Genes with FDR-corrected p-values < 0.05 were considered significant. GSEA was subsequently performed on the resulting DEGs using the same clusterProfiler and msigdb settings described above. Due to an insufficient number of DEGs in some detection subgroups, GSEA was performed only for the p16+, HPV16 DNA+, and any-positive cohorts.

2.6.5. Correlation of TP53 and CT Gene Expression

I performed Spearman correlation analysis between TP53 expression and the expression of CT genes identified in the ODP, using the `cor.test()` function in R. This analysis was conducted across the p16+, HPV16 DNA+, and any-positive detection groups in the DTKK-ROG cohort to assess the consistency of the TP53-CT gene relationship across detection modalities.

2.7. Code availability

All scripts used for the analyses presented in this thesis are available at the following GitHub repository: <https://github.com/DKFZ-E220/Biomarker-AI-Public>. This includes code for data preprocessing, integration, annotation, and downstream analyses performed on the whole dissertation.

3. Results: HPV-Positive HNSCC & Radiosensitivity

HPV-positive HNSCC represents a biologically distinct tumour entity characterised by distinct transcriptional regulation and increased radiosensitivity compared to HPV-negative cases. Although gene expression differences associated with HPV status have been extensively reported in patient cohorts, it remains unclear to what extent these transcriptional programs are conserved in preclinical xenograft models. Demonstrating concordance between preclinical and clinical datasets is essential to justify the use of these models for biomarker discovery and mechanistic investigation. Therefore, I aimed to define shared HPV-associated transcriptional alterations by integrating RNA sequencing data from treatment-naïve HPV-positive and HPV-negative HNSCC xenografts with RNA sequencing data from the treatment-naïve TCGA-HNSCC cohort.

3.1. Integration of preclinical and clinical transcriptomics data

3.1.1. Preclinical Cohort

To identify HPV-associated transcriptional differences in the preclinical cohort, I performed DGE analysis based on HPV status in treatment-naïve xenograft samples ($n = 18$). This analysis identified 782 significantly differentially expressed genes (DEGs) between HPV-positive and HPV-negative tumours. These genes represent HPV-associated transcriptional alterations observed in the xenograft models.

3.1.2. TCGA Cohort

To assess whether these transcriptional differences were also present in patient tumours, I applied the same DGE analysis to treatment-naïve samples from the TCGA-

HNSCC cohort (n = 487). This analysis identified 3,987 significantly differentially expressed genes associated with HPV status.

Comparison of the DEG lists from the preclinical and TCGA cohorts revealed 172 overlapping genes that were consistently differentially expressed in both datasets. I defined this shared gene set as the “Overlapping Differential Panel” (ODP). I used this overlapping gene set as the basis for subsequent analyses in this study (Table 2).

Table 2. Overlapping Differential Panel (ODP): 172 genes consistently differentially expressed between HPV positive and HPV-negative samples in both the preclinical xenograft cohort and the TCGA-HNSCC cohort. Positive log2 fold change values indicate upregulation in HPV-positive samples.

gene_symbol	log2FoldChange	gene_symbol	log2FoldChange	gene_symbol	log2FoldChange
RNF212	11.64391599	CD19	3.51950125	ANKRD31	-2.9153656
TMED11P	10.25313599	KCTD19	3.51202047	AFAP1-AS1	-3.1118738
ZNF541	8.657656472	ARHGEF33	3.48919025	COL28A1	-3.1800409
ESRG	8.61043621	SLC7A11-AS1	3.40621599	DGKG	-3.2078545
WDR87	8.223006766	YBX2	3.36665716	PRIMA1	-3.2748774
CALB1	8.220847282	GGT7	3.33231607	IL24	-3.4846283
ZNF214	8.094891038	PNMA2	3.31882397	ADRA2C	-3.5369221
TDRD9	7.973331731	ABCA17P	3.25336827	CORO6	-3.5748563
TCP11	7.708674071	C3orf70	3.24012703	LIPC	-3.5767524
FAM182A	7.703589779	NKAIN2	3.22080192	HTR1D	-3.5947773
MYO3A	7.482951766	RAMP2-AS1	3.21038322	ZSCAN23	-3.8054652
SHCBP1L	7.258593573	UCP2	3.19218407	GALNT13	-3.950334
SCN2A	7.119813878	SYNGR3	3.18221764	FGF5	-4.1181794
HMG2N2P10	6.826084254	WNK3	3.16104351	VAX1	-4.1207759
NLRP14	6.609251305	TAF7L	3.14624169	GRIA3	-4.2258902
ANKRD36B P2	6.451282698	SCARF2	3.11468508	STXBP6	-4.2390582
TCAM1P	6.429898291	SNRPFP2	3.08664673	MMP3	-4.2819269
MEI1	6.288491917	SMC1B	3.04592936	TERT	-4.399795
ENPP4	6.166303543	TCEA3	3.00083894	TYRP1	-4.5283788
CSH2	6.134370256	RIBC2	2.98345822	INSC	-4.5906803
ZNF280B	6.051078704	FUZ	2.96113264	CD22	-4.6079968
HAS2	6.007141553	C5	2.9280611	PCNPP3	-4.6608657
G0S2	5.718346524	PBX1	2.89964273	COLCA1	-4.761402
LGALS7	5.667535117	PDE6B	2.80501605	AKR1B10	-4.8327933
FAM180A	5.599240202	SOX30	2.79930854	GTSF1	-4.8791687
CYP2T3P	5.557501258	SLC7A11	2.75241866	SCG2	-4.9802359

ZFR2	5.447336394	ZNF540	2.74186847	TRIM55	-4.9825462
CSPG4P13	5.331613743	CCDC158	2.58614307	EREG	-5.0810869
GIPC2	5.192283748	CBY3	2.57601462	CYP1B1-AS1	-5.108974
GBX1	5.03173518	CBS	2.41976895	FREM2	-5.1480557
SYCP2	4.921276645	PLEKHG4	2.40451044	IRX6	-5.1818485
LDHC	4.880562071	GPC2	2.40394175	EFHC2	-5.4390087
COL1A2	4.876661213	KLHL35	2.37730382	LINC00973	-5.5022505
IKZF3	4.869887724	LYPD6	2.37711417	PAPPA2	-5.5307858
XKR5	4.867979803	AKR1C3	2.32960474	CXCL14	-5.5418688
DMBT1	4.801496063	RBFADN	2.2667698	NOVA1	-5.8077647
C5orf46	4.750339489	EPB41L4A	2.22128112	CALB2	-5.8425003
TEX15	4.73940418	DNAH7	2.16601576	KRT75	-6.052503
DPY19L2P4	4.713710425	FAM182B	2.16520868	LINC00601	-6.0711523
NAPSB	4.704512394	CD37	2.09172008	LINC00355	-6.2722487
SLC13A5	4.701479094	STAG3	2.08885769	RPL30P11	-6.7230326
ZNF732	4.675913226	GRIN2C	1.99185527	TRIML2	-6.7505816
GCK	4.672753264	FAM221A	1.84186596	STK33	-6.8636126
PODXL2	4.576338666	SYCE2	1.81174082	ABCA4	-7.1430996
RCN3	4.424659947	EPHX2	1.67028631	BCHE	-7.2745382
CLCA4	4.406873153	ZCWPW1	1.57539967	GPR158	-7.5863728
A3GALT2	4.406638021	CCDC150	1.43240593	PTPRD	-7.5904357
LY75	4.400371452	CCDC28B	1.30427102	MAGEB2	-8.5378755
SP9	4.312761433	MYH3	-1.7793443	BRDT	-9.4273745
ARHGAP4	4.269163195	SPRY4	-1.7924551	MAGEA4	-10.546046
MEIOB	4.06800527	SLC7A5	-1.7934731	MAGEA3	-22.879174
KCNH4	3.988868439	CCND1	-2.1023269	MAGEA6	-23.94545
PLAC8	3.848767874	MT2A	-2.3055389	SALL1	-24.275791
TACR3	3.827756168	TTC7B	-2.379451	NDN	-24.499316
SPOCK3	3.7399687	REPS2	-2.5099764		
ESPNP	3.739897515	KRT17P1	-2.5330527		
KEL	3.648039262	LMX1B	-2.5588689		
HMX2	3.631864947	CTTN	-2.5594638		
SLC10A4	3.588737994	PPFIA1	-2.57437		

3.2. Integration of preclinical and clinical epigenomics data

To investigate whether epigenetic changes contribute to the observed transcriptional differences between HPV-positive and HPV-negative samples, I performed methylation array analysis on the preclinical and TCGA-HNSCC cohorts. Methylation array analysis of the preclinical cohort identified 3078 significant DMPs between HPV-negative and HPV-positive samples. Nine genes (*EREG*, *RIBC2*, *TCAM1P*, *SMC1B*, *ZSCAN23*, *SP9*, *MEI1*, *TMED11P*, and *LY75*) exhibited consistent overlap between transcriptomic (DEG) and epigenomic (DMP) analysis. However, *MEI1*, *TMED11P*, and *SP9* were excluded following cell line-specific quality control due to inconsistent expression patterns (Figure 4). Samples displaying inconsistent expression trends relative to their HPV group were excluded during this quality control step; hence I excluded these three genes from further

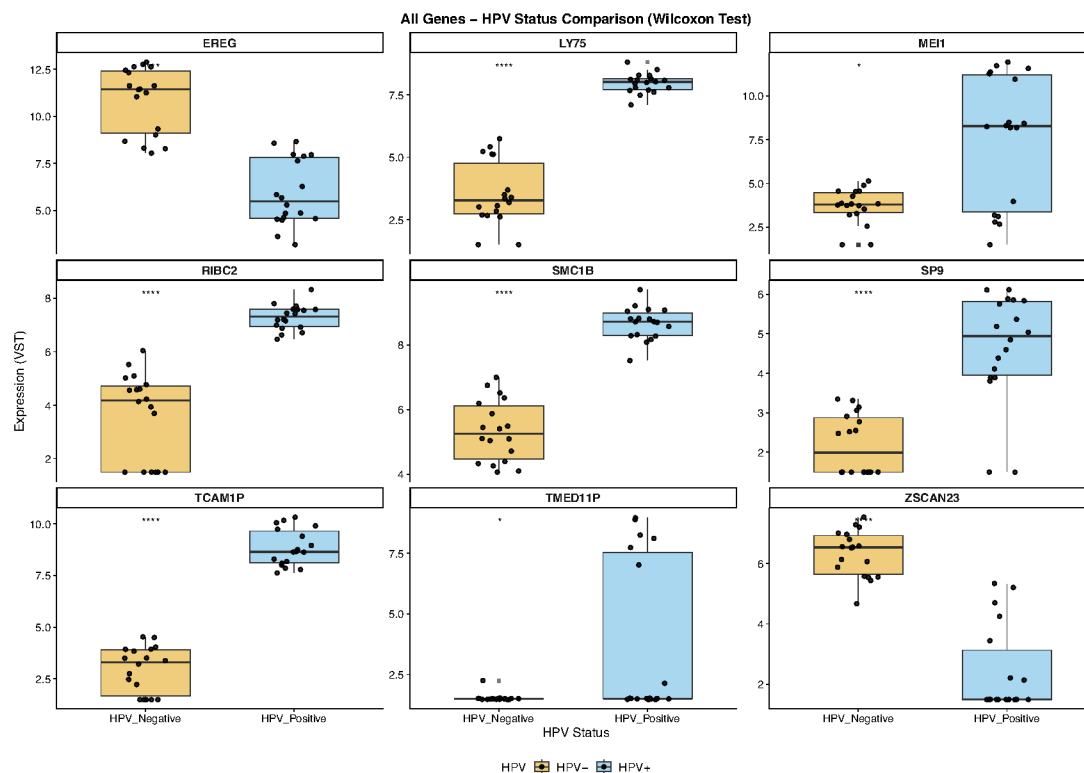


Figure 4. Normalised expression (VST) levels of nine multiomics candidate genes across HPV-negative and HPV-positive HNSCC samples. Wilcoxon test used to compare the groups. P-value: Significance is indicated as < 0.05 (*), < 0.01 (**), and < 0.001 (***).

analysis. To further characterise the regulatory context of the identified DMPs, I performed Locus Overlap Analysis (LOLA) on the methylation array data. This analysis

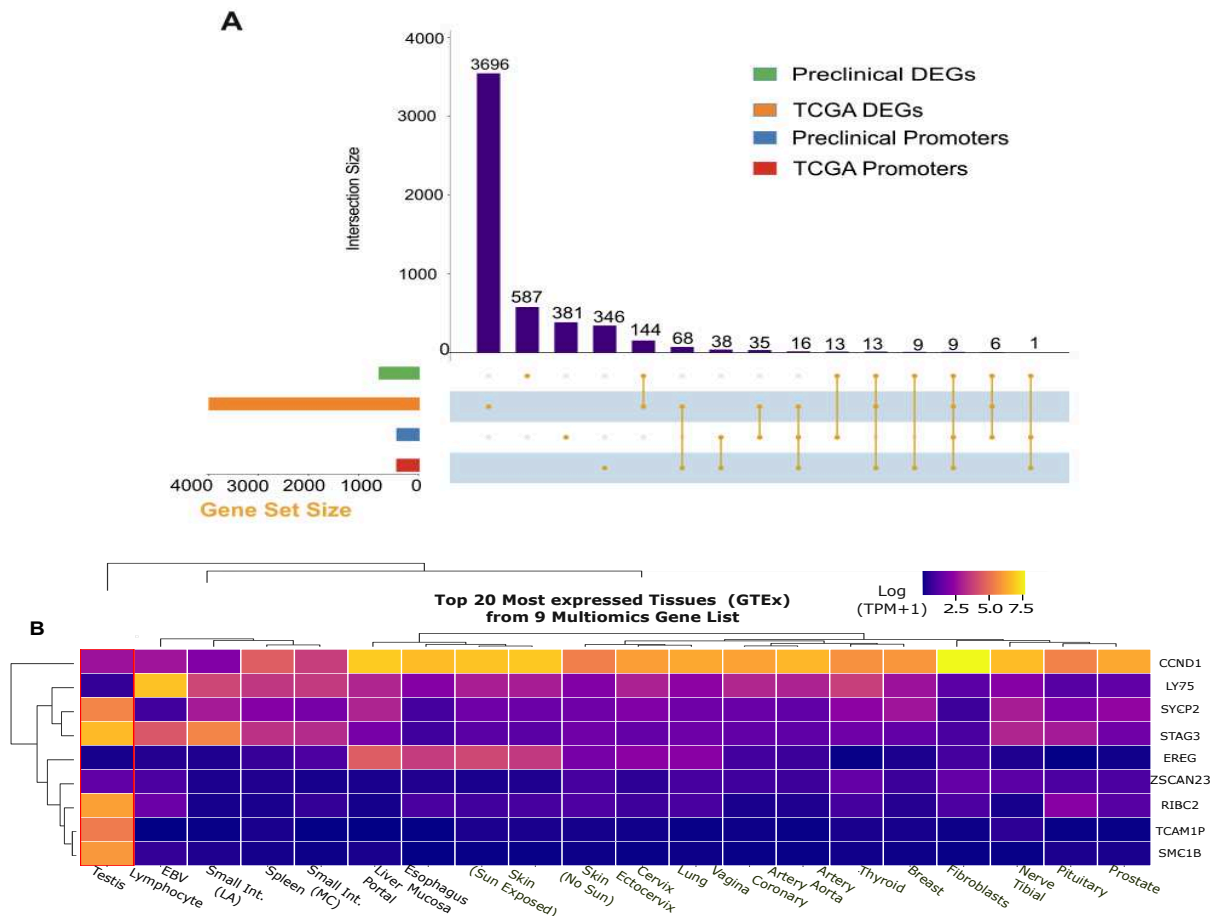


Figure 5. Multi-omics-based development of the cancer-testis (CT) gene panel. (A) Genes from the Overlapping Differential Panel (ODP) showing concordant transcriptomic and epigenomic profiles (DEG + DMP overlap) in HPV-positive vs. HPV-negative samples. (B) GTEx expression of the top 20 tissues from the initial 9 multi-omics genes.

further identified three histone modifications, $H3K27me3$, $H3K9me3$, and $H3K4me3$, as significantly enriched in HPV-positive samples across both preclinical and TCGA cohorts. These findings identify a set of HPV-associated genes which might be linked to histone modifications. Immunofluorescence staining of $H3K27me3$, $H3K9me3$, $H3K4me3$ in FFPE xenograft sections did not reveal significant differences between HPV-positive and HPV-negative tumours (Supplementary Figures 1-6). This likely reflects the global nature of these histone marks and the limited sensitivity of immunofluorescence for detecting locus-specific chromatin modifications.

3.3. Cancer-Testis (CT) gene panel

3.3.1. Development of cancer-testis gene panel

Given the limited resolution of immunofluorescence for detecting locus-specific histone changes, I focused the downstream analysis on the gene panel derived from transcriptomic and epigenomic evidence. I refined candidate genes from the ODP by integrating multi-omics evidence with functional relevance derived from the literature. I prioritised six genes (*EREG*, *RIBC2*, *TCAM1P*, *SMC1B*, *ZSCAN23*, *LY75*) based on concordant transcriptomic and epigenomic profiles (Figure 6). I extended the gene list with three additional genes: *STAG3*, *SYCP2*, and *CCND1*, previously implicated in HPV related biomarker studies with transcriptomics and epigenomics relevance in

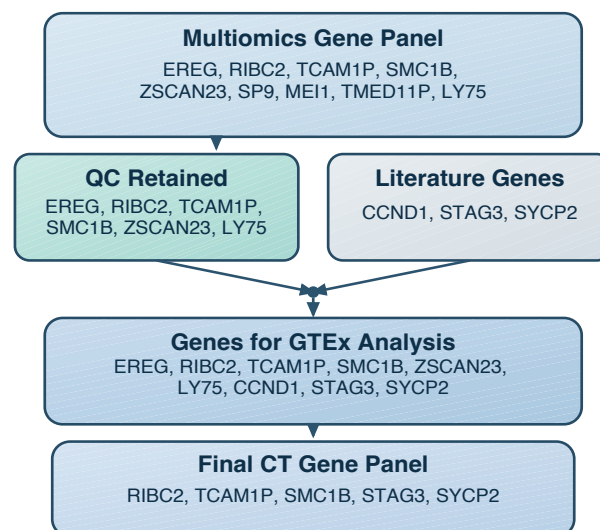


Figure 6. Flowchart of the development of the final CT gene panel. The initial multi-omics gene panel (*EREG*, *RIBC2*, *TCAM1P*, *SMC1B*, *ZSCAN23*, *SP9*, *MEI1*, *TMED11P*, *LY75*) was reduced by cell-line quality control to the QC-retained set (*EREG*, *RIBC2*, *TCAM1P*, *SMC1B*, *ZSCAN23*, *LY75*). Combined with three literature-curated genes (*CCND1*, *STAG3*, *SYCP2*), these formed the set taken to GTEx analysis, from which the final five-gene CT panel (*RIBC2*, *TCAM1P*, *SMC1B*, *STAG3*, *SYCP2*) was derived.

HNSCC (Figure 6). These three genes were also part of the initial ODP panel but failed to show significance in the methylation array data. Given their strong transcriptomic evidence and prior relevance in HPV-related HNSCC studies, I included them in the panel based on the transcriptomic signal alone.

GTEx analysis revealed testis-specific expression in five of the nine genes (*RIBC2*, *TCAM1P*, *SMC1B*, *STAG3*, and *SYCP2*), consistent with CT classification (Figure 5)(Cheng Wang et al. 2016). Together, these findings define a set of HPV-associated CT genes supported by multi-omics evidence and relevant literature, providing a focused panel for subsequent functional or translational evaluation.

3.3.2. CT Panel stratifies the TCGA patients receiving RT into two risk groups

To investigate the relevance of the CT gene panel (*RIBC2*, *TCAM1P*, *SMC1B*, *STAG3*, and *SYCP2*), I analysed 293 patients from the TCGA-HNSCC cohort treated with RT. Patients were grouped into low ($n = 239$) and high ($n = 54$) CT panel expression clusters based on K-means expression groups. Patients with HPV-positive tumours were primarily distributed across the high CT panel expression group, while the low-expression group included a higher proportion of HPV-negative patients. I then selected HPV-positive patients from the two CT panel expression groups identified in the TCGA-RT cohort (high, $n = 45$; low, $n = 5$). I observed differences in PFS between the groups, with the high CT expression group showing more favourable outcomes (Figure 7). However, the survival curves crossed, indicating that this separation did not remain consistent over time. Because the low-expression group contained only a small number of patients, I considered this analysis exploratory and therefore validated the CT panel in an independent cohort. Silhouette analysis favoured a two-cluster solution in TCGA (average silhouette width 0.6 vs 0.35 for $k=3$), exceeding the conventional >0.5 benchmark for reasonably well-separated clusters, so I selected

k=2 for downstream analyses (Figure 7).

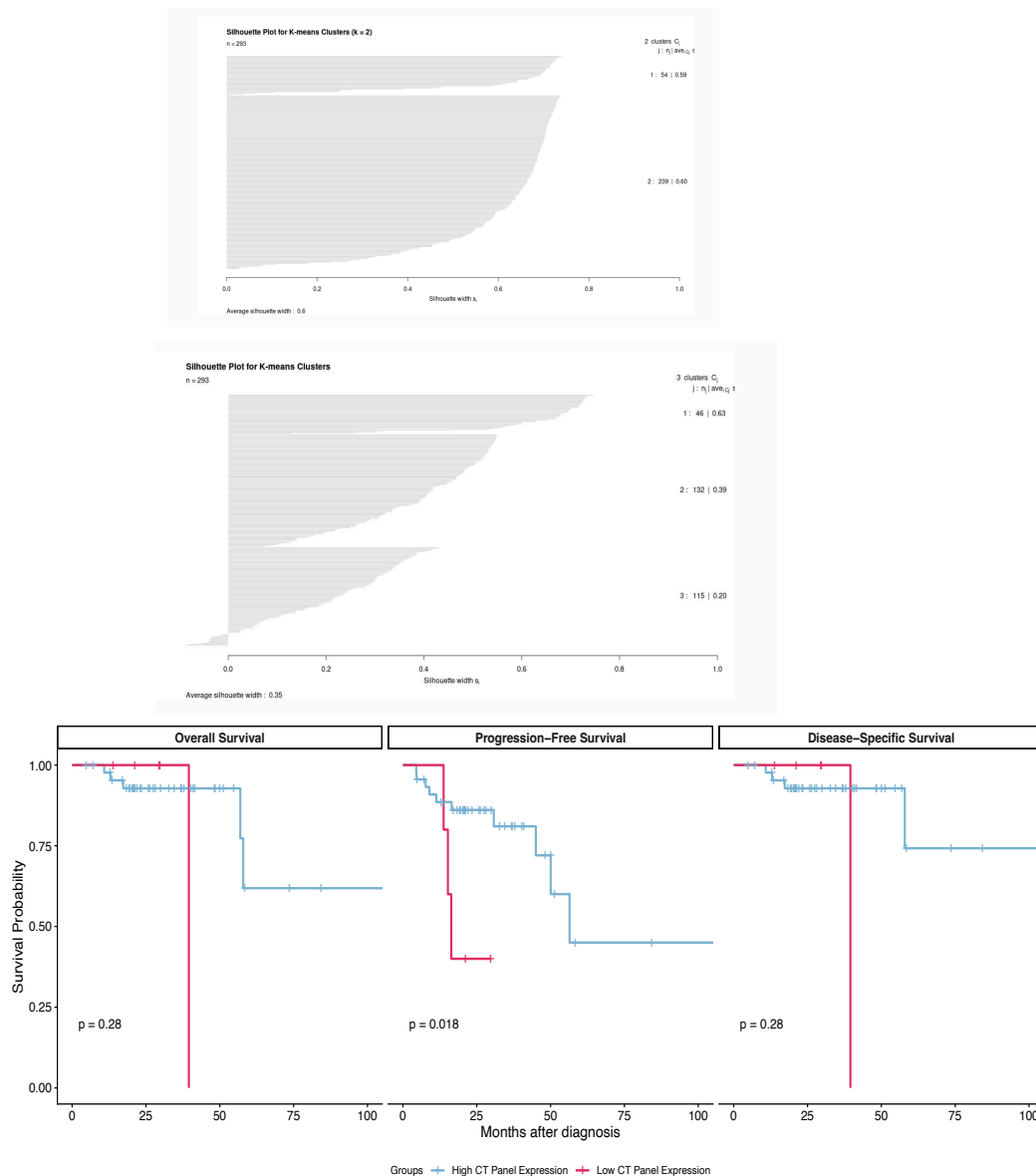


Figure 7. CT panel expression stratifies HPV-positive TCGA-HNSCC patients treated with radiotherapy. (Top) Silhouette analysis of K-means clustering of all 293 RT-treated TCGA-HNSCC patients on the five CT panel genes (*RIBC2*, *TCAM1P*, *SMC1B*, *STAG3*, *SYCP2*). (Top) two-cluster solution ($k = 2$): cluster 1 (high), $n = 54$, mean silhouette width 0.59; cluster 2 (low), $n = 239$, mean width 0.60; overall average silhouette width 0.60. (Middle), three-cluster solution ($k = 3$): cluster 1, $n = 46$, 0.63; cluster 2, $n = 132$, 0.39; cluster 3, $n = 115$, 0.20; overall average width 0.35. The higher average width for $k = 2$ (0.60 vs 0.35), above the 0.5 benchmark for well-separated clusters, supported selection of $k = 2$. (Bottom) Kaplan–Meier curves for overall survival (OS), progression-free survival (PFS) and disease-specific survival (DSS) for the HPV-positive patients within these clusters ($n = 50$; high, blue, $n = 45$; low, red, $n = 5$). Log-rank test: OS $p = 0.28$, PFS $p = 0.018$, DSS $p = 0.28$.

3.4. Validation of CT panel in the independent DTKK cohort

3.4.1. K-means clustering identifies three risk groups cohort in HPV-positive patients in an independent cohort (DTKK-ROG)

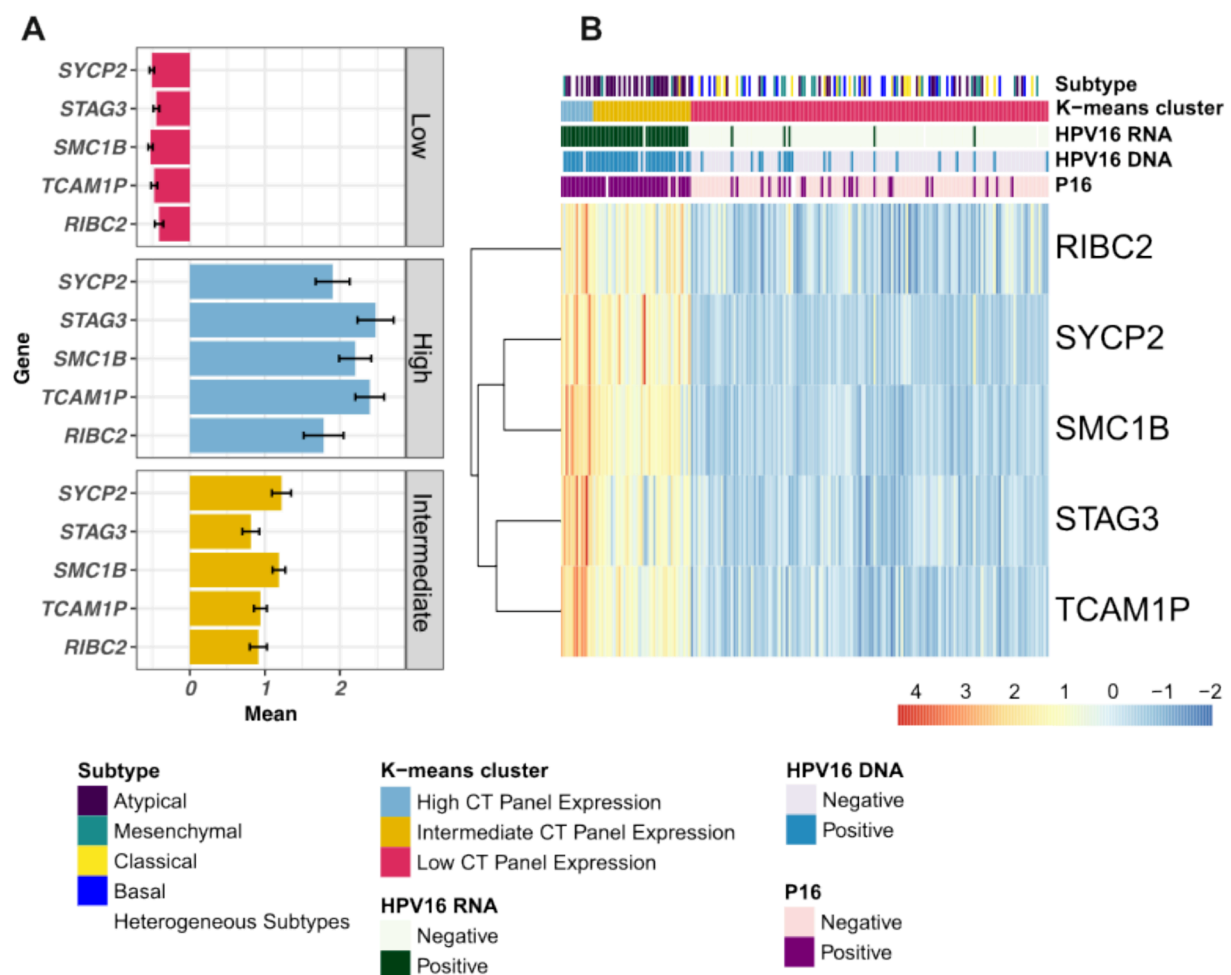


Figure 8. CT panel-based stratification of the DTKK adjuvant cohort. (A) Mean expression of five CT panel genes (RIBC2, TCAM1P, SMC1B, STAG3, and SYCP2) in each of the three K-means clusters (low, intermediate, high). Bars represent the mean $\pm$ standard deviation of normalised expression values across patients in each cluster. (B) Heatmap showing unsupervised clustering of the five CT panel genes across all patients in the DTKK cohort. Rows represent genes, and columns represent patients. Annotations above the heatmap include CT expression group, detection method (p16, DNA, RNA), and HPV status.

To further evaluate the robustness of my gene panel for stratifying patients with HPV-positive tumours, I performed K-means clustering on gene expression data from the independent DTKK-ROG cohort using the five CT panel genes (RIBC2, TCAM1P,

SMC1B, *STAG3*, and *SYCP2*). This analysis delineated three distinct expression clusters, corresponding to low, intermediate, and high expression groups (Figure 8). Patients with HPV-positive tumours were primarily distributed across the high and intermediate groups, while the low-expression group included a higher proportion of HPV-negative patients (Figure 8). The average silhouette width was 0.53 for the three clusters (Figure 9). These results demonstrate that the CT panel stratifies patients into distinct risk groups (Figure 8).

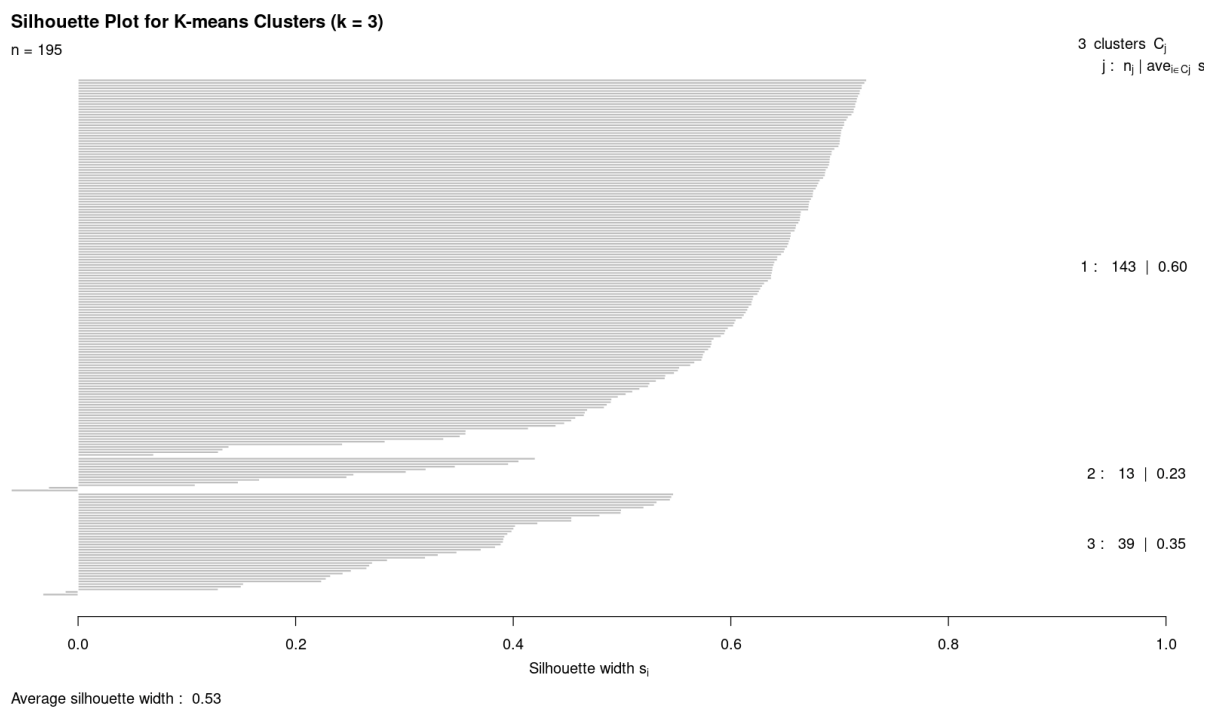


Figure 9. Silhouette analysis of K-means clustering ($k = 3$) of the DTK-ROG cohort. Silhouette plot for the three-cluster solution across all 195 post-operative RCT-treated patients, clustered on the five CT panel genes (*RIBC2*, *TCAM1P*, *SMC1B*, *STAG3*, *SYCP2*). Cluster 1, $n = 143$, mean silhouette width 0.60; cluster 2, $n = 13$, mean width 0.23; cluster 3, $n = 39$, mean width 0.35; overall average silhouette width 0.53. x-axis, silhouette width s_i .

3.4.2. Low CT panel expression predicts poor prognosis in patients with p16-positive and HPV-positive tumour

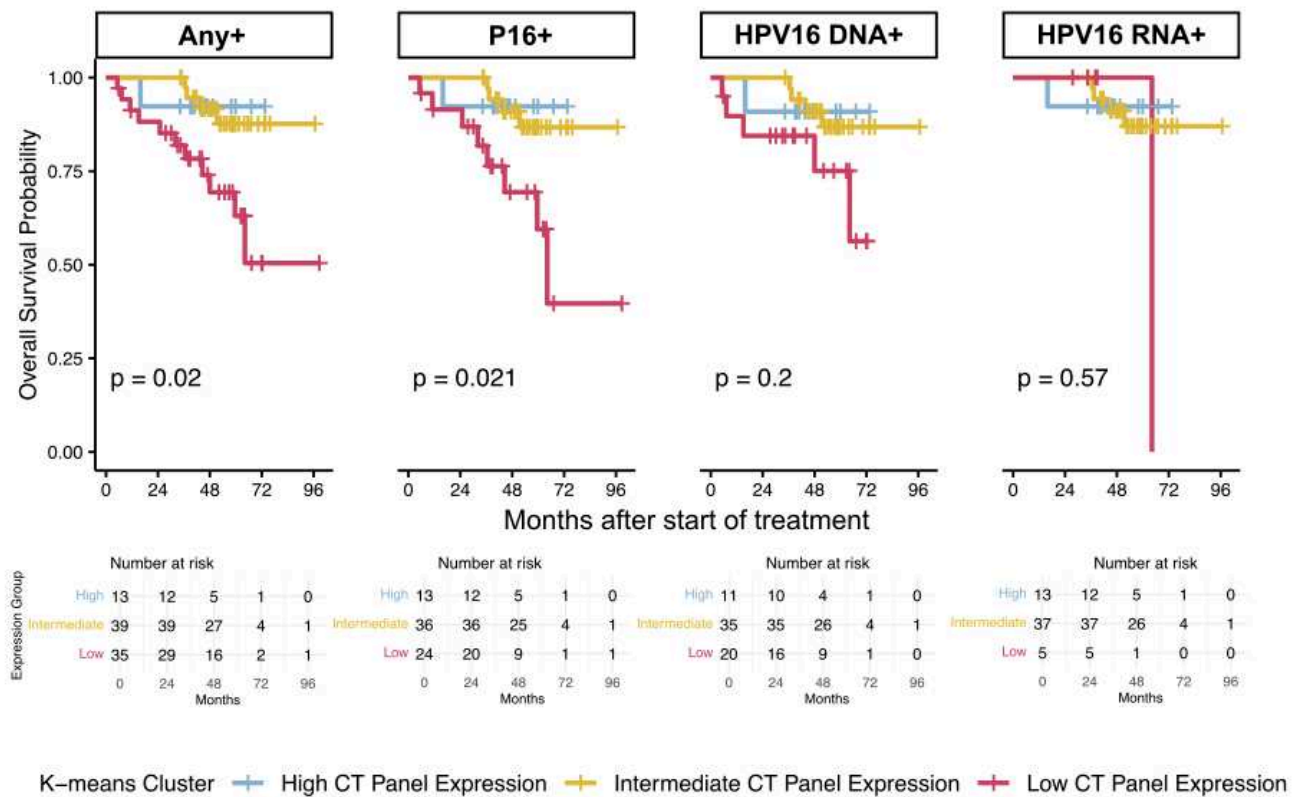


Figure 10. Kaplan-Meier overall survival of HPV-positive patients stratified by CT panel expression. K-means expression groups: high (blue), intermediate (yellow), low (pink). Panels show four HPV detection-method cohorts: any-positive (Any+), p16-positive (P16+), HPV16 DNA-positive and HPV16 RNA-positive. y-axis, overall survival probability; x-axis, months after start of treatment. Log-rank test: Any+ p = 0.02, P16+ p = 0.021, HPV16 DNA+ p = 0.2, HPV16 RNA+ p = 0.57. Number-at-risk tables are shown beneath each plot.

To evaluate whether molecular subgroups defined by CT panel expression are prognostically relevant, I performed survival analysis in patients with HPV-positive tumours across detection methods. This cohort included HPV-positive patients identified through different detection methods, resulting in variable sample sizes across analyses. Kaplan-Meier survival analysis of HPV-positive patients (positive in any detection method, n=87) revealed significant survival differences among the three risk groups (p = 0.02), with the low CT panel expression group exhibiting the poorest prognosis (Figure 10). Within the p16-positive cohort (n=73) (defined by p16 staining),

a similar trend was observed, with the low-expression group associated with significantly worse survival outcomes ($p = 0.021$). In HPV16 DNA-positive ($n=66$) cohort same trend was observed but without statistical significance ($p=0.2$). In contrast, the HPV16 RNA-positive cohort ($n=55$) an irregular pattern was observed where the low-risk group showed no significant survival distinction ($p = 0.58$). Detailed survival analyses for single positives (HPV16 DNA, HPV16 RNA, or p16), double positives (HPV16 DNA + p16, HPV16 RNA + HPV16 DNA, or p16 + HPV16 RNA), and triple positives are provided in the Supplementary section.

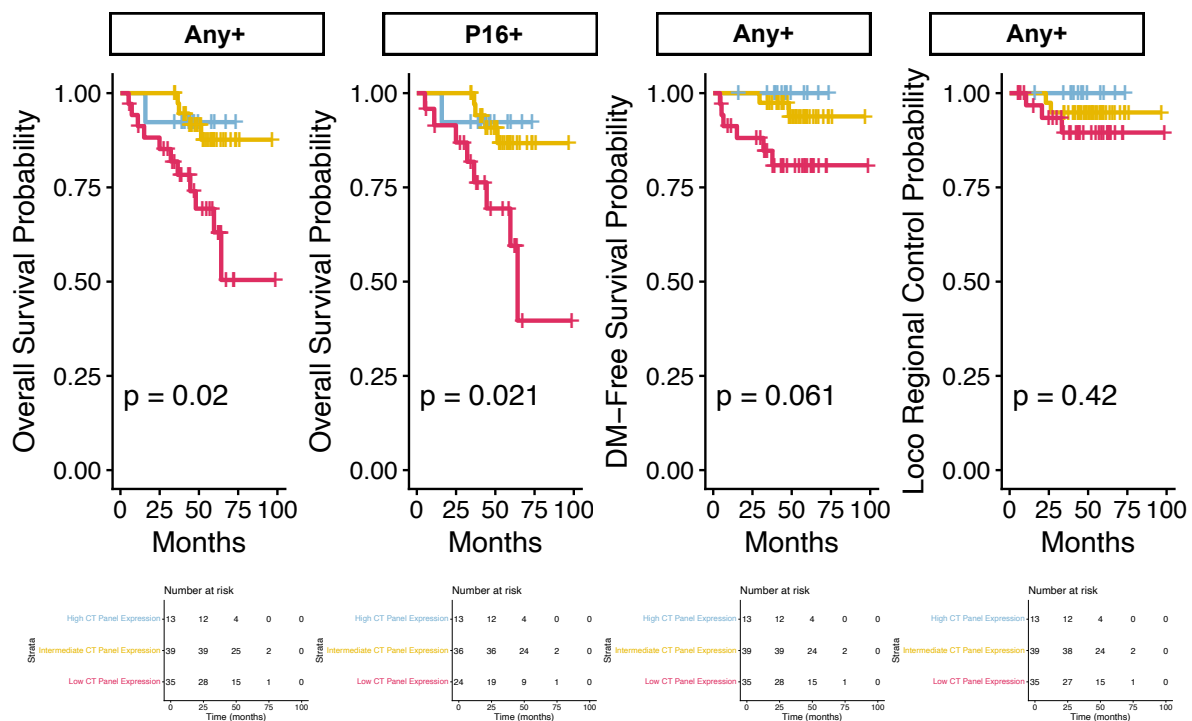


Figure 11. Kaplan–Meier analysis of HPV-positive patients stratified by CT panel expression, for additional survival endpoints. K-means expression groups: high (blue), intermediate (yellow), low (pink). Panels, left to right: overall survival in the any-positive cohort (Any+, $p = 0.02$) and the p16-positive cohort (P16+, $p = 0.021$); distant-metastasis-free survival in the any-positive cohort ($p = 0.061$); and loco-regional control in the any-positive cohort ($p = 0.42$). y-axis, survival/control probability; x-axis, months; log-rank test; number-at-risk tables shown beneath each plot.

I observed a trend towards worse DMFS probability ($p=0.061$) among the three risk groups, with the low CT panel expression group exhibiting the poorest prognosis and increased metastasis (Figure 8). Only 5 HPV+ patients had a tumour relapse, all of them were part of the intermediate and low CT panel expression groups (Figure 11).

3.5. TP53 Biology and Pathway analysis

3.5.1. TP53 alterations, p53 staining, and molecular subtype distribution vary across the risk groups in the DKTK cohort

To characterise the biological and clinical relevance of the risk groups, I compared available clinical and molecular variables across the three risk groups in the DKTK cohort (table 3). The low CT panel expression group, which is associated with high risk, exhibited higher frequencies of *TP53* mutations, positive p53 staining, and distinct distributions of molecular subtypes defined in Walter et al. (Walter et al. 2013). The high CT panel group associated to low risk was predominantly characterised by wild-type *TP53*, negative p53 staining, and enrichment of atypical molecular subtypes. No differences were observed in other clinical parameters like tumour staging, alcohol consumption, smoking history, etc. Due to having a lot of unknown variables in alcohol consumption and smoking history, I took caution in the interpretation of the results and tried to do an analysis independent of them. This also impacted my decision of not going forward with a multivariate cox analysis. The number of patients would not have been enough to achieve a significant test result. Data imputation was a choice, but I chose to avoid it because of a small number of patients. However, these results suggest that biological differences linked to TP53 status may drive the prognostic separation of CT panel subgroups.

Table 3. Distribution of clinical data across CT panel expression groups (low, intermediate, high) in the DKTK-ROG cohort.

Variable	"level"	"High CT Panel Expression"	"Intermediate CT Panel Expression"	"Low CT Panel Expression"	"p"
n		13	39	35	
P53	Negative	13 (100.0)	33 (84.6)	21 (60.0)	0.004
	Positive	0 (0.0)	6 (15.4)	14 (40.0)	
TP53 status	altered	1 (9.1)	2 (5.6)	15 (53.6)	<0.001
	wt	10 (90.9)	34 (94.4)	13 (46.4)	
HPV16 DNA	Negative	1 (8.3)	4 (10.3)	15 (42.9)	0.002
	Positive	11 (91.7)	35 (89.7)	20 (57.1)	
HPV16 RNA	Negative	0 (0.0)	1 (2.6)	30 (85.7)	<0.001
	Positive	13 (100.0)	37 (97.4)	5 (14.3)	
P16	Negative	0 (0.0)	2 (5.3)	9 (27.3)	0.007
	Positive	13 (100.0)	36 (94.7)	24 (72.7)	
Subtype	Atypical	6 (46.2)	26 (66.7)	4 (11.4)	<0.001
	Basal	0 (0.0)	0 (0.0)	2 (5.7)	
	Classical	0 (0.0)	1 (2.6)	7 (20.0)	
	Heterogeneous Subtypes	6 (46.2)	9 (23.1)	16 (45.7)	
	Mesenchymal	1 (7.7)	3 (7.7)	6 (17.1)	
Gender	Female	1 (7.7)	10 (25.6)	8 (22.9)	0.391
	Male	12 (92.3)	29 (74.4)	27 (77.1)	
Tumor stage	T1	3 (23.1)	4 (10.3)	5 (14.3)	0.132
	T2	4 (30.8)	27 (69.2)	19 (54.3)	
	T3	5 (38.5)	6 (15.4)	5 (14.3)	
	T4	1 (7.7)	2 (5.1)	6 (17.1)	
Age mean SD		59.54 (8.18)	59.36 (9.30)	54.80 (8.96)	0.071
Chemotherapy dose Gy mean SD		48.80 (12.76)	45.89 (16.72)	41.95 (19.32)	0.412
Smoking	Ex-smoker since 1 Year	2 (15.4)	4 (10.3)	2 (5.7)	0.928
	No	2 (15.4)	6 (15.4)	4 (11.4)	
	Unknown	5 (38.5)	13 (33.3)	13 (37.1)	
	Yes	4 (30.8)	16 (41.0)	16 (45.7)	

Alcohol	No	4 (30.8)	9 (23.1)	6 (17.1)	0.880
	Non-Alcoholic since 1 Year	0 (0.0)	4 (10.3)	3 (8.6)	
	Unknown	6 (46.2)	17 (43.6)	18 (51.4)	
	Yes	3 (23.1)	9 (23.1)	8 (22.9)	
N status	N0	0 (0.0)	3 (7.7)	4 (11.4)	0.404
	N1	1 (7.7)	5 (12.8)	5 (14.3)	
	N2	4 (30.8)	5 (12.8)	9 (25.7)	
	N2a	1 (7.7)	1 (2.6)	2 (5.7)	
	N2b	5 (38.5)	18 (46.2)	7 (20.0)	
	N2c	1 (7.7)	0 (0.0)	3 (8.6)	
	N3	1 (7.7)	7 (17.9)	5 (14.3)	
R status	R0	8 (61.5)	24 (61.5)	16 (47.1)	0.417
	R1	5 (38.5)	15 (38.5)	18 (52.9)	
Grading	G1	0 (0.0)	1 (2.6)	2 (5.9)	0.399
	G2	5 (38.5)	15 (39.5)	19 (55.9)	
	G3	8 (61.5)	22 (57.9)	13 (38.2)	
UICC status	II	0 (0.0)	2 (5.1)	1 (2.9)	0.596
	III	1 (7.7)	4 (10.3)	7 (20.0)	
	IV	12 (92.3)	33 (84.6)	27 (77.1)	
ECE status	No	5 (38.5)	19 (48.7)	15 (42.9)	0.776
	Yes	8 (61.5)	20 (51.3)	20 (57.1)	

3.5.2. Comparison of CT genes of ODP panel

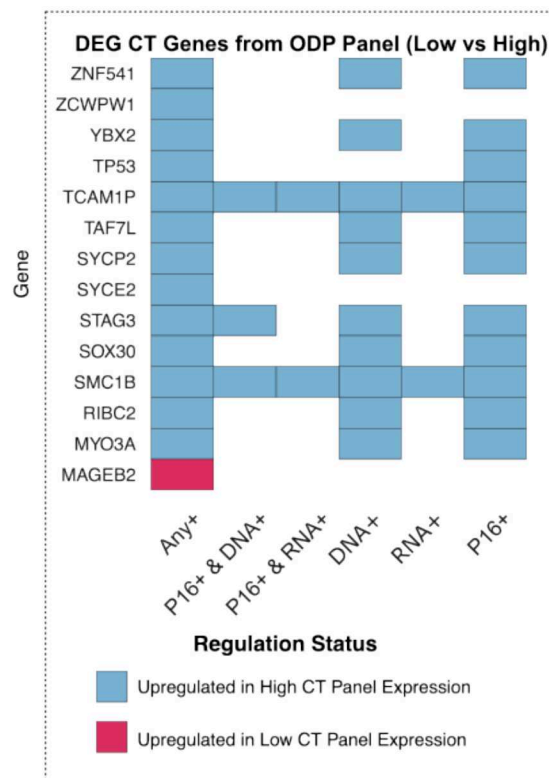


Figure 12. Significant cancer-testis DEGs from the ODP panel between high and low CT panel expression groups across HPV detection methods. Matrix in which rows are genes (ZNF541, ZCWPW1, YBX2, TP53, TCAM1P, TAF7L, SYCP2, SYCE2, STAG3, SOX30, SMC1B, RIBC2, MYO3A, MAGEB2) and columns are HPV detection cohorts (Any+, P16+ & DNA+, P16+ & RNA+, DNA+, RNA+, P16+). Coloured cells indicate genes significantly differentially expressed in that cohort: blue, upregulated in the high CT panel expression group; red, upregulated in the low CT panel expression group; blank, not significant.

To evaluate the transcriptional profiles of patients stratified by CT panel expression, I performed differential gene expression analysis between high and low CT expression groups across multiple HPV detection methods. I excluded the Intermediate expression groups were excluded to enable focused analysis of the signature's high- and low-expression extremes. Several significant DEGs were observed in the p16 (n = 954), HPV16 DNA (n = 142), and Any-Positive (n = 1195) detection cohorts. Apart from the five genes (RIBC2, STAG3, SMC1B, SYCP2, TCAM1P) from my CT panel, additional CT genes from the ODP as well as TP53 gene were also differentially

expressed between the two risk groups (Figure 12). These CT genes were derived from Wang et al ((Cheng Wang et al. 2016).

I compared the expression of TP53 among the three CT panel groups in p53 positive and negative groups along with mutational data (Figure 13). I observed that the low CT expression group exhibited higher frequencies of TP53 mutations and positive p53 staining, while the high CT expression group was predominantly wild-type TP53 and p53-negative patients. There were some irregularities. I observed that some mutations are grouped in the P53 negative patients, while some p53 positive patients had wild type properties.

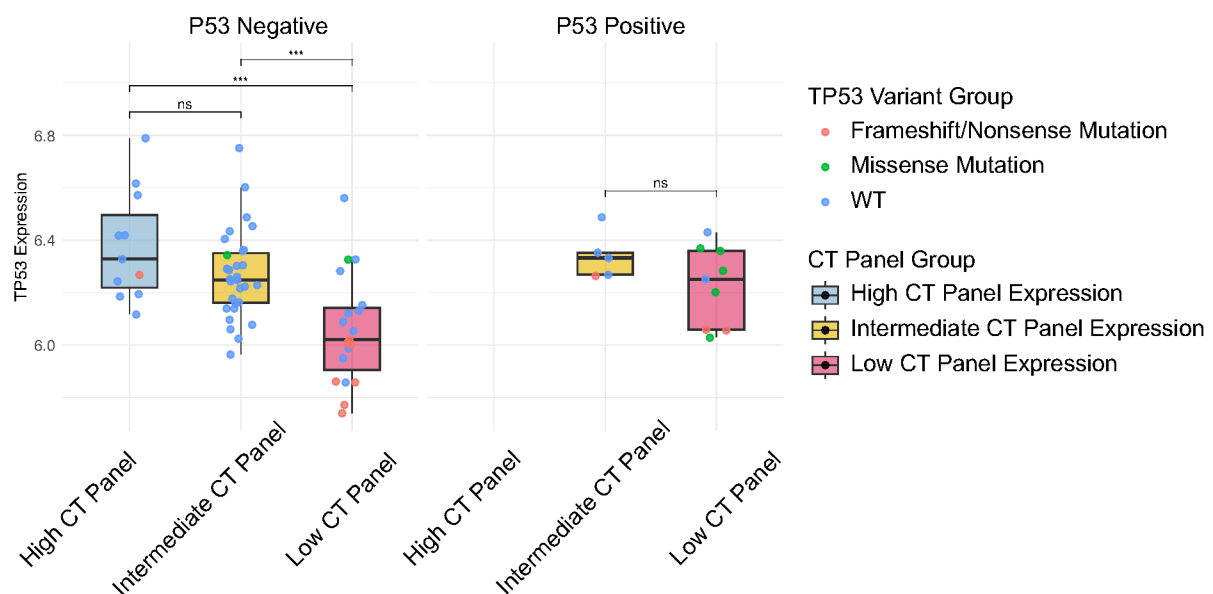
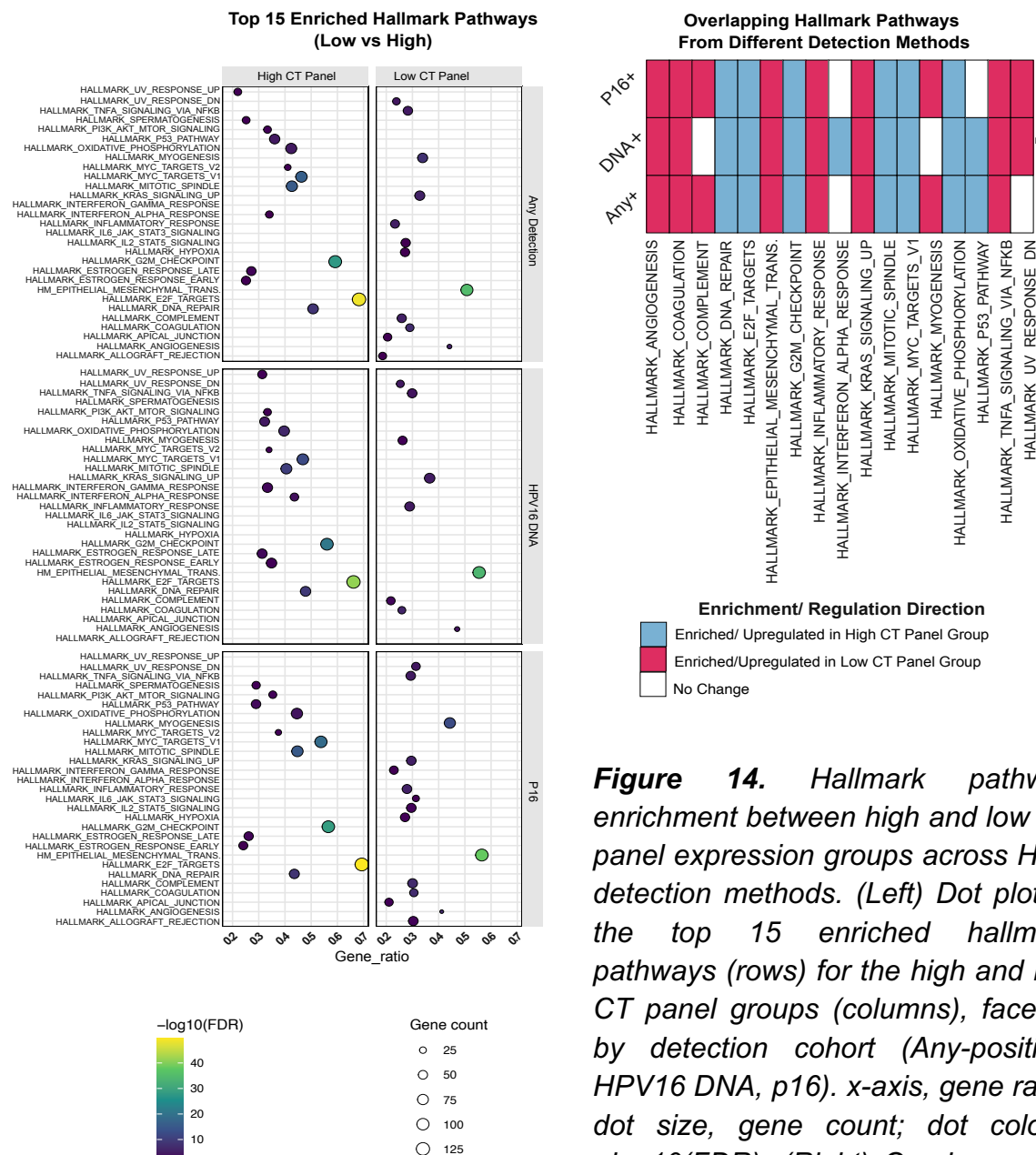


Figure 13. TP53 expression across CT panel expression groups, split by p53 immunohistochemistry status. Boxplots of TP53 expression for the high (blue), intermediate (yellow) and low (pink) CT panel expression groups in the any-positive cohort ($n = 87$), faceted into p53-negative (left) and p53-positive (right) tumours by IHC. Overlaid points are coloured by TP53 variant status: frameshift/nonsense mutation, missense mutation, wild-type. Pairwise comparisons by Wilcoxon rank-sum test (Mann–Whitney U): ns, not significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.5.3. CT panel-defined subgroups show hallmark pathway enrichment in p16, HPV16 DNA, and Any-positive patients



I observed Hallmark pathway enrichment, including Epithelial to mesenchymal transition (EMT), in the low CT expression high-risk group, while DNA repair pathways and P53 pathways were activated in the high CT panel low-risk group across all three cohorts (Figure 14). I mostly focused my analysis on the Any positive cohort for this study but also compared the HPV 16DNA and P16 cohort for the pathway enrichment analysis. In all three cohorts, the analysis generated similar results. These findings demonstrate that the CT panel captures biologically meaningful gene expression differences linked to tumour aggressiveness and DNA repair capacity across HPV-positive HNSCC cohorts.

3.5.4. TP53 expression correlates with CT panel genes, while CT panel provides complementary prognostic information

Spearman correlation analysis between TP53 and CT genes (ODP, n=13) revealed

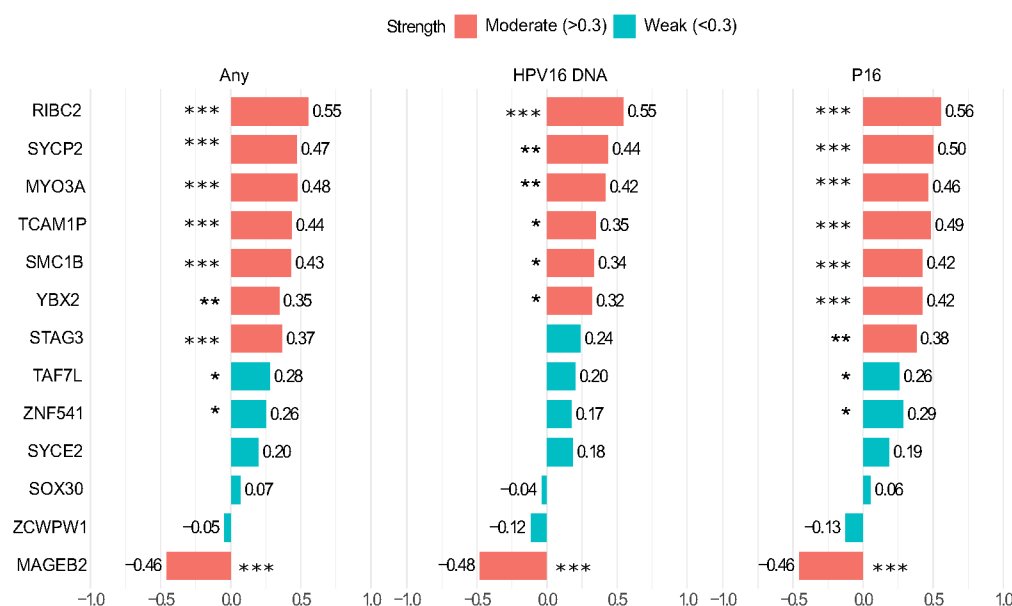


Figure 15. Correlation of TP53 expression with cancer-testis/ODP genes across HPV detection cohorts. Bar plots of Spearman correlation coefficients (ρ) between TP53 and 13 CT/ODP genes (rows) in the Any-positive, HPV16 DNA-positive and p16-positive cohorts (panels); the p value appears beside each bar; x-axis, -1.0 to 1.0 . Bar colour denotes correlation strength: weak ($|\rho| < 0.3$, teal) and moderate ($|\rho| \geq 0.3$, red). Significance (FDR): * < 0.05 , ** < 0.01 , *** < 0.001 .

significant positive correlations ($p < 0.05$) across HPV detection cohorts, with all five CT panel genes correlating with TP53 expression in the Any-positive (n=87), HPV16

DNA (n=66) and p16 (n=73) detection groups (Figure 15). To assess prognostic complementarity, I performed C-index and likelihood-ratio test (LRT) analyses (Table 4). As a single marker, the CT panel outperformed TP53 status alone for both endpoints (OS: C-index 0.651 vs 0.462, n=75; DM: 0.736 vs 0.55, n=75). The strongest prognostic performance, however, was achieved by combining the two: adding TP53/p53 to the CT panel gave the highest C-index for OS (0.691 vs 0.667 for the panel alone, n=87) and for DM (0.757, n=75; 0.781, n=87). The LRT confirmed that this combination carried significant independent prognostic information (LRT p=0.042 for OS and p=0.049 for DM with TP53 mutation), while p53 staining added only borderline value (LRT p=0.072 for OS). Together these results indicate that the CT panel and TP53 status are complementary rather than redundant: the CT panel is the stronger single predictor, but combining it with TP53 status gives the best overall prognostic performance.

Table 4. Concordance index (C-Index) and likelihood ratio test (LRT) results comparing prognostic models with and without TP53-based markers in combination with CT panel grouping, for OS and DM endpoints in the DKTK-ROG cohort.

Analysis	Model	C_Index	LRT_p
OS (n=75)	CT Panel	0.651	
	TP53 Mutation	0.462	
	CT Panel + TP53	0.623	0.042
OS (n=87)	CT Panel	0.667	
	P53 Staining	0.623	
	CT Panel + P53	0.691	0.072
DM (n=75)	CT Panel	0.736	
	TP53 Mutation	0.55	
	CT Panel + TP53	0.757	0.049
DM (N=87)	CT Panel	0.733	
	P53 Staining	0.669	
	CT Panel + P53	0.781	0.131

Combined CT panel and TP53 status enhance overall survival stratification

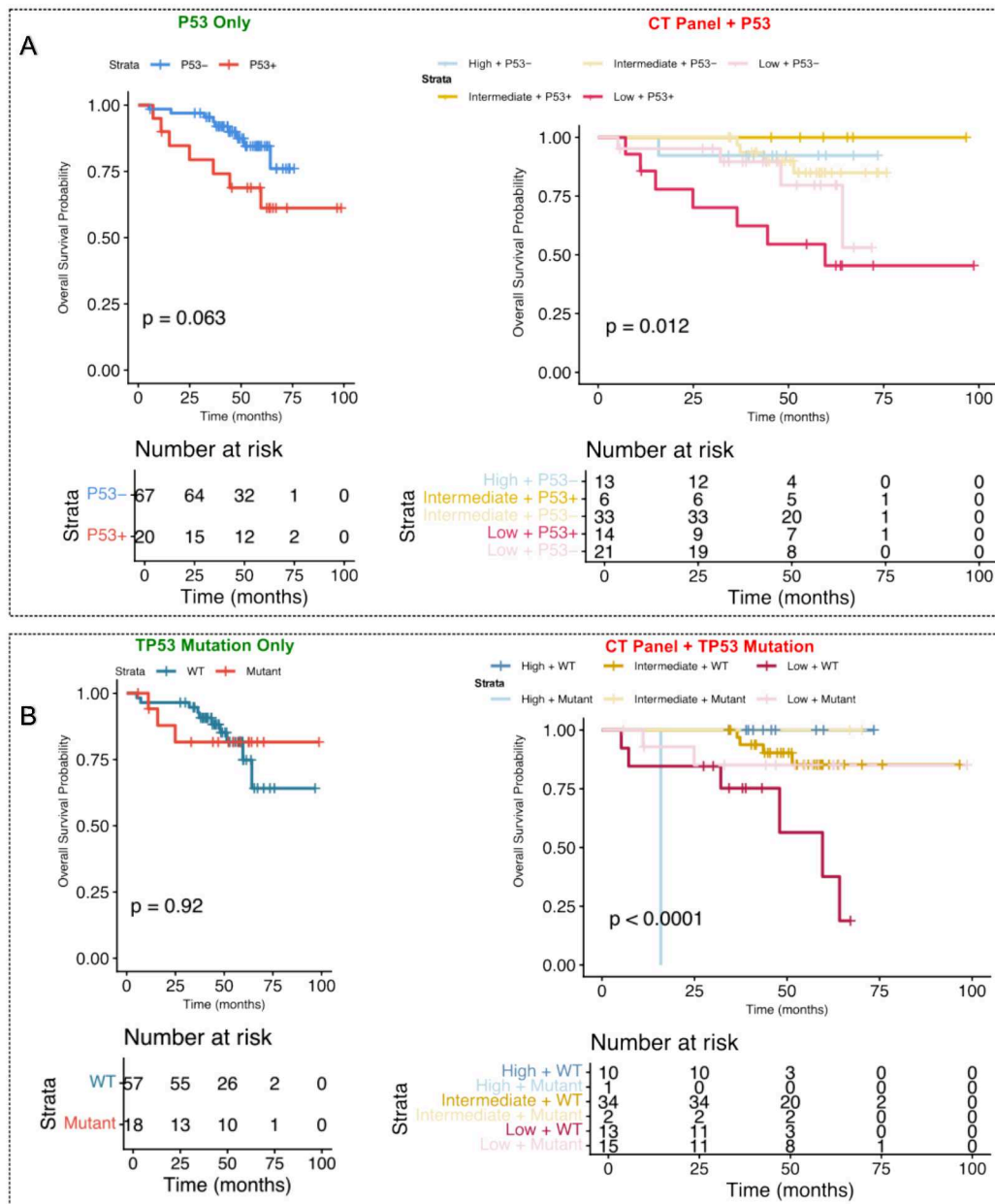


Figure 16. Kaplan–Meier overall survival for combined CT panel and TP53 stratification (Any-positive cohort, $n = 87$). (A) Overall survival by p53 immunostaining alone (left: P53-, P53+; $p = 0.063$) and by CT panel expression combined with p53 staining (Right: High CT + P53-, Intermediate CT + P53-, Intermediate CT + P53+, Low CT + P53+, Low CT + P53-; $p = 0.012$). (B) Overall survival by TP53 mutation status alone (left: wild-type, mutant; $p = 0.92$) and by CT panel expression combined with TP53 mutation status (right: High CT + WT, High CT + Mutant, Intermediate CT + WT, Intermediate CT + Mutant, Low CT + WT, Low CT + Mutant; $p < 0.0001$). y-axis, overall survival probability; x-axis, months; log-rank test; number-at-risk tables beneath each plot.

I observed that Integration of CT panel expression with TP53 status enhanced OS discrimination compared to single markers. Kaplan-Meier analysis of combined CT panel + TP53 mutations (Figure 12B, $p < 0.0001$) and CT panel + p53 staining (Figure 16, $p = 0.012$) showed distinct survival separation. In the Any-Positive cohort ($n=87$), the high CT expression group with WT TP53 or p53-negative status ($n=13$) demonstrated prolonged OS, whereas the low CT expression group ($n=35$) showed reduced OS. Across HPV detection methods, the high CT expression group consistently comprised p53-negative and TP53 wild-type tumours, while the low CT expression group (WT $n=13$, Mutant $n=15$) demonstrated heterogeneity in TP53 mutation type and p53 staining. The CT panel captures molecular differences not explained by TP53 mutation or p53 staining alone. The CT panel-based stratification identifies distinct risk groups in HPV-positive HNSCC, with TP53 status providing additional biological context.

4. Discussion: HPV-Positive HNSCC & Radiosensitivity

4.1. Multi-omics Identification and Biological Relevance of the CT Gene Panel

In this study, I identified a CT gene panel with prognostic significance for OS and LRC/PFS in patients with HPV-positive HNSCC treated with postoperative cisplatin-based RCT in DTK or RT in TCGA. My primary goal was to identify subgroups of HPV-positive patients, who may qualify for de-escalation protocols and those who may receive standard treatment similar to HPV-negative HNSCC patients. To achieve this, I integrated multi-omics data from preclinical models with patient data from TCGA followed by literature curation of previous HPV-HNSCC biomarker studies.

My multi-omics analysis revealed six genes (EREG, RIBC2, TCAM1P, SMC1B, ZSCAN23, and LY75) that consistently overlapped across both transcriptomic and epigenomic datasets, in both the preclinical and the TCGA cohort. To this core set, I added three additional candidates- CCND1, STAG3, and SYCP2, from the ODP panel, previously implicated in HPV-related studies with relevance in transcriptomics and epigenomics (Pyeon et al. 2007; Schlecht et al. 2007; Tonella et al. 2017; Koneva et al. 2018; Y. Zhang et al. 2016; Slebos et al. 2006; Wichmann et al. 2015; Wood et al. 2016; Lohavanichbutr et al. 2009). These three genes were significantly differentially expressed between HPV subgroups in both the preclinical and TCGA cohorts but did not show significant differential methylation, and I therefore excluded them from the final gene set. GTEx analysis confirmed that five genes (RIBC2, TCAM1P, SMC1B, STAG3, and SYCP2) exhibit pronounced testis-specific expression, further validated by an independent pan cancer study confirming them as CT genes (Cheng Wang et al. 2016). CT genes are aberrantly re-expressed in HNSCC and other malignancies, correlate with dysplasia and clinical outcome, and represent immunogenic, targetable biology, underscoring their functional relevance as biomarkers and therapeutic

candidates (Cuffel et al. 2011; Piotti et al. 2013; Simpson et al. 2005; Xie et al. 2019; Wu and Munger 2021).

To specifically evaluate the treatment response of my gene panel in the TCGA cohort, I focused on those patients who had received RT and performed K-means clustering using the five-gene CT panel. This analysis revealed two significant distinct gene expression clusters and HPV-negative patients predominantly clustered in the low-expression group, whereas HPV-positive cases were enriched in the high-expression group. Despite the limited number of HPV-positive patients who received RT (n = 50), I still observed a difference in PFS between the CT expression groups, as patients in the low CT panel (n=5) expression group showed poorer outcomes than those in the high CT group (n=45). To validate the CT panel in a larger cohort with comprehensive survival endpoints, I analysed the independent DTKK-ROG cohort comprising 87 HPV-positive patients treated with postoperative cisplatin-based RCT. Silhouette analysis validated a three-group structure in the DTKK cohort (Figure 9), reflecting the biological strategy to focus on the distinct extreme phenotypes (high and low CT expression) rather than intermediate states. The analysis revealed that HPV-negative patients predominantly clustered in the low-expression group, whereas HPV-positive cases were enriched in intermediate or high-expression groups. Kaplan-Meier survival analysis further demonstrated that HPV-positive patients with low CT panel expression had poorer outcomes.

The five CT genes encode proteins with roles in chromosomal organisation and DDR pathways. SMC1B, STAG3, and SYCP2 are established components of the meiotic cohesin complex regulating chromosomal organisation and DNA repair processes (Ishiguro 2019; Rankin 2015). Although preliminary studies suggest a potential link between these genes in DNA damage response (DDR) and HNSCC, their roles remain insufficiently characterised and require further investigation (Tian et al. 2021; Masterson et al. 2015). In contrast, RIBC2, which encodes a microtubule-associated protein, lacks a confirmed role in HNSCC or HPV biology (GeneCards Human Gene Database n.d.). Although TCAM1P is expressed in HPV-positive cervical cancer, its direct relevance to HPV-positive HNSCC remains to be fully elucidated (Zhu et al. 2022). Cilia as microtubule-based structures are involved in DDR pathways, and the previously described roles of TCAM1P and RIBC2 in cilia formation suggest that these five genes may be linked with DDR and treatment response (Morrison 2021). Given

that radiosensitivity is strongly influenced by tumour DNA repair capacity, and that ionizing radiation primarily induces DNA double-strand breaks, these processes may be relevant to treatment outcome under RT or RCT (Fabbrizi and Parsons 2020). High CT panel expression was associated with improved OS in both cohorts (Figure 10,11). This raises the possibility that coordinated expression of genes linked to chromosomal organisation and stress response marks a biologically distinct subgroup of HPV-positive tumours with more favourable treatment outcomes and may help explain why some HPV-positive patients have worse prognosis than others.

4.2. Prognostic Validation in the Independent DKTK-ROG Cohort

To stratify HPV-positive patients within the DKTK-ROG cohort, I examined my CT gene panel across different HPV detection methods (Lohaus et al. 2014). The panel showed robust prognostic separation in the p16+ subgroup, a weaker trend in the HPV16 DNA+ cohort, and no prognostic value in the HPV16 RNA+ group. Some of this discrepancy is likely explained by the fact that these assays capture different aspects of HPV biology. HPV16 DNA detection identifies the presence of viral genomic material but does not distinguish between active and latent infections, potentially including cases where the virus is present without driving oncogenesis (Lohaus et al. 2014). HPV16 RNA detection targets the transcriptional activity of the viral oncogenes E6 and E7, thereby providing a more direct measure of viral activity; however, this method is susceptible to RNA degradation in stored samples, which may lead to misclassification of active cases (Venuti and Paolini 2012). In contrast, p16 IHC detects overexpression of the p16 protein, a surrogate marker for HPV-driven oncogenesis that reflects E7-mediated degradation of the pRb protein (“Comparison of Detection Methods for HPV Status as a Prognostic Marker for Loco-Regional Control after RCT in Patients with HNSCC - PubMed” 2018). Its broad availability and ability to identify HPV-driven oncogenesis make it a practical choice for clinical routine, though its limited specificity, sometimes capturing HPV-negative tumours via alternative pathways, may influence its prognostic strength (Mehanna et al. 2023).

Additionally, some of the observed discrepancy may reflect differences in sample size and therefore statistical power across subgroups.

4.3. Molecular Pathway Landscape of CT Panel Risk Groups

GSEA provided additional insight into the biological differences between CT panel-defined risk groups. Tumours with low CT expression, corresponding to the high-risk group, were enriched in pathways related to proliferation and oncogenic signalling, suggesting aggressive tumour growth and disturbed cell cycle control. High CT expression tumours with low risk showed activation of DDR pathways as well as enhanced immune activation and oestrogen signalling (W. Zhang et al. 2016; Wichmann et al. 2015). When examined in the context of established HNSCC molecular subtypes (Walter et al. 2013), the atypical subtype, which is most commonly associated with HPV-positive tumours, was predominantly enriched in the intermediate and high CT groups, whereas low CT tumours showed marked heterogeneity without a clear subtype assignment. Patients with the atypical subtype have previously been reported to show more favourable outcomes in HNSCC ("Comprehensive Genomic Characterization of Head and Neck Squamous Cell Carcinomas" 2015). The enrichment of this subtype within the high CT expression group suggests that these tumours represent a favourable-risk population in the context of cisplatin-based RCT.

4.4. TP53 Alterations and Pathway Enrichment in CT Panel Risk Groups

My analysis revealed that low-CT tumours show enrichment of TP53 alterations (Figure 13), a finding that appears paradoxical given HPV's canonical E6-mediated p53 degradation (Scheffner et al. 1990). Mutation profiling identified two classes of TP53 alterations enriched in the low-CT group: gain-of-function stabilising missense

variants (e.g., R175H, G245D, R273C, R280G, R290H) that produce conformationally altered p53 resistant to E6 degradation, and truncating loss-of-function mutations (e.g., Q167*, Q192*, F54fs) that abolish p53 transcriptional activity (Freed-Pastor and Prives 2012). This may reflect a tumour population in which these TP53 mutations co-occur with altered DDR capacity and reduced expression of genes linked to chromosomal organisation and stress response. Notably, within the low-CT expression group, patients with WT TP53 demonstrated worse OS compared to those with TP53 mutations (Figure 16), suggesting that low CT expression itself is a dominant prognostic driver that supersedes individual TP53 mutation status. Model comparison suggested that the CT panel adds prognostic information beyond TP53 status (Table 4), indicating that the molecular state captured by the CT genes extends beyond individual TP53 alterations alone.

The enrichment of gain-of-function TP53 mutations in the low-CT group may have implications for tumour behaviour. Such variants have been associated with increased tumour aggressiveness and treatment resistance in HPV-positive HNSCC models (Prasad et al. 2026). In my cohort, this is in line with the observed trend toward reduced DMFS in the low-CT expression group within the DTK-ROG cohort (Figure 11), although the limited number of events precludes definitive conclusions regarding underlying mechanisms. These findings suggest that, beyond loss of canonical tumour suppressor function, altered p53 activity may contribute to a more aggressive phenotype in this subgroup. The distribution of relapse events in LRC is consistent with this interpretation. All five observed relapse events in the HPV-positive DTK-ROG cohort occurred in the intermediate and high-risk low CT-expression groups, while I observed none in the low-risk high-CT (favourable risk) group (Figure 10). Nevertheless, the absence of local relapse in the low-CT group, despite inferior overall and distant metastasis-related outcomes, may indicate a tendency toward systemic rather than locoregional progression, consistent with a more aggressive disease phenotype.

4.5. Limitations

Taken together, these findings support the CT panel as a biologically grounded stratification framework whose prognostic signal reflects a molecular state involving genomic organisation, DDR, and p53 pathway perturbation. However, dedicated functional studies will be required to establish the mechanistic relationships underlying these associations.

I acknowledge several limitations in this study. Independent external validation is currently limited by the scarcity of large, well-annotated HPV-positive cohorts, and prospective multi-centre studies will be essential to establish the prognostic robustness and clinical utility of the CT panel. The CT panel could be readily implemented in clinical practice using a small FFPE-compatible expression assay, such as a targeted RT-qPCR or RNA-seq panel, enabling robust measurement from standard diagnostic biopsy material. Future investigations should aim to dissect the mechanisms driving aggressive behaviour in low-CT tumours, including specific TP53 alterations, DDR dysfunction, and immune evasion strategies, employing spatially resolved and targeted molecular technologies. Elucidating whether and how CT genes actively contribute to these processes will clarify their role in modulating tumour biology and inform the design of more precise therapeutic strategies, ultimately enabling rational treatment de-escalation for the favourable-risk majority while ensuring that patients at highest risk continue to receive standard treatment.

4.6. Conclusion

In conclusion for the first section, my integrated multi-omics analysis identifies a CT gene panel that stratifies survival risk within HPV-positive HNSCC. In the independent DTK-ROG cohort, the panel consistently defined a favourable-outcome group across HPV detection methods, with the strongest prognostic separation observed in the clinically relevant p16-positive subgroup. Low CT expression was associated with inferior survival and enrichment of TP53 alterations, suggesting that a subset of HPV-

positive tumours harbours biologically aggressive features despite HPV positivity. These findings support the CT panel as a promising framework for refining risk stratification in HPV-positive HNSCC and for distinguishing patients who may be

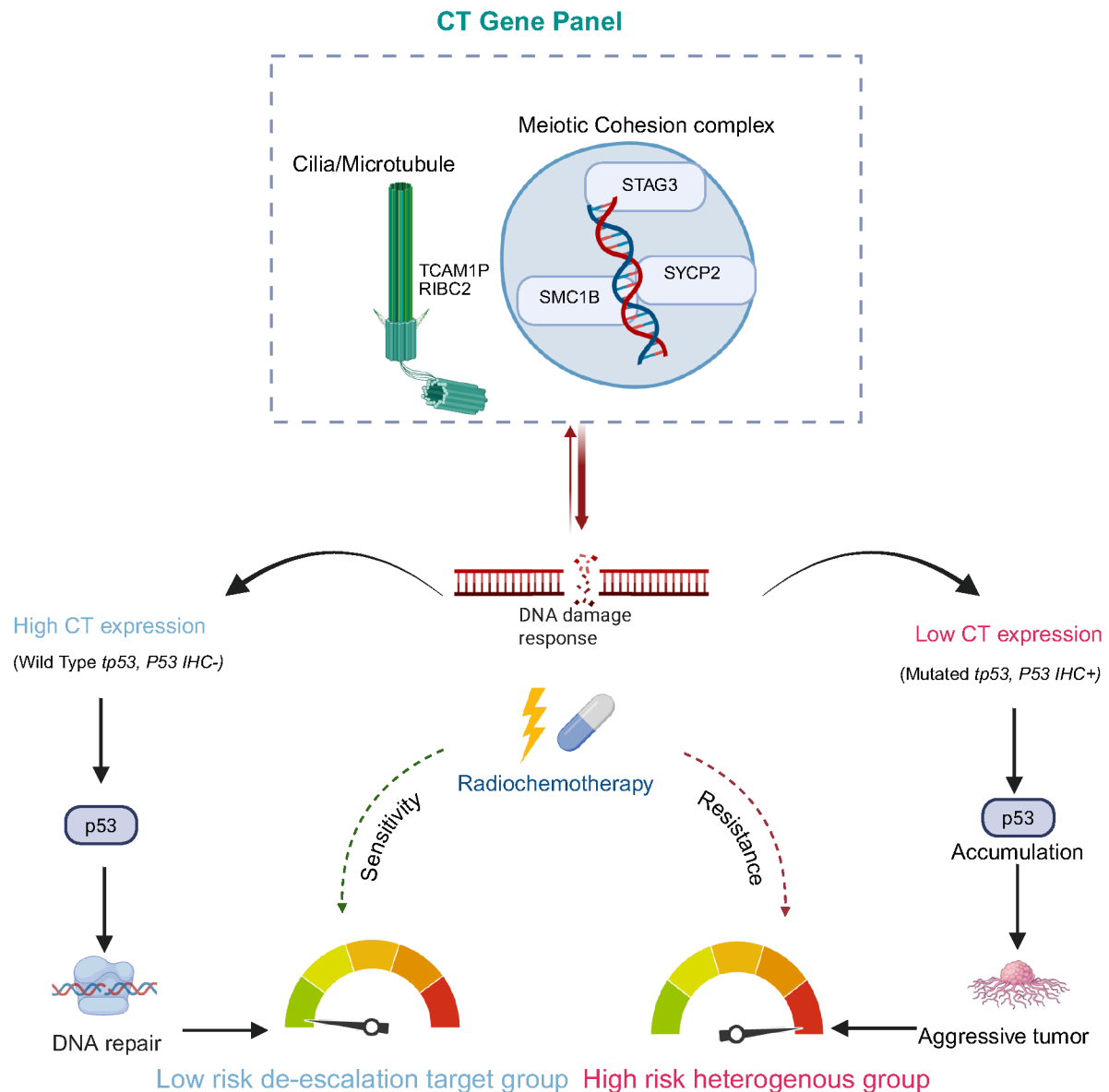


Figure 17. Schematic model illustrating how the five-gene CT panel (*SYCP2*, *STAG3*, *SMC1B*, *RIBC2*, *TCAM1P*) may modulate DNA damage response, *p53* status, and treatment sensitivity, distinguishing low-risk (high CT expression, DDR-proficient) from high-risk (low CT expression, *p53*-mutant, DDR-deficient) HPV-positive HNSCC riskgroups. (Created in <https://BioRender.com>.)

considered for treatment de-escalation from those who may benefit from standard therapy. To my knowledge, this is the first study to define a CT gene-based stratification approach specifically for HPV-positive HNSCC, providing a rationale for prospective and mechanistic validation of its clinical utility. Because it requires only a small, FFPE-compatible expression readout (targeted RT-qPCR or RNA-seq), it is in principle a practical routine diagnostic biopsy material. It has two translational promises: sparing the favourable-risk majority the long-term toxicity of full-intensity chemoradiotherapy, while flagging the biologically aggressive low-CT minority for standard or intensified treatment. Realising this will require prospective, multi-centre validation and functional dissection of how CT-gene loss, DDR dysfunction, and altered p53 activity jointly shape radioresistance; the step that would move the panel from a prognostic signal to a treatment-guiding biomarker.

Part II: Tumour Heterogeneity and Spatial OMICS

Bulk molecular subtyping assigns a single label to each tumour and, in doing so, averages over the different cell states that coexist within it; in practice a large fraction of tumours cannot be confidently classified at all. This obscures the intratumoral heterogeneity thought to drive treatment resistance, including the hypoxic and mesenchymal states linked to radioresistance in HNSCC.

In this second part I move from bulk to spatial heterogeneity and ask how transcriptional programmes are organised within HNSCC tissue. Using Visium FFPE spatial transcriptomics of xenograft tumours, with co-registered histology and PIMO hypoxia labelling, I apply an unsupervised NMF approach to decompose each tumour into recurrent MPs, then relate these to hypoxia, tissue architecture, bulk molecular subtype, and the intrinsic radioresistance of the models. The aim is to build a spatially resolved map of HNSCC tumour states that goes beyond what bulk subtyping captures.

5. Methods: Tumour Heterogeneity and Spatial OMICS

5.1. Preclinical Cohort and Experimental Design

To characterise transcriptional heterogeneity across HNSCC xenograft models, I first performed bulk molecular subtype assignments across multiple independent xenograft projects using centroid-based classification (Walter et al. 2013). This cross-project survey revealed that the Heterogenous subtype was the predominant assignment across cell lines, indicating that the majority of HNSCC xenograft samples lack a

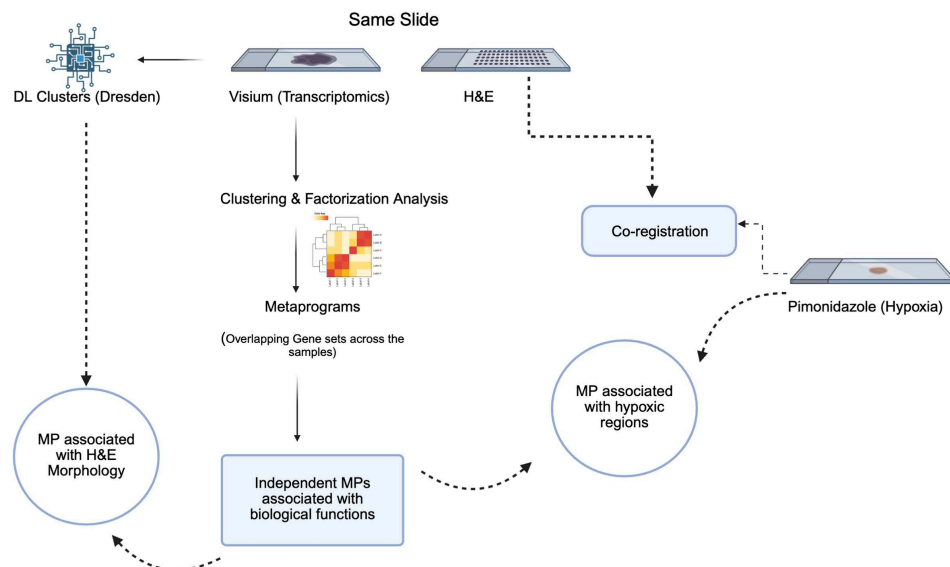


Figure 18. Overview of the spatial transcriptomics analysis workflow. Visium FFPE slides provide simultaneous transcriptomic and morphological (H&E) readouts from the same tissue section. Transcriptomic data undergo NMF-based factorisation to yield consensus metaprograms (MPs), subsequently associated with pathologist-annotated tissue classes (QuPath) and pimonidazole-based hypoxia labels in a consecutive section (co-registration). (Created in <https://BioRender.com>.)

dominant transcriptional centroid at the bulk level. From this broader cohort, I selected twenty treatment-naïve Visium FFPE sections for paired bulk RNA-sequencing and spatial transcriptomics; these sections were derived from seven human HNSCC cell lines: FaDu (oropharyngeal carcinoma), SAS (tongue carcinoma), Cal33 (laryngeal carcinoma), UT-SCC-5 (laryngeal carcinoma), UT-SCC-8 (pharyngeal carcinoma),

UT-SCC-45 (laryngeal carcinoma), and SAT. Xenograft tumour sections were prepared and co-stained with PIMO by Maria Jose Besso (post-doctoral researcher, DKFZ); PIMO staining was performed on a consecutive section adjacent to the Visium section. PIMO is a 2-nitroimidazole compound that forms stable protein adducts specifically under hypoxic conditions ($pO_2 < 10$ mmHg) and is visualised by IHC, providing a direct, spatially resolved map of oxygen status. Of the 20 sections, all had matched bulk RNA-sequencing profiles, 17 had complete PIMO hypoxia annotations, and 19 had pathologist H&E annotations available; 16 sections had complete data across all three modalities. H&E annotations were done by Dr. Thomas Metzler (Preclinical and Translational Pathology, Core facility, DKFZ).

5.2. RNA Extraction and Bulk RNA Sequencing from the Preclinical Cohort

I isolated total RNA from 20 formalin-fixed, paraffin-embedded (FFPE) preclinical samples using the AllPrep DNA/RNA FFPE Kit (Qiagen, Cat# 80234), following the manufacturer's protocol. RNA sequencing libraries were subsequently prepared at the DKFZ GPCF using the Illumina RiboZero Stranded Kit. Libraries were quality-checked with a Qubit fluorometer and Agilent TapeStation prior to sequencing on the Illumina NovaSeq 6000 platform (paired-end 2×100 bp), yielding a minimum of 63 million reads per sample.

5.3. RNA Sequencing Data Alignment and Processing

RNA sequencing (RNA-seq) data from xenograft samples were processed using the Roddy RNA-seq Workflow (v1.3.0) maintained by the Omics IT and Data Management Core Facility (ODCF) at the DKFZ. Sequencing reads were aligned to a hybrid reference genome composed of human (hs37d5) and mouse (GRCm38mm) assemblies, supplemented with a PhiX control sequence, using the STAR aligner with two-pass mapping enabled (--twopassMode Basic). Aligned reads were output as sorted and indexed BAM files. Post-alignment, BAM files were merged and duplicate

reads were marked using Sambamba. Further processing and indexing were performed with SAMtools. Gene-level expression quantification was performed using featureCounts, counting only reads aligned to human genes; mouse-derived reads were excluded from the final count matrices.

5.4. Molecular Subtyping of Bulk RNA-sequencing Data

5.4.1. Data Preparation and Normalisation

I performed all analyses in R (v4.4.3). I loaded bulk RNA-sequencing count matrices and applied VST using the DESeq2 package (v1.40.2;(Love, Huber, and Anders 2014)). Following VST, I applied median-centring to each gene across all samples:

$$x_{\text{centred}} = x_{\text{original}} - \text{median}(x)$$

5.4.2. Centroid-Based Subtype Assignment

I computed Pearson correlation coefficients between each sample's median-centred gene expression profile and four pre-defined molecular subtype centroids (Atypical, Basal, Classical, and Mesenchymal), derived from prior transcriptomics studies of head and neck cancer. These centroids represent the average expression pattern of each subtype across a reference training set, computed from 838 genes shared between the bulk RNA-sequencing data and the reference signatures (Walter et al., 2013).

For each sample, the subtype with the highest Pearson correlation was assigned as the candidate subtype. Assignment was confirmed only when two stringent criteria were simultaneously satisfied:

1. The maximum correlation coefficient must exceed 0.2, ensuring at least minimal similarity to the best-matching centroid.
2. The margin between the top-ranked and second-ranked correlation values must exceed 0.2, ensuring sufficient separation between the most and second-most likely subtypes.

This method was adapted from (Patil et al. 2022). Samples failing either criterion were designated *Heterogenous Subtypes*, operationally defined as tumours exhibiting mixed transcriptional signatures with no single dominant molecular phenotype.

5.4.3. Spatial Resolution of Subtypes

To assess whether bulk-level subtype assignments could be resolved at single-spot spatial resolution, I applied the identical centroid correlation method to individual Visium spots (log-normalised, median-centred; minimum correlation threshold 0.2, minimum margin 0.2).

5.5. Visium Spatial Transcriptomics Tissue Preparation

Visium spatial transcriptomics libraries were prepared from FFPE tissue sections following the 10x Genomics Visium Spatial Gene Expression for FFPE protocol (10x Genomics, User Guide CG000407). Tissue sectioning, slide preparation, permeabilisation, and library construction were performed by Dr. Maria Jose Besso (post-doctoral researcher, E220, DKFZ), with technical assistance from the Single-cell Open Lab at the DKFZ. Briefly, FFPE tissue sections were deparaffinised, rehydrated, and mounted onto Visium capture areas. Following haematoxylin and eosin (H&E) staining and imaging, decrosslinking was performed to reverse formalin-induced crosslinks and improve probe accessibility, after which tissue permeabilisation was performed and hybridisation-based cDNA capture was carried out using the Visium Human Transcriptome Probe Set v1.0. Sequencing-ready libraries were constructed through extension, ligation, and PCR amplification steps as specified in the manufacturer's protocol. Libraries were sequenced in an Illumina Novaseq6000 platform (pair end 50bp, S1 flowcell) at the Next Generation Sequencing DKFZ core facility.

5.6. Processing and Quality Control of Spatial Transcriptomics Data

5.6.1. SpaceRanger Processing

Spatial transcriptomics data from Visium FFPE slides were processed using the Space Ranger pipeline (v3.1.3, 10x Genomics). Verena Bitto (post-doctoral researcher, DKFZ) developed the initial processing script and completed alignment for a subset of samples; I performed SpaceRanger processing for the remaining samples using the same pipeline. Space Ranger performed demultiplexing of sequencing reads, alignment of cDNA reads to the human genome reference (GRCh38 2020-A) using STAR, assignment of reads to spatial barcodes, generation of feature-barcode matrices (UMI counts per gene per spot), and image registration linking transcriptomics data to high-resolution H&E histology images. For one sample (N155b200_UT8_SPT_D1), automated fiducial spot detection failed due to poor fiducial spot quality on the capture area; manual tissue alignment and spot selection were therefore performed using the 10x Genomics Loupe Browser.

For each of the 20 samples, Space Ranger count was executed with the Visium Human Transcriptome Probe Set v1.0 (17,943 human genes).

5.6.2. Quality Control Assessment

I performed post-processing quality control in R (v4.4.3) using the Seurat package (v5.0.1; (Hao et al. 2024)). I loaded processed spatial transcriptomics data into Seurat objects using the `Load10X_Spatial()` function. For each sample, the following metrics were systematically calculated:

- **Total UMI counts per spot** (*nCount_Spatial*): the sum of UMI counts across all genes, reflecting sequencing depth and transcript abundance.
- **Number of detected genes per spot** (*nFeature_Spatial*): the count of genes with at least one UMI, reflecting capture complexity.
- **Percentage of mitochondrial genes** (*percent_mt*): calculated using `PercentageFeatureSet()` with pattern `^MT-`. Absence was verified, as Space Ranger's FFPE probe set excludes mitochondrial genes by default.

- **Percentage of ribosomal genes** (*percent_ribo*): similarly verified absent using patterns [^]RPL and [^]RPS.

Image registration was verified for all samples by confirming correct scale factors and visual overlay of H&E histology with expression-based metrics. Per-spot quality metrics (UMI counts, detected genes) were assessed across the cohort and inspected visually.

5.7. Metaprogram Generation via NMF and Leiden Clustering

I applied a four-stage computational pipeline adapted from (Greenwald et al. 2024), combining unsupervised gene program discovery via NMF, spatial validation via Leiden community detection, inter-sample integration, and consensus MP definition. All analyses were performed in R (v4.4.3) using Seurat (v5.0.1) and NMF (v0.25). I performed MP discovery on all 20 Visium sections; I restricted downstream biological and statistical analyses comparing hypoxic and normoxic regions to the 17 sections with complete PIMO hypoxia annotations.

5.7.1. Stage 1: Per-Sample Gene Program Discovery via NMF

Prior to NMF, I applied hierarchical gene filtering to each sample independently to remove genes unlikely to capture true biological signals:

1. **Organellar gene removal:** genes matching [^]MT-, [^]mt-, [^]Mt-, [^]RPL, [^]RPS, [^]rpl, or [^]rps were excluded. Mitochondrial and ribosomal genes are highly abundant but reflect housekeeping functions rather than cell-state-specific biology; their inclusion would dominate factorisation and obscure programs of interest.
2. **Zero-variance gene removal:** genes with variance = 0 after log-normalisation were excluded, as they provide no discriminative information.
3. **Low-expression filtering:** genes with mean expression ≤ 0.4 on the log-normalised scale were excluded. This removes consistently lowly-expressed genes that represent noise or detection artefacts.

4. **Post-centring filtering:** after applying mean-centring and clipping negative values to zero (reflecting the biological non-negativity constraint of NMF), genes with summed centred expression = 0 were excluded.

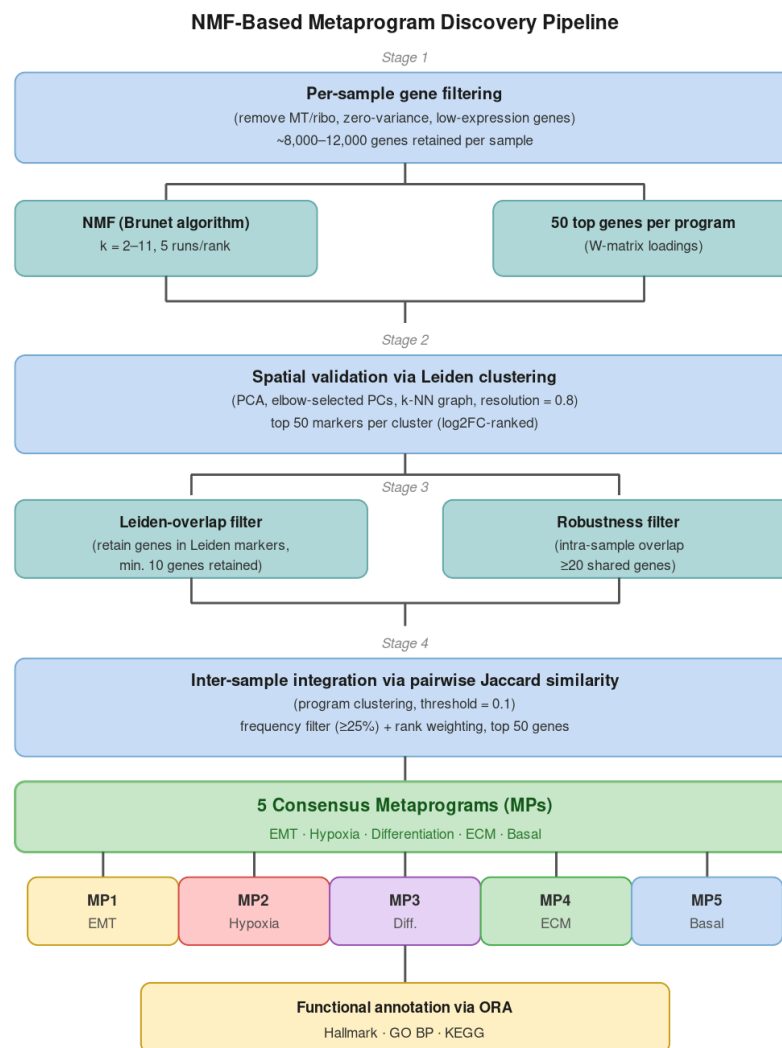


Figure 19. Four-stage NMF-based metaprogram discovery pipeline. Stage 1: per-sample gene filtering and rank-sweep NMF (Brunet algorithm, $k=2-11$, 5 runs/rank). Stage 2: independent Leiden clustering for spatial validation. Stage 3: Leiden-overlap and robustness filters. Stage 4: pairwise Jaccard similarity and greedy clustering to 5 consensus metaprograms, annotated by ORA (Overrepresentation analysis).

I then performed rank-based NMF using the Brunet algorithm (method = "brunet", NMF R package v0.25; Brunet et al., 2004) across ranks $k = 2$ to 11, with 5 independent runs per rank ($nrun = 5$), each initialised from sample-specific random seeds. NMF decomposes the gene expression matrix $\mathbf{X}$ (genes $\times$ spots) into two non-negative matrices:

$$X \approx W \times H$$

where **W** (genes × programs) contains gene loadings and **H** (programs × spots) contains program activities. The top 50 genes per program, ranked by loading strength in **W**, were extracted as the gene set representing each program. This yielded a total of 1,000 NMF factorisations (20 samples × 10 ranks × 5 runs).

5.7.2. Stage 2: Spatial Validation via Leiden Clustering

I independently processed each spatial sample for graph-based clustering to provide a spatially-grounded reference gene set for NMF program validation. The workflow comprised:

1. Log-normalisation using `NormalizeData(method = "LogNormalize")` with scale factor 10,000.
2. Variable feature selection using `FindVariableFeatures(selection.method = "vst", nfeatures = total_genes)`, using all expressed genes as variable features to avoid restricting marker detection to a subset.
3. PCA with 50 components using `RunPCA(assay = "Spatial", npcs = 50, features = all_genes)`.
4. A fixed threshold of 15 PCs was applied uniformly across all samples for neighbour graph construction.
5. K-nearest neighbour graph construction in PCA space using `FindNeighbors(dims = 1:15)`.
6. Leiden clustering (Traag, Waltman, and van Eck 2019) using `FindClusters(algorithm = 4, resolution = 0.8)`, yielding 8-15 clusters per sample.
7. UMAP visualisation for spatial topology assessment using `RunUMAP(dims = 1:15)`.

Cluster-specific marker genes were identified using `FindAllMarkers()` (Wilcoxon rank-sum test; `only.pos = TRUE`, `min.pct = 0.25`, `logfc.threshold = 0.25`). The top 50 genes ranked by $\log_2$ fold-change per cluster were selected as cluster-specific markers.

5.7.3. Stage 3: Program Filtering and Robustness Selection

I pooled all NMF programs from all 20 samples and all ranks ($k=2-11$) into a single collection. Two sequential filtering steps were then applied following the (Greenwald et al. 2024) methodology.

First, each program's gene set was filtered to retain only genes present in the Leiden cluster marker set for that sample (top 50 genes per cluster by average $\log_2$ fold-change):

$$p_{\text{filtered}} = p_{\text{NMF}} \cap M_s$$

where M_s is the union of Leiden marker genes from all clusters in sample s .

Second, a robustness filter was applied: only programs that shared at least 20 genes with at least one other program from the same sample ($\text{intra_min} = 20$) were retained. This criterion ensures that each program represents a reproducible transcriptional state (identifiable across multiple NMF ranks or runs within the same sample) rather than a single-run artefact.

5.7.4. Stage 4: Inter-Sample Integration and Consensus Metaprogram Definition

I computed Pairwise Jaccard similarities among all retained robust programs:

$$J_{ij} = \frac{\text{shared genes}}{\text{total unique genes}}$$

This yielded a symmetric ($n_{\text{programs}} \times n_{\text{programs}}$) Jaccard similarity matrix. Programs with $J \geq 0.1$ were grouped into clusters using a greedy agglomeration algorithm. Within each cluster, consensus genes were defined using a frequency $\times$ rank-weighting strategy:

1. **Frequency criterion:** genes appearing in $\geq 25\%$ of programs within a cluster were designated consensus candidates.
2. **Rank weighting:** for each consensus candidate, the average rank position across programs where it appears was calculated (rank 1 = highest loading). Genes were sorted by average rank position (ascending).
3. **Top gene selection:** the top 50 genes from this rank-weighted list constituted the final consensus MP.

Program clusters with fewer than 10 consensus genes or contributing fewer than 5 input programs were excluded as unstable. This yielded five validated consensus MPs, designated MP1-MP5.

5.7.5. Metaprogram Scoring and Dominant Assignment

Each of the five consensus MPs was scored per spot by computing the mean log-normalised expression across all MP signature genes present in the spot's expression profile. Only MPs with at least five genes detected per spot were scored; otherwise the score was set to NA. This approach was applied using `colMeans()` on the log-normalised expression submatrix for each MP gene set.

I assigned each spot to the MP with the highest score (dominant MP), determined by `which.max()`. Assignment confidence was quantified by the dominance margin (top score – second-highest score). All five MP scores, the dominant MP assignment, and the dominance margin were stored per spot in the master spot table.

To characterise the transcriptional fingerprint of each cell line and each individual section, I aggregated spot-level dominant MP assignments at the sample and cell-line level. For each cell line (pooling all sections) and for each individual section separately, the percentage of spots assigned to each of the five MPs was computed. The MP with the highest percentage was designated the dominant MP for that cell line or section; the MP with the second-highest percentage was designated the secondary program.

5.8. Functional Annotation of Metaprograms

I performed functional annotation of each MP via over-representation analysis (ORA) using the `clusterProfiler` package (v4.8.3; (Yu 2024)) in R. The input gene list for each MP comprised the top 50 consensus genes ranked by average NMF loading position (W-matrix rank weighting). These gene names were tested for over-representation against three reference collections:

- **MSigDB Hallmark gene sets**
- **GO Biological Process**
- **KEGG pathways**

Statistical significance was assessed at $FDR < 0.05$ (Benjamini-Hochberg correction).

5.9. Pathologist Annotation via Area-Based Majority Assignment

5.9.1. Polygon Drawing and Export

Tissue class annotations were manually drawn on the H&E images by Dr. Thomas Metzler (Preclinical and Translational Pathology, Core facility, DKFZ) using QuPath. Tissue classes annotated included: Tumour, Necrosis, Stroma, Keratinization, Blood Vessels, Inflammatory Infiltrate, Immune Cells, Oedema, Muscle, Epidermis, and Bacteria. Completed annotations were exported as GeoJSON files, one per sample. Since the same H&E images were used in both QuPath and SpaceRanger, no additional image co-registration was required.

One Cal33 sample (N154a038_Cal33_SPT_B1) had no pathologist annotation available and was excluded from all annotation-dependent analyses. The remaining 19 of 20 samples were annotated.

5.9.2. Area-Based Spot Assignment

I followed an area-based majority rule for spot-label assignment, adapting the approach of (Dong et al. 2025), who established that “when 70% of the Visium spot’s area was overlapping with a GeoMx segment, it was considered matching to that segment’s AOI label.” All geometric operations were performed using the `sf` package (v1.0; (“Simple Features for R” n.d.)) in R.

The procedure for each sample was as follows:

1. **Spot centroid extraction:** spot centre coordinates (`pxl_col_in_fullres`, `pxl_row_in_fullres`) were read from the master spot table.
2. **Spot radius retrieval:** the per-sample spot radius in full-resolution pixels was retrieved from SpaceRanger’s `scalefactors_json.json` file (`spot_diameter_fullres / 2`).

3. **Spot circle construction:** each spot centroid was buffered to its measured radius using `st_buffer()`, representing the 55 μm -diameter Visium capture area as a circle in pixel space.
4. **Tissue class union:** for each tissue class, all QuPath annotation polygons of that class were merged into a single unified geometry using `st_union()`. This prevents double-counting when a spot circle overlaps multiple non-adjacent polygons of the same class.
5. **Intersection area computation:** the geometric intersection between each spot circle and each tissue class geometry was computed using `st_intersection()`, and its area calculated with `st_area()`. This was expressed as a fraction of the theoretical spot area (πr^2):

$$f_{\text{class}} = \frac{\text{area}(\text{spot} \cap \text{class polygon})}{\pi r^2}$$

6. **Label assignment:** the tissue label whose fraction was largest and met or exceeded the 70% threshold was assigned. Spots with no overlap with any annotation polygon were labelled *Unannotated*. Spots with some overlap but no single class reaching the 70% threshold were labelled *Ambiguous*, representing boundary spots between two tissue classes. Both categories were excluded from annotation-dependent downstream analyses.

5.10. Hypoxia Label Co-registration

PIMO immunohistochemical staining (Raleigh et al., 1998) provided spot-level hypoxia labels. Hypoxia images were co-registered by Cylia Ouadah (PhD Student, OncoRay, Dresden).

5.10.1. Co-registration of Immunofluorescence to H&E Images

To enable complementary analysis of SPT data with respect to hypoxic regions, IF images from consecutive tissue slices were registered to their corresponding H&E images of the SPT data. This alignment allowed the spatial mapping of PIMO staining-derived hypoxia segmentation masks onto histological tissue.

Due to inherent differences between modalities (IF and H&E), as well as the tissue variability introduced with consecutive tissue sections and separate staining procedures (holes, folding, cellular differences), intensity-based and multi-resolution registration methods were excluded for simplification. Instead, the registration strategy relied on the overall tumour section shape and reference landmarks. A coarse landmark-based approach was adopted with manual selection of reference points pairs (landmark pairs) on both the moving and fixed images.

The DAPI channel from the same section as the pimonidazole staining, was considered to be the moving image, to be aligned to the H&E image, which served as the fixed image. To improve computational efficiency, both images were downsampled by a factor of 5 using PIL (12.2.0) and LAZCONS (lossless compression). The DAPI image, originally acquired at a resolution of $0.3248 \mu\text{m} \times 0.3248 \mu\text{m}$, was subsequently upsampled by a factor of 1.19 to match the H&E image resolution (Original pixel size: $0.2731 \mu\text{m} \times 0.2731 \mu\text{m}$).

Corresponding points across the DAPI and H&E images were manually identified using a custom Python (3.10) GUI built with Tkinter, enabling manual selection of landmark pairs ($n_pairs \geq 3$). Two transformation models were evaluated: Rigid (translation, rotation) and Affine (translation, rotation, scaling and shearing).

The transform matrix was computed for the chosen landmark pairs with SimpleITK (2.5.2) `LandmarkBasedTransformInitializerFilter`, which determines the optimal fit (in a least-squares sense) mapping the moving points to the fixed ones. Both Rigid and Affine transformations were then applied to the moving image through resampling with linear interpolation, and the one providing the best visual alignment of the images was selected.

Due to the coarse nature of the landmark-based registration and inherent tissue variability across consecutive sections, pixel-perfect alignment between the IF and H&E images was not achieved. However, the spatial correspondence was considered to be sufficient for spot-based and region-based analysis.

5.10.2. Hypoxia labels for SPT spots

Hypoxia masks derived from pimonidazole staining were pre-processed similarly to the DAPI image and were transformed to the H&E-SPT coordinates (5x downsampled)

using the resulted transform from the landmark-based registration and nearest-neighbour interpolation.

For each spot, a circular region centred on its coordinates was defined using the diameter from the SPT metadata, rescaled to match the x5 downsampled resolution. The percentage of pixels within this circle that overlapped with they hypoxic regions (i.e. white pixels) was calculated. Spots with >25% hypoxic pixel coverage were labelled as hypoxic; all others were considered to be normoxic. The threshold of 25% was chosen empirically based on visual validation of spot alignment with pimonidazole staining on H&E slides.

5.11. Statistical Analysis of Metaprogram-Hypoxia Associations

I performed all statistical analyses in R (v4.4.3) and generated all figures using ggplot2 (v3.4.4) and patchwork (v1.2.0).

5.11.1. Spot-Level MP Score Visualisation

I summarised per-spot MP score distributions in hypoxic versus normoxic regions as boxplots stratified by cell line for visualisation only. I did not use spot-level inferential testing (e.g., the Wilcoxon rank-sum test, `wilcox.test`, `exact = FALSE`) for inference because spots within a section are not independent (shared library preparation, batch, and strong spatial autocorrelation), which inflates test statistics and apparent significance. Statistical inference on hypoxia-MP associations was therefore performed at the sample level using a one-sample t-test on per-sample composition shifts, as described below.

5.11.2. Composition Shift: One-Sample t-Test and Cohen's d

I quantified per-sample dominant MP composition as the percentage of spots assigned to each MP in hypoxic versus normoxic regions separately. For each MP, the per-sample shift was defined as:

$$\Delta_{MP} = \%_{\text{hypoxic}} - \%_{\text{normoxic}}$$

I applied a one-sample t-test against zero to the distribution of Δ_{MP} values across samples, testing whether the mean shift differed significantly from no change. Cohen's d was calculated as:

$$d = \frac{\bar{\Delta}}{SD(\Delta)}$$

and interpreted as a standardised effect size independent of sample size ($|d| > 0.8$ = large effect). Because the five dominant-MP proportions are compositional, with per-sample shifts summing to approximately zero, the five tests are not statistically independent; p-values are therefore reported without multiplicity correction and interpreted together with the effect sizes.

5.12. Master Spot Table and Comprehensive Spatial Integration

I constructed a master data table containing one row per spatial spot across all 17 samples (42,184 spots total), integrating: spatial identifiers (sample, barcode), pixel coordinates, hypoxia labels, MP scores for MP1-MP5, dominant MP assignment, dominance margin, and pathologist annotation label. This table served as the primary data structure for all downstream comparative analyses.

6. Results: Tumour Heterogeneity and Spatial OMICS

6.1. Multi-Project Cohort Overview

The HNSCC molecular subtypes defined by Walter et al. (2013) were derived from bulk transcriptomics data obtained from clinical samples (or biopsy specimens). The Walter subtypes were derived from bulk clinical transcriptomes, where averaging

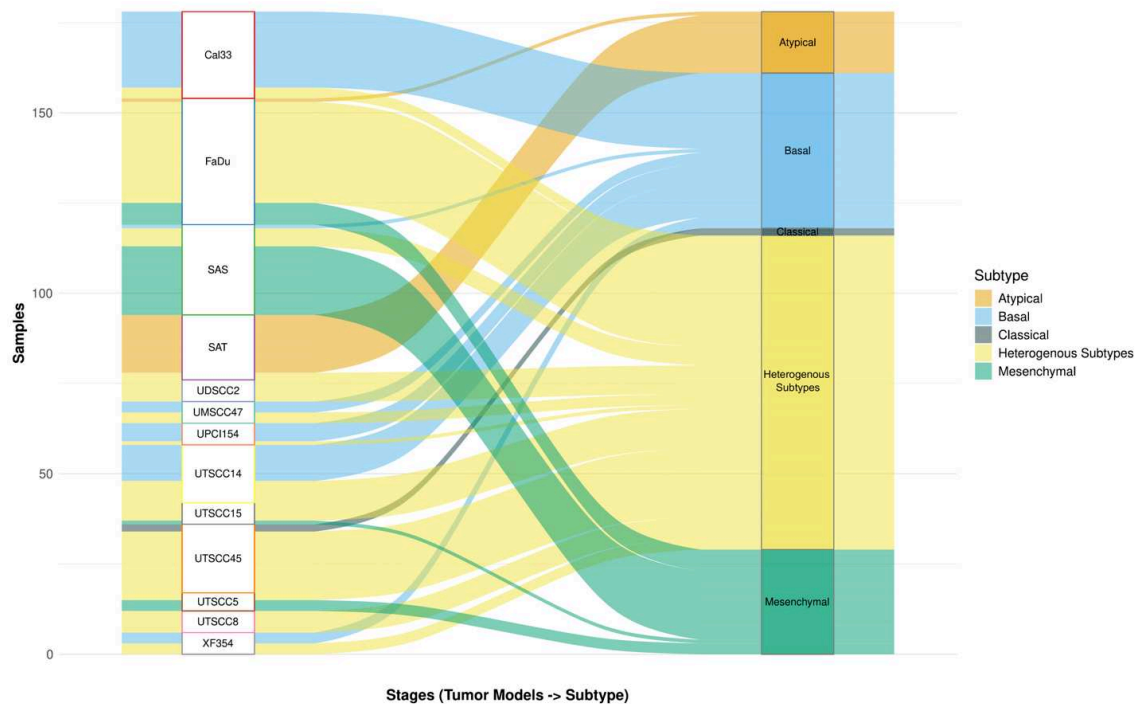


Figure 20. Bulk molecular subtype assignments across multiple xenograft projects. Alluvial diagram showing the flow from raw subtype assignment. Each ribbon represents one sample; width is proportional to the number of samples sharing that transition.

across mixed cell populations means a substantial fraction of tumours fall into ambiguous or unclassified calls and subtype assignment can be unstable. I therefore asked whether this classification could be applied reliably to the more defined preclinical models available in the lab.

To test whether this classification also applies to preclinical material, I assigned bulk molecular subtypes to xenograft samples from several independent studies in our lab. I drew these samples from different preclinical projects, giving a diverse set of HNSCC models suitable for this analysis.

In Figure 20 I show the flow of bulk molecular subtype assignments across all xenograft projects ($n = 178$). I assigned 55.6% of samples to one of the four Walter subtypes, Mesenchymal (20.8%), Basal (20.2%), Atypical (13.5%) or Classical (1.1%).

I labelled the remaining 44.4% Heterogeneous, meaning I could not match them to any single reference centroid and so could not give them a dominant subtype. Heterogeneous was the single largest category, showing that a substantial fraction of HNSCC xenograft samples lack a dominant transcriptional centroid at the bulk level, which indicates that the classification needs refinement for preclinical material. The high proportion of unclassified samples indicates that bulk profiling lacks the resolution to capture the transcriptional heterogeneity of these tumours. To resolve this heterogeneity directly, I analysed 20 FFPE sections from model tumours for which matched bulk subtype data were available, relating the spatially resolved metaprograms to the established bulk classification and to radiosensitivity.

6.2. Bulk Molecular Subtyping Characterises the Cohort but Cannot Resolve Spatial Heterogeneity

The study cohort for this chapter comprises 20 FFPE sections from seven HNSCC xenograft cell lines, each profiled by both bulk RNA-seq and Visium spatial transcriptomics, enabling direct comparison of bulk-level and spatially resolved transcriptional identity within the same tissue material. Of the 20 sections, 17 had complete PIMO hypoxia annotations and 19 had pathologist H&E annotations available; 16 sections had complete data across all three modalities. Analytical subsets are explicitly stated where they apply (e.g., $n = 17$ for hypoxia-MP analyses, $n = 16$ for tissue-class-stratified hypoxia analyses). Centroid-based classification of the bulk RNA-seq profiles from these 20 sections yielded the following subtype assignments: FaDu, UT5, UT8, and the majority of Cal33 sections as Heterogeneous; SAS as Mesenchymal; SAT as Atypical; and one Cal33 section (N154a.38) as Basal.

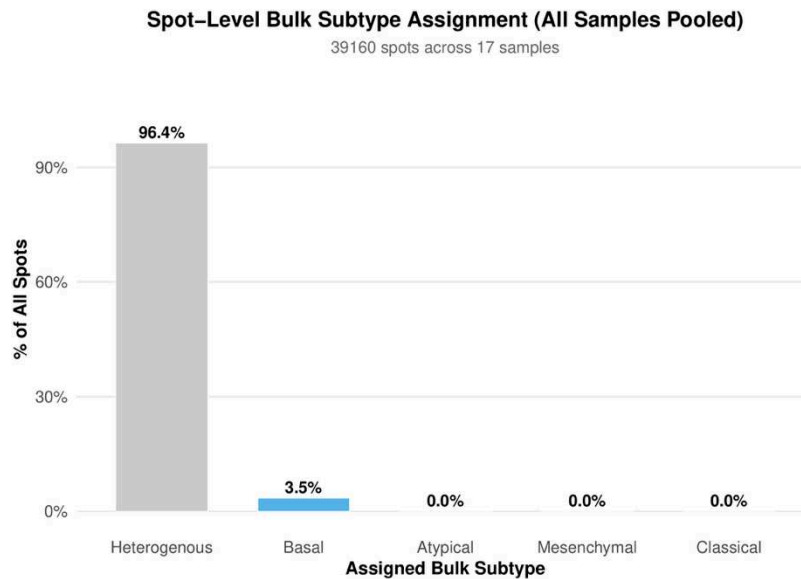


Figure 21. Spot-level bulk molecular subtype assignment, pooled across sections. Bar chart of the percentage of Visium spots assigned to each bulk subtype (39,160 spots across 17 samples), using the centroid-based classifier of Walter et al. applied to the spot profiles. x-axis, assigned bulk subtype; y-axis, % of all spots. Heterogeneous, 96.4%; Basal, 3.5%; Atypical, Mesenchymal and Classical, 0.0% each.

To assess whether the bulk subtype labels could be resolved at single-spot spatial resolution, I applied the same centroid correlation method to individual Visium spots using log-normalised, median-centred expression values and identical assignment thresholds. Across all 20 sections, most spots received the Heterogenous classification; fewer than 5% of spots achieved sufficient centroid margin for confident subtype assignment (Figure 21). The low per-spot library depth relative to bulk profiles, the multi-cell composition of individual 55 µm capture areas, and the sensitivity of centroid correlation to gene dropout all contribute to this result. Reference-based top-down subtype projection is therefore not applicable at single-spot resolution, and an unsupervised approach was required to characterise spatial transcriptional heterogeneity in these sections (Section 6.3).

6.3. NMF and Leiden Community Detection Identify Five Consensus Metaprograms

In order to evaluate spatial transcriptional heterogeneity, I performed unsupervised transcriptional program discovery on all 20 Visium FFPE sections using NMF combined with Leiden cluster-based validation. All 20 sections were included at the program discovery stage to maximise the available program pool; downstream biological and statistical analyses involving PIMO hypoxia labels were restricted to the 17 sections with complete PIMO annotations.

Per-sample UMI distributions and gene detection rates across all 20 sections are shown in Figure 22. In the second stage, I computed pairwise Jaccard similarities among all filtered programs, and a greedy clustering algorithm grouped programs with Jaccard similarity ≥ 0.1 into consensus communities. Five stable groups emerged, each representing a consensus MP. I defined consensus gene signatures by frequency $\times$ rank weighting across contributing programs, taking the top 50 genes per community by this composite score. Because the pool of candidate consensus genes is constrained by both the frequency threshold and the upstream Leiden-overlap filtering, the number of signature genes per MP reflects only genes consistently recovered across contributing programs; the final consensus gene list for each MP therefore contains fewer than 50 genes: MP1 (23 genes), MP2 (28 genes), MP3 (37 genes), MP4 (36 genes), and MP5 (35 genes).

The output of the NMF-Leiden integration pipeline is summarised in Figure 19. The Jaccard similarity heatmap (Figure 23) shows five distinct high-similarity blocks confirming that the five consensus MPs are stable and internally coherent; each MP drew contributing programs from most sections, confirming cross-sample reproducibility.

One section, N153a083 (UT5), contributed zero programs to the consensus MP definitions: its NMF outputs did not pass the Jaccard similarity and robustness filters during Leiden community detection.

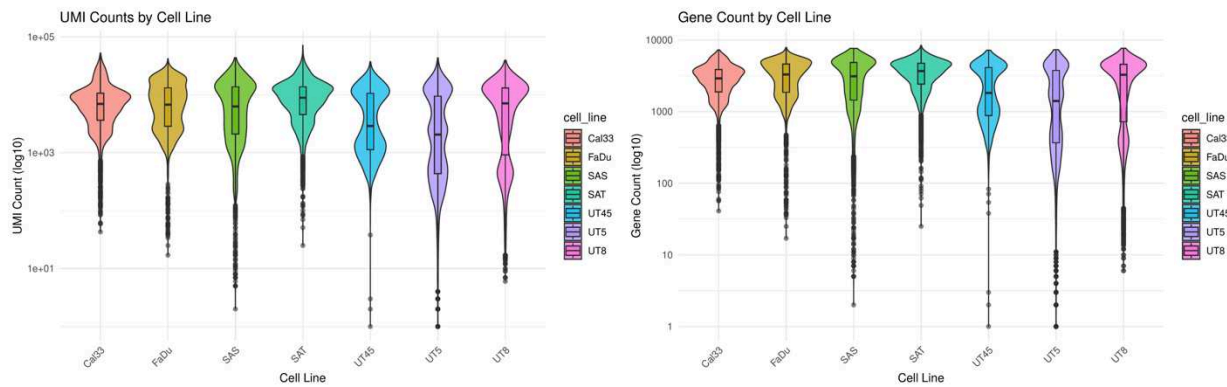


Figure 22. Quality control of the Visium FFPE dataset. Violin plots with embedded boxplots of per-spot UMI counts (left) and detected genes per spot (right) on a log10 scale, grouped by cell line (Cal33, FaDu, SAS, SAT, UT45, UT5, UT8) across the 20 sections; colour denotes cell line.

I computed spot-level MP scores as the mean log-normalised expression of each MP's signature genes present in each spot (colMeans on the log-normalised submatrix). Each spot received five continuous MP scores (MP1-MP5). I assigned dominant MP using `which.max()` - the program with the highest mean expression score. The resulting dominant MP assignment covered 35,511 spots; spots where all five scores were NA were excluded from composition analyses ($n = 6,673$, 15.8%).

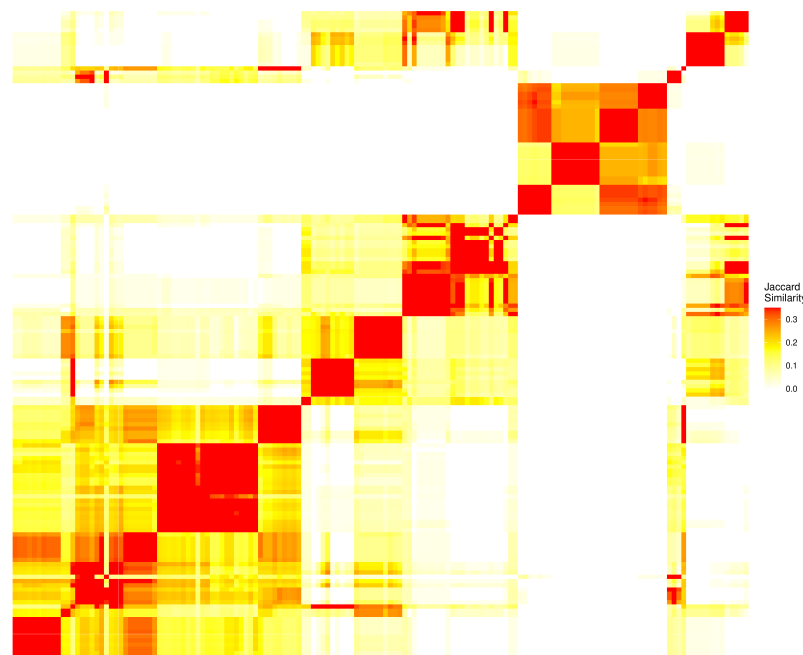


Figure 23. NMF program integration: program-similarity heatmap. Pairwise Jaccard similarity across all filtered NMF programs, ordered by community (colour scale 0.0–0.3). Greedy clustering at threshold ≥ 0.1 demarcates five stable communities (MP1–MP5) as diagonal blocks.

6.4. Biological Identity of the Five Consensus Metaprograms

I determined the biological identity of each MP from two complementary lines of evidence: marker gene identity and hallmark ORA. The combined evidence yields the following program identities:

MP1 (EMT/Mesenchymal)- The top marker genes for MP1 include *VIM*, *AXL*, *F3*, and *EMP3*, all well-established markers of epithelial-to-mesenchymal transition and mesenchymal cell identity (Gjerdrum et al. 2010; Chunyan Wang et al. 2025; Vasaikar et al. 2021; Bourcy et al. 2016). ORA against the MSigDB Hallmark gene set collection identified `HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION` as the most significantly over-represented pathway (adjusted $p = 0.001$).

MP2 (Hypoxia Response)- MP2 marker genes include *DDIT4*, *ADM*, *SERPINB2*, *NDRG1*, *ANGPTL4*, and *EGLN3*, all of which are established transcriptional targets of HIF-1 α (Semenza 2003; Benita et al. 2009; Inoue et al. 2014). This identity is the most confidently assigned of the five programs. ORA confirmed `HALLMARK_HYPOXIA` as the most significantly over-represented pathway (adjusted $p = 3.6 \times 10^{-18}$), with `HALLMARK_MTORC1_SIGNALLING` and `HALLMARK_P53_PATHWAY` as secondary enrichments.

MP3 (Squamous Differentiation / Cornification)- MP3 is defined by cornified-envelope and terminal squamous-differentiation genes, including *CNFN*, *SPRR2A*, *LCE3D*, *IVL* and *TGM1* (the transglutaminase that cross-links the cornified envelope) (Kypriotou, Huber, and Hohl 2012; Candi, Schmidt, and Melino 2005), together with the differentiated-squamous marker *ECM1* (Chan 2004), and an inflammatory, barrier-associated epithelial component (*LCN2*, *S100A7*, *IL36G*) (Chiricozzi et al. 2011; Pfaff et al. 2017). ORA identified keratinisation and cornification terms as significantly enriched.

MP4 (ECM/Stromal-like)- MP4 marker genes include *FSTL1*, *FBN1*, *TPM1*, *TMSB4X*, and *IGFBP4* (Sanders, Goldstein, and Wang 1992; Sakai et al. 2016; Voutsadakis 2020; Gunning et al. 2015; Allard and Duan 2018), which are characteristic of ECM/matrix-remodelling and cytoskeletal gene programmes. The top ORA enrichment for MP4 is `HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION`

(adjusted $p = 0.009$). This reflects shared ECM and cytoskeletal remodeling genes between EMT and stromal programs, but the marker gene profile distinguishes MP4 clearly from MP1: where MP1 carries canonical EMT drivers (VIM, AXL, EMP3), MP4 is defined by FSTL1, FBN1, TPM1, TMSB4X, and IGFBP4; genes associated with ECM scaffolding and cytoskeletal organisation rather than active epithelial-to-mesenchymal plasticity.

MP5 (Keratinocyte / Squamous-epithelial differentiation)- MP5 is characterised by the keratin genes *KRT4*, *KRT13*, *KRT10* and *KRTDAP* (Moll, Divo, and Langbein 2008), markers of keratinocyte and suprabasal squamous-epithelial differentiation (*KRT4/KRT13*, non-keratinised mucosal squamous epithelium; *KRT10*, keratinising suprabasal epithelium), together with differentiating-keratinocyte genes such as *KLK10*, *KLK13*, *SERPINB3* and *AQP3* (Guo et al. 2013; Komatsu et al. 2003; Saidak et al. 2018).

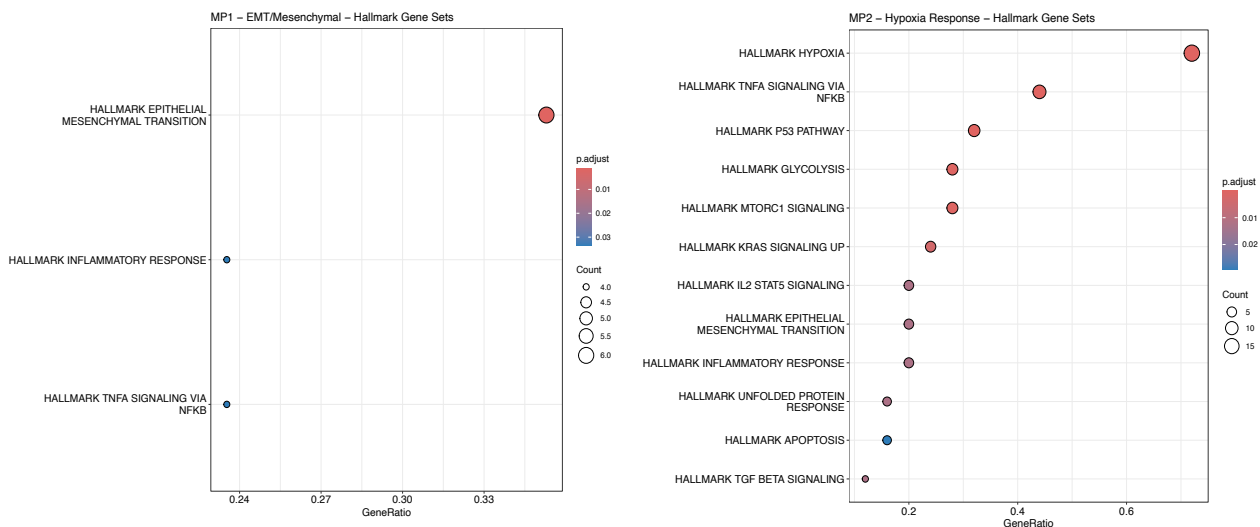


Figure 24. Over-representation analysis (ORA) of hallmark gene sets for MP1 and MP2. Dot plots of enriched hallmark pathways (rows). (Left) MP1 (EMT/Mesenchymal), top hit *HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION*. (Right) MP2 (Hypoxia Response), top hit *HALLMARK_HYPOXIA*. x-axis, gene ratio; dot size, gene count; dot colour, adjusted p -value ($p.adjust$).

6.5. Cell-Line-Specific Transcriptional Fingerprints

Each of the HNSCC cell lines expressed a characteristic spatial transcriptional fingerprint, defined by the dominant and secondary MP contributions across its spots. The six fingerprints fall into three biologically interpretable groups.

6.5.1. MP4-Dominant Lines: FaDu, UT5, UT8, Cal33

Four cell lines are primarily MP4-dominant, but differ substantially in their secondary program composition:

FaDu (n = 5,891 spots; Heterogenous bulk subtype) is the most MP4-dominated line in the cohort. MP4 accounts for 63.1% of FaDu spots, with meaningful secondary contributions from MP1 (18.4%, EMT/Mesenchymal) and MP2 (14.9%, Hypoxia Response).

UT5 (n = 8,041 spots; Heterogenous) exhibits a more distributed MP fingerprint than FaDu, with MP4 leading at 35.9% but with substantial MP1 (14.9%), MP2 (11.1%), and MP5 (1.2%).

UT8 (n = 9,545 spots; Heterogenous) is the most balanced line in the cohort. MP4 remains the leading program at 35.7%, followed by MP2 (15.1%), MP3 (9.6%), MP1 (9.8%), and MP5 (3.0%). UT8 is notable for the highest MP3 fraction (Squamous Differentiation, 9.6%) of any MP4-dominant line and for maintaining the second highest MP2 fraction (15.1%) after Cal33.

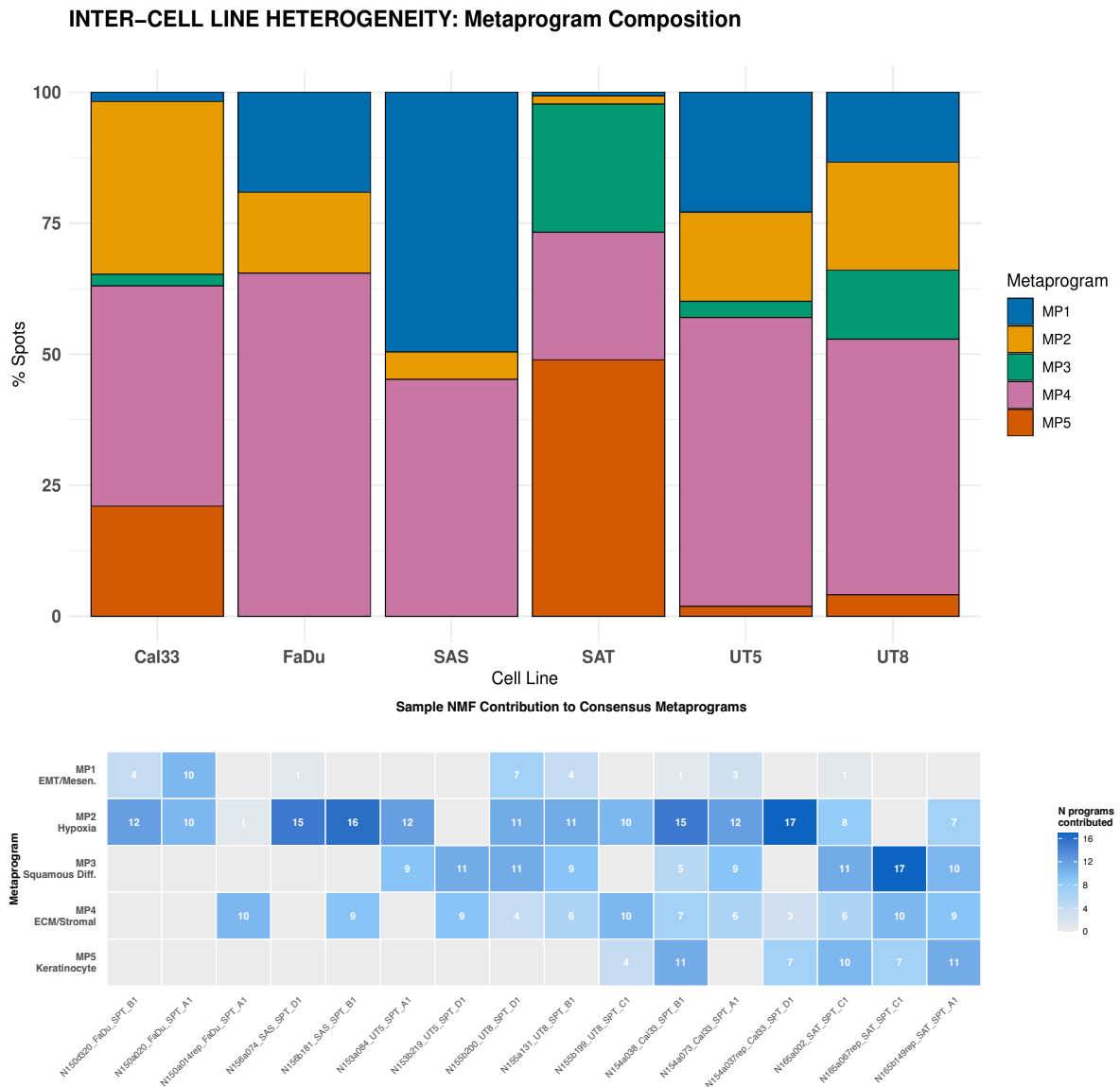


Figure 25. Cell-line transcriptional fingerprints. (Top) Stacked bar plot of the percentage of spots assigned to each metaprogram (MP1, blue; MP2, gold; MP3, green; MP4, pink; MP5, orange) per cell line (Cal33, FaDu, SAS, SAT, UT5, UT8), aggregated across sections; y-axis, % spots. (Bottom) Heatmap of the number of per-sample NMF programs contributing to each consensus metaprogram (rows: MP1 EMT/Mesenchymal, MP2 Hypoxia, MP3 Squamous Differentiation, MP4 ECM/Stromal, MP5 Keratinocyte; columns: individual sections). Cell values give the count of contributing programs (colour scale 0–16); grey indicates no contributing program.

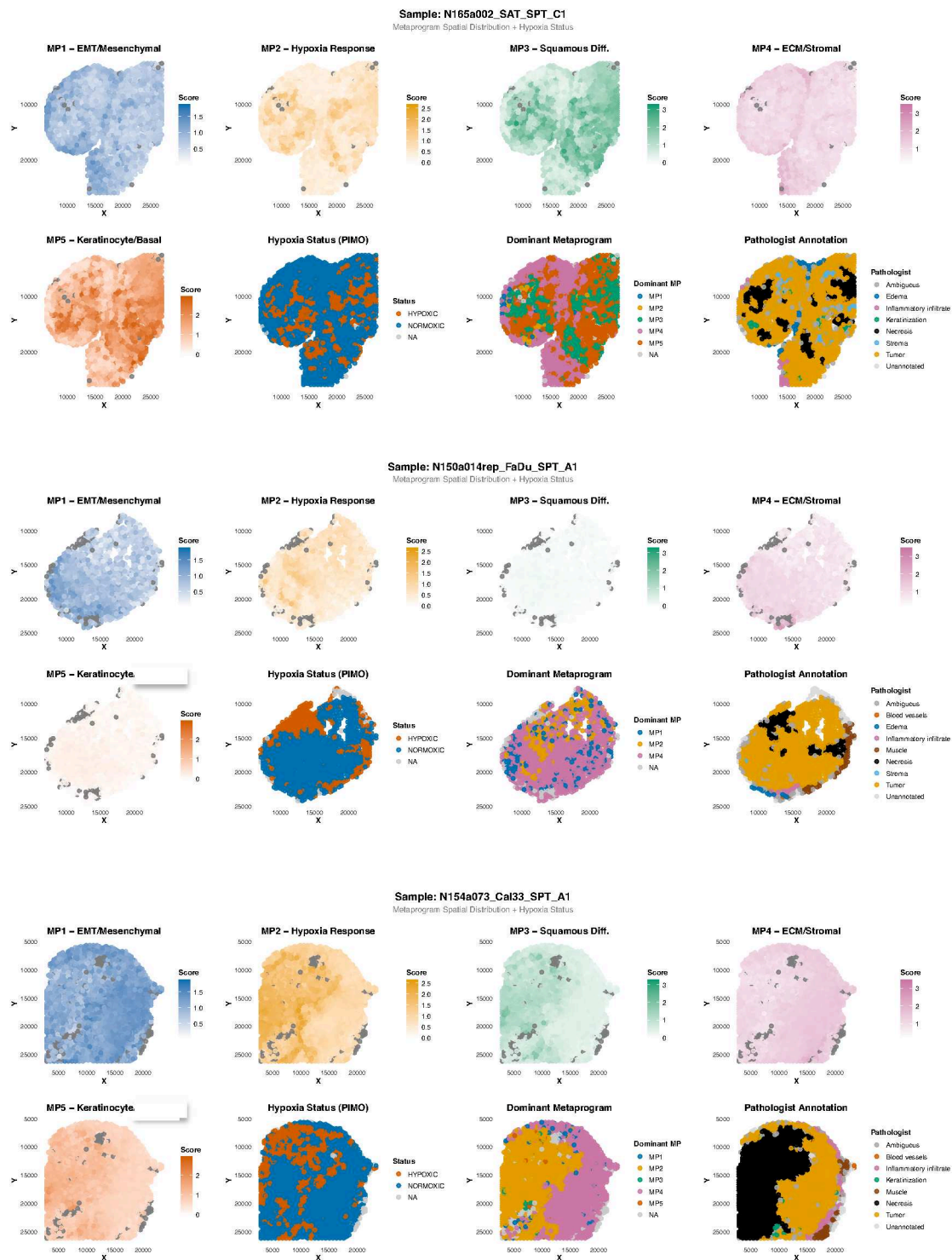


Figure 26. Representative spatial metaprogram (MP) maps. Dominant MP assignment colour-coded per spot and overlaid on the tissue section for (Top) SAT N165a002, (Middle) FaDu N150a014, (Bottom) Cal33 N154a073. Hypoxia and Pathologist annotation are added to the MP assignments.

Cal33 (n = 7,396 spots across three sections) is the most internally heterogeneous cell line in the cohort, and the most instructive case for interpreting how bulk subtype labels relate to spatial transcriptional identity. At the cohort level, Cal33 is described by MP4 (40.0%), MP2 (31.4%), and MP5 (20.0%), but these aggregate statistics conceal intra-line variation across its three sections. Section N154a.37rep (Heterogenous subtype) is MP4-dominant (62.3%), with secondary MP2 (25.7%); Section N154a.73 (Heterogenous subtype) is MP2-dominant (48.3%), with secondary MP4 (42.5%): this single sample drives the high Cal33 MP2 average and represents one of the most hypoxia-enriched sections in the cohort. Section N154a.38 (Basal subtype) is MP5-dominant (49.1%), with secondary MP4 (23.9%): here the bulk Basal subtype label maps directly onto a Keratinocyte/Squamous-epithelial spatial transcriptional identity. The stacked bar composition across all cell lines and the per-section heatmap are shown in Figure 25. The heatmap reveals intra-line variability: Cal33 sections span the widest range of MP compositions, with one section showing near-complete MP2 dominance (N154a.73), while FaDu and SAT sections cluster tightly within their respective fingerprints, indicating high intra-line consistency (Figure 25).

6.5.2. The Mesenchymal Split: SAS

SAS (n = 5,122 spots; Mesenchymal bulk subtype) is the only line where MP1 has identical dominance like MP4: MP1 = 44.8%, MP4 = 41.0%, with minimal MP2 (4.7%) (Figure 25). SAS is the only line in the cohort where the EMT/Mesenchymal program approaches co-equality with the stromal program. This is directly and specifically predicted by its bulk Mesenchymal subtype assignment -the Mesenchymal subtype label is characterised by high expression of EMT-associated genes (*VIM*, *AXL*, *EMP3*) at the bulk level, and this translates spatially into near-ubiquitous MP1 dominance across SAS tissue sections. SAS is the only cell line in the cohort where the dominant spatial program (MP1) directly matches the dominant bulk subtype label (Mesenchymal).

6.5.3. The Unique Outlier: SAT

SAT (n = 6,189 spots; Atypical bulk subtype) is categorically different from all other lines. MP5 dominates at 47.0%, followed by MP3 (23.5%) and MP4 (23.4%), with negligible contributions from MP1 (0.7%) and MP2 (1.5%).

Representative spatial MP maps for three samples, illustrating the program distributions described above, are shown in Figure 26.

6.6. Bulk Molecular Subtype Maps onto Spatial Metaprograms

To characterise the relationship between bulk-level molecular subtype assignments and spatial transcriptional identity, I examined two complementary views of the data (Figure 27). At the section level, I inspected the dominant spatial MP composition for each of the 17 sections with paired bulk and spatial data. A consistent and interpretable pattern was observed across all 17 samples: the Mesenchymal subtype (SAS) mapped to MP1 dominance, the Atypical subtype (SAT) and the single Basal-classified section (Cal33 N154a.38) both mapped to MP5 dominance, and samples without a confident bulk subtype assignment (Heterogenous) predominantly showed MP4 dominance. Heterogenous is not a distinct molecular subtype; it is a default classification for transcriptionally mixed samples that did not meet both the Pearson

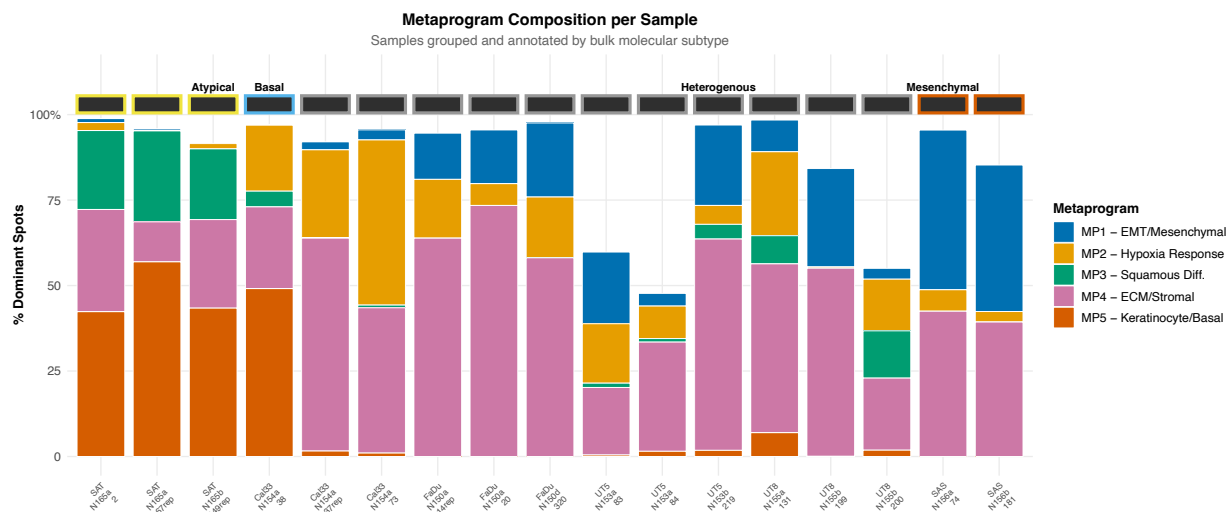


Figure 27. Metaprogram composition per sample ($n = 17$), grouped by bulk molecular subtype. Stacked bars give the percentage of dominant-MP spots per section (MP1 EMT/Mesenchymal, blue; MP2 Hypoxia Response, gold; MP3 Squamous Differentiation, green; MP4 ECM/Stromal, pink; MP5 Keratinocyte, orange); y-axis, % of dominant spots. The bulk molecular subtype of each sample (Atypical, Basal, Heterogeneous, Mesenchymal) is annotated above the bars.

correlation threshold and the inter-centroid margin threshold. At the spot level, applying the same centroid correlation method to individual Visium spots and pooling assignments across all 17 sections confirmed that fewer than 5% (Figure 21) of spots achieved a confident subtype classification; the remainder were assigned Heterogenous, consistent with the finding in Section 6.2 that reference-based subtype projection is not applicable at single-spot resolution. Both views are shown in Figure 27.

The Mesenchymal subtype mapped to MP1 dominance (SAS), Atypical to MP5 dominance (SAT), and Basal to MP5 dominance (Cal33 N154a.38). Unclassified samples ('Heterogenous') were predominantly MP4-dominant, with variable secondary programs across lines and sections.

6.7. Hypoxia Reorganizes the Spatial Transcriptional Landscape

I focused on hypoxia because it is the principal driver of radioresistance and treatment failure in HNSCC (Section 1.3.3), and the only TME marker which I could use as a direct tissue-resolved readout. I defined hypoxic regions from PIMO labelling on a consecutive section adjacent to the Visium slide, which Cylia Ouadah co-registered to the spots so that each spot carried a hypoxic or normoxic label. I analysed the hypoxia-MP relationship in two complementary views. The first is a descriptive, spot-pooled comparison of dominant-MP composition in normoxic versus hypoxic spots (Table 5, Section 6.7.1). The second is a sample-level inference across the 17 sections that underpins all statistical claims: for each section I measured how the dominant-MP composition shifted between normoxic and hypoxic regions and tested these shifts with one-sample t-tests and Cohen's d effect sizes (Figure 28, Table 6, Section 6.7.2). I did not run statistics at the spot level, because spots within a section are not independent, and treating them as if they were would inflate the significance. Because the spot-pooled view is weighted by spot number while the sample-level view weights each section equally, the two give slightly different shift magnitudes for the same programme.

6.7.1. Spot-Level Composition Shifts

Examining the dominant MP composition separately in normoxic and hypoxic spots reveals a reorganization of the transcriptional landscape under hypoxia:

Table 5. Spot-level dominant MP composition in normoxic versus hypoxic regions across the cohort. Percentages indicate proportion of classifiable spots assigned to each dominant MP; Shift (pp) = hypoxic minus normoxic percentage points.

Metaprogram	Normoxic Spots	Hypoxic Spots	Shift (pp)
MP1 (EMT/Mesenchymal)	17.0%	12.0%	−5.0
MP2 (Hypoxia Response)	12.9%	30.6%	+17.7
MP3 (Squamous Diff.)	5.7%	14.9%	+9.2
MP4 (ECM/Stromal-like)	51.2%	28.4%	−22.8
MP5 (Keratinocyte/Squamous-epithelial)	13.3%	14.0%	+0.7

MP4 is the dominant program in normoxic tissue (51.2% of normoxic classifiable spots) and drops by nearly half in hypoxic spots (28.4%; −22.8 percentage points). Conversely, MP2 more than doubles its representation in hypoxia (12.9% → 30.6%), becoming the second most common program in hypoxic spots behind a substantially diminished MP4. MP3 undergoes a proportional doubling from 5.7% to 14.8% in hypoxia. MP1 decreases moderately. MP5 is essentially unchanged at the cohort level, though this stability conceals meaningful intra-line variation.

6.7.2. Sample-Level Shift Statistics: All Five Metaprograms

The one-sample t-tests on per-sample dominant-MP composition shifts (n = 17 samples with hypoxia annotations) yielded the following results:

Table 6. Sample-level dominant MP composition shifts between hypoxic and normoxic regions ($n = 17$ sections). One-sample t -test p -values and Cohen's d effect sizes shown for each MP.

Metaprogram	Identity	Mean Shift	p-value	Cohen's d	Direction
MP1	EMT/Mesenchymal	-4.59%	0.0114	-0.693	↓ depleted
MP2	Hypoxia Response	+16.47%	+0.955	↑↑ strongly enriched	MP2
MP3	Squamous Differentiation	+9.26%	0.0146	+0.662	↑ enriched
MP4	ECM/Stromal-like	-18.51%	0.0006	-1.033	↓↓ strongly depleted
MP5	Keratinocyte/Squamous-epithelial	-2.64%	0.079	-0.452	→ not significant

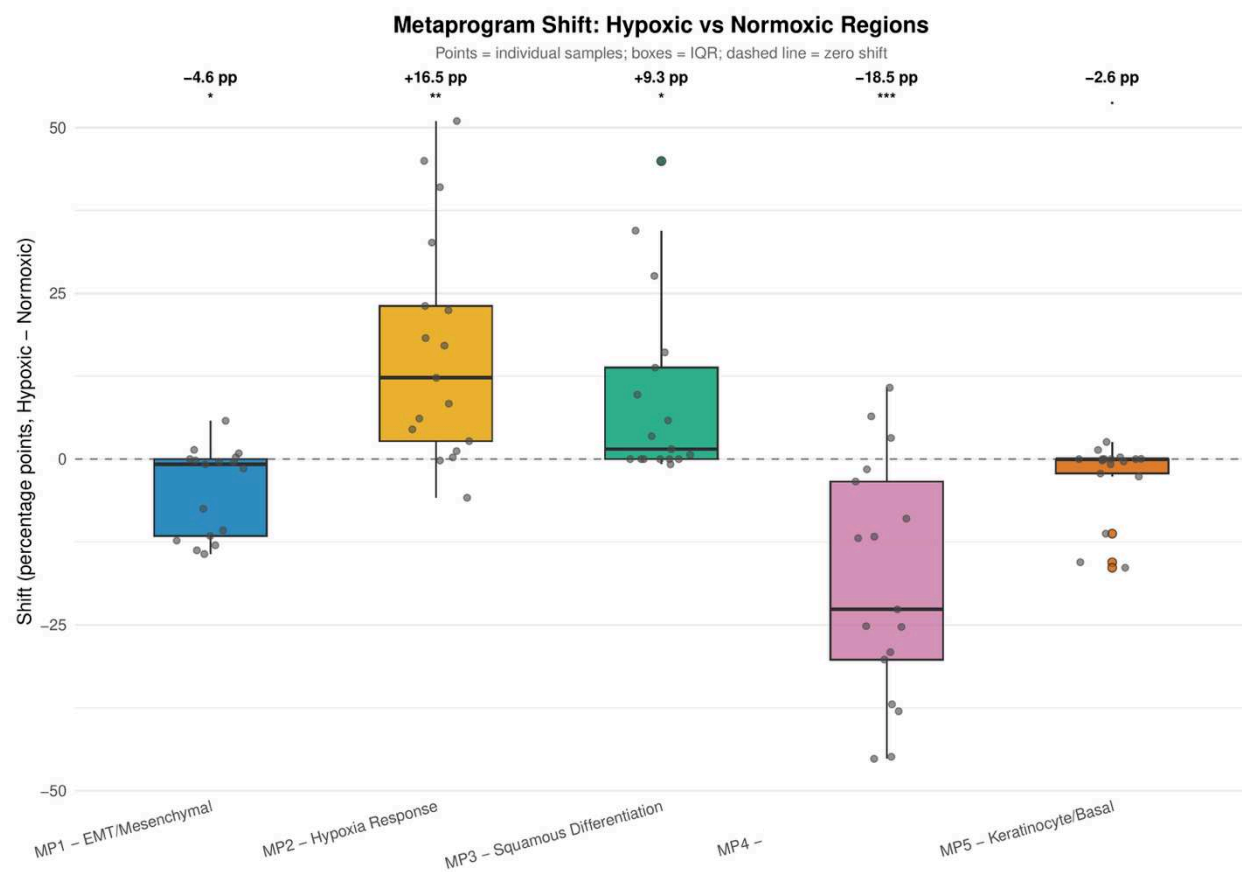


Figure 28. Per-metaprogram composition shift between hypoxic and normoxic regions. Boxplots (box = IQR) of the per-sample shift in dominant-MP composition (hypoxic minus normoxic, percentage points) for each metaprogram (MP1 EMT/Mesenchymal, MP2 Hypoxia Response, MP3 Squamous Differentiation, MP4 ECM/Stromal, MP5 Keratinocyte) across 17 sections; each point is one section; the dashed line marks zero shift. Mean shift (percentage points) and one-sample t-test significance appear above each metaprogram: MP1 -4.6, MP2 +16.5, MP3 +9.3, MP4 -18.5, MP5 -2.6; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

MP2 (Hypoxia Response; mean shift +16.47%, $p = 0.0012$, $d = +0.955$). MP2 was the most strongly enriched MP in hypoxic spots. The shift was directionally consistent across 15 of 17 samples. Top marker genes include DDIT4, ADM, SERPINB2, and NDRG1; ORA confirmed HALLMARK_HYPOXIA as the top over-represented pathway (adjusted $p = 3.6 \times 10^{-18}$).

MP4 (ECM/Stromal-like; mean shift -18.51%, $p = 0.0006$, $d = -1.033$). MP4 showed the largest absolute effect size in the dataset and was depleted in hypoxic spots. I observed directional consistency in 14 of 17 samples. The per-sample composition shifts for all five MPs are shown in Figure 28.

MP3 (Squamous Differentiation; mean shift +9.26%, $p = 0.0146$, $d = +0.662$). MP3 was enriched in hypoxic spots with a medium-to-large effect. Directional consistency was lower than for MP2 and MP4; 10 of 17 samples showed a positive shift, indicating the t-test effect is driven by a subset of samples with large shifts rather than uniform directionality.

MP1 (EMT/Mesenchymal; mean shift -4.59%, $p = 0.0114$, $d = -0.693$). MP1 was significantly depleted in hypoxic spots with a medium-to-large effect. The depletion was directionally consistent across 12 of 17 samples.

MP5 (Keratinocyte/Squamous-epithelial; mean shift -2.64%, $p = 0.079$, $d = -0.45$). MP5 showed no significant composition shift between hypoxic and normoxic spots at the cohort level.

6.8. Tissue Microarchitecture Anchors Metaprogram

Expression

In order to test whether the MPs and the hypoxic signal are organised by tissue architecture rather than scattered at random, I related both to the pathologist's H&E annotations. Tissue-class labels were assigned to 39,282 spots across 16 of the 17 hypoxia-annotated sections by area-weighted majority assignment; one Cal33 section (N154a038_Cal33_SPT_B1) lacked annotation and was excluded. For the analyses below I used the 29,774 annotated spots that carried a confident dominant metaprogram, excluding unannotated and ambiguous spots.

6.8.1. PIMO-positive fraction across tissue classes

I first quantified the PIMO-positive fraction of each annotated tissue class (Table 7; Figure 29). Among the major compartments, keratinisation carried the highest fraction of hypoxic spots (38.5%), followed by necrosis (25.4%), inflammatory infiltrate (24.2%) and muscle (23.7%). Tumour was intermediate (16.6%) and stroma was the most oxygenated compartment (3.6%). Two small classes, epidermis (51.5%, $n = 66$) and bacteria (66.7%, $n = 6$), showed higher fractions but contained too few spots to interpret. Hypoxia was therefore not confined to a single morphological compartment but distributed across several tissue types, with the greatest burden in keratinised and necrotic regions.

Table 7. Tissue microarchitecture summary: PIMO-positive fraction, spot count, and dominant metaprogram (MP) by annotated tissue class across 16 annotated sections.

<i>Tissue Type</i>	<i>PIMO⁺ Fraction</i>	<i>n Spots</i>	<i>Dominant MP</i>	<i>Second MP</i>
<i>Keratinization</i>	38.5%	291	MP5 (39.9%)	MP3 (30.2%)
<i>Inflammatory infiltrate</i>	24.2%	627	MP4 (75.8%)	MP2 (10.4%)
<i>Necrosis</i>	25.4%	7,607	MP2 (32.4%)	MP4 (27.1%)
<i>Muscle</i>	23.7%	186	MP4 (87.1%)	MP1 (10.2%)

<i>Tumour</i>	16.6%	20,376	MP4 (54.8%)	MP1 (16.4%)
<i>oedema</i>	8.8%	160	MP4 (83.1%)	MP5 (11.2%)
<i>Stroma</i>	3.6%	421	MP4 (55.1%)	MP5 (35.9%)

6.8.2. Dominant metaprogram by tissue class

I next examined the dominant MP within each tissue class (Table 7; Figure 30). MP4 (ECM/stromal-like) was the dominant programme in most compartments, including tumour (54.8%), stroma (55.1%), inflammatory infiltrate (75.8%), muscle (87.1%) and oedema (83.1%). Necrosis was the only class in which MP2 (hypoxia response) was dominant (32.4%, with MP4 second at 27.1%), consistent with its high hypoxic burden. Keratinisation was distinct from all other classes: it was the only compartment dominated by the squamous-differentiation programmes, with MP5 (39.9%) and MP3 (30.2%) together accounting for 70.1% of its spots and MP2 largely absent. Each tissue class therefore carried a characteristic metaprogram, with MP2 confined to necrosis and the squamous programmes to keratinised regions.

6.8.3. Metaprogram composition of keratinised regions

Because keratinisation combined the highest hypoxic fraction with a non-canonical (non-MP2) programme, I examined it in more detail (Table 8). Keratinisation-annotated spots were rare across the cohort (291 spots, 1.0% of MP-assigned spots) and occurred as small, discrete areas in most lines, reaching an appreciable fraction only in the three SAT sections (2.3%). Where present, these spots were dominated by the squamous-differentiation programmes MP5 and MP3 (together 70.1% of keratinisation spots overall, and 99.2% in SAT). The overlap was weaker in Cal33 (25.3%), so the coupling between keratinisation and the squamous programmes was clearest in SAT and, to a lesser extent, UT8 (64.0%). Despite their high PIMO-positive fraction, keratinised regions thus expressed squamous-differentiation programmes rather than the canonical hypoxia programme MP2.

Table 8. Keratinisation-annotated Visium spots per cell line and their metaprogramme composition. Cell lines ordered by intrinsic radioresistance (TCD50, 30 fractions/6

weeks, radiotherapy alone; Koi et al. 2017). Keratin spots = spots annotated as keratinisation by the pathologist; % of section spots = keratinisation spots as a percentage of all QC-passing spots for that line. MP5+MP3 (%) = percentage of a line's keratinisation spots whose dominant metaprogramme is MP5 or MP3 (squamous-differentiation programmes). – = not applicable (no keratinisation spots).

Cell line	TCD50 (Gy)	Keratin. spots (n)	% of section spots	MP5+MP3 (%)	MP5 (%)	MP3 (%)
UT5	121.7	21	0.4	85.7	19.0	66.7
SAS	118.8	0	0.0	–	–	–
FaDu	64.8	0	0.0	–	–	–
Cal33	59.0	75	1.9	25.3	16.0	9.3
UT8	44.0	75	1.2	64.0	8.0	56.0
SAT	37.8	120	2.3	99.2	78.3	20.8
All	–	291	1	70.1	39.9	30.2

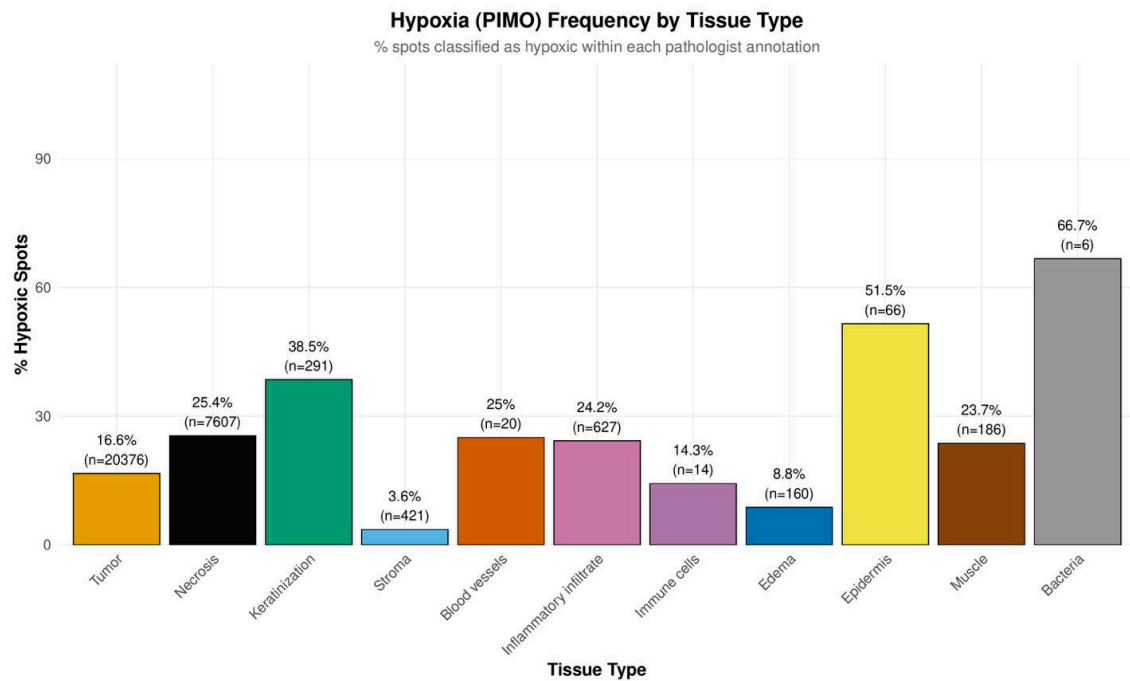


Figure 29. Hypoxia (PIMO) frequency by tissue type. Bar chart of the percentage of spots classified as hypoxic (PIMO-positive) within each pathologist-annotated tissue class. Each bar is labelled with the percentage and number of spots (n): Tumour 16.6% (n = 20,376), Necrosis 25.4% (n = 7,607), Keratinisation 38.5% (n = 291), Stroma 3.6% (n = 421), Blood vessels 25% (n = 20), Inflammatory infiltrate 24.2% (n = 627), Immune cells 14.3% (n = 14), Edema 8.8% (n = 160), Epidermis 51.5% (n = 66), Muscle 23.7% (n = 186), Bacteria 66.7% (n = 6). y-axis, % hypoxic spots; bar colour denotes tissue class.

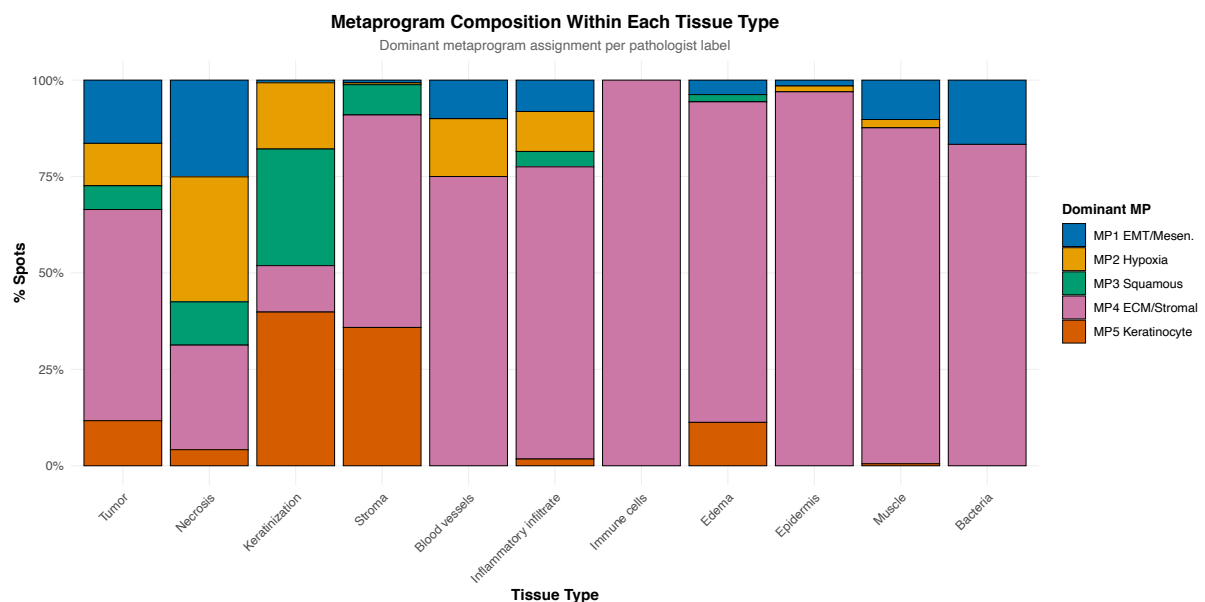


Figure 30. Dominant metaprogram composition within each annotated tissue class. Stacked bars give the percentage of spots assigned to each dominant metaprogram

(MP1 EMT/Mesenchymal, blue; MP2 Hypoxia, gold; MP3 Squamous, green; MP4 ECM/Stromal, pink; MP5 Keratinocyte, orange) within each pathologist-annotated tissue class; y-axis, % of spots.

6.8.4. Hypoxia-associated metaprogram shifts within the tumour compartment

Finally, I asked how hypoxia reshapes MP composition within the tumour compartment itself, which was the largest annotated class (n = 20,376; 16.6% PIMO-positive) and MP4-dominant (54.8%) with secondary MP1 (16.4%). Separating tumour spots by PIMO status revealed a consistent redistribution: PIMO-positive spots showed reduced MP4 and elevated MP2 relative to PIMO-negative spots. The mean metaprogram scores stratified by tissue class and hypoxia status are shown in Figure 32, which confirms that MP2 elevation and MP4 depletion in hypoxic spots occur across tissue classes, with the strongest MP2 signal in necrotic zones regardless of PIMO status, while keratinised regions retain their MP5/MP3 identity in both normoxic and hypoxic spots.

6.9. Spatial Metaprogram Composition Covaries with Radioresistance

To examine whether MP composition is consistent with radiosensitivity, I re-ordered the per-sample composition by the intrinsic radioresistance of each cell line, defined by TCD50 (the total dose in Gy required for 50% local tumour control after 30 fractions in 6 weeks; RT alone), from most radioresistant to most radiosensitive, left to right (UT5 > SAS > FaDu > Cal33 > UT8 > SAT; Figure 31) (Koi et al. 2017). Along this axis, MP1 (EMT/mesenchymal) showed the clearest gradient, with the largest fraction of MP1 spots in the most radioresistant lines and a progressive decline towards the radiosensitive end. The radiosensitive lines instead carried more of the squamous-differentiation programmes MP3 (squamous differentiation) and MP5 (keratinocyte/squamous-epithelial), although this was concentrated in SAT and, to a lesser extent, UT8, rather than a smooth cohort-wide trend. When the same composition was split by oxygenation (Supplementary Figure 10), the MP1 and MP5 components were broadly comparable between hypoxic and normoxic regions,

whereas the MP3 contribution differed; this oxygenation-dependence of MP3 was again concentrated in the SAT and UT8 sections, consistent with the cell-line-specific keratinisation pattern described in Section 6.8.3.

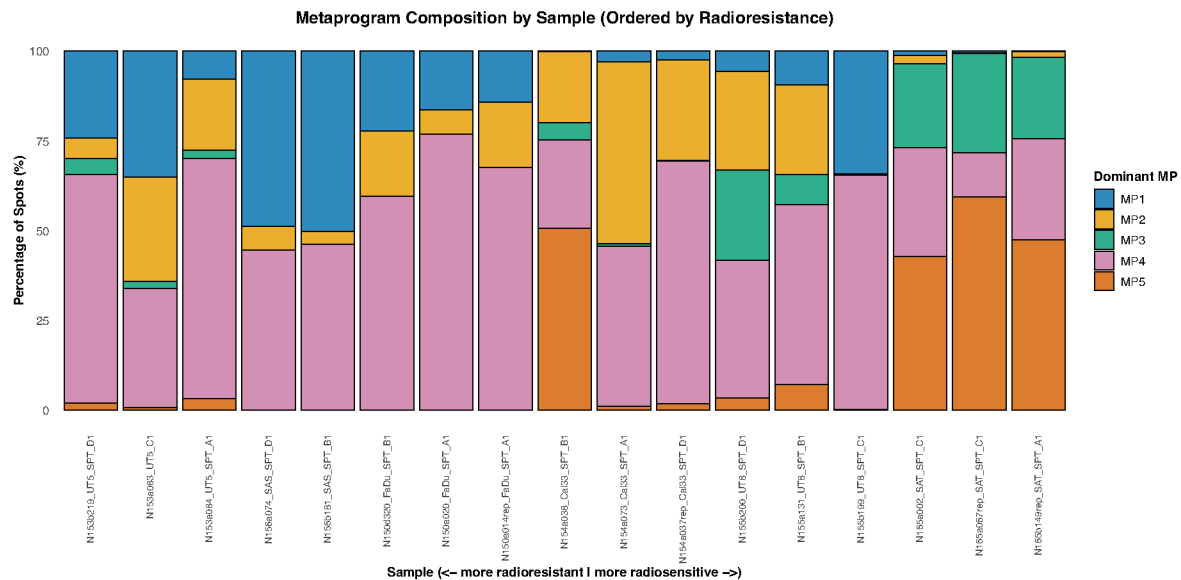


Figure 31. Metaprogram composition by sample, with cell lines ordered by radioresistance (most radioresistant to most radiosensitive, left to right) Stacked bars give the percentage of spots assigned to each dominant metaprogram (MP1-MP5) per section.

6.10. Three Recurrent Hypoxia-Associated Spatial Patterns in the Cohort

Integrating the spot-level composition shifts, sample-level statistics, tissue architecture data, and ORA results, I identified three recurrent hypoxia-associated spatial patterns.

6.10.1. Pattern 1 -Canonical HIF-1 α Response (MP2 Enriched)

MP2 was enriched in hypoxic spots across all six cell lines and 15 of 17 sections (mean shift +16.47%, $p = 0.0012$, $d = +0.955$; directionally consistent in 15 of 17 samples). MP2 marker genes include DDIT4, ADM, SERPINB2, NDRG1, ANGPTL4, and

EGLN3. MP2 enrichment was strongest in necrotic border zones and hypoxic tumour spots.

6.10.2. Pattern 2 -Structural Hypoxia and Squamous Commitment (MP3 and MP5 Enriched)

MP3 (Squamous Differentiation) was enriched in PIMO-positive spots, that is, spots defined as hypoxic by PIMO binding rather than by MP2 score (mean shift +9.26%, $p = 0.0146$, $d = +0.662$). The shift was medium-to-large but less directionally consistent

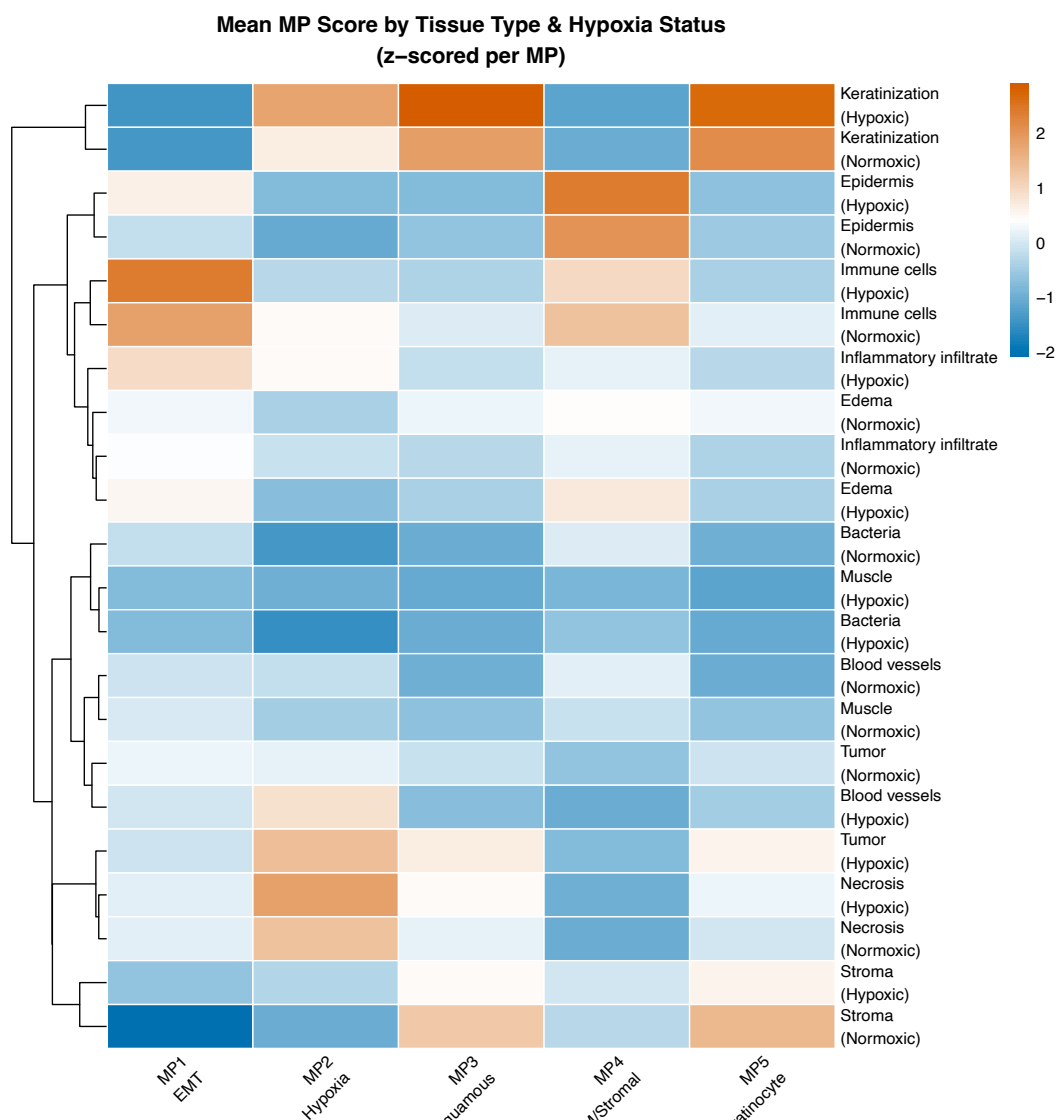


Figure 32. Tissue- and hypoxia-stratified metaprogram scores. Heatmap of the mean score of each metaprogram (columns: MP1 EMT, MP2 Hypoxia, MP3 Squamous, MP4 ECM/Stromal, MP5 Keratinocyte), z-scored per metaprogram, for each pathologist-annotated tissue class split by PIMO hypoxia status (rows: each tissue class shown as hypoxic and normoxic). Colour scale -2 to +2 (z-score); rows ordered by hierarchical clustering.

than for MP2 or MP4, with 10 of 17 samples showing a positive shift. MP5 (Keratinocyte/Squamous-epithelial) was not significantly shifted across the cohort (mean shift -2.6%, not significant), but co-dominated with MP3 within the keratinised tissue class, which carried the largest PIMO-positive fraction of any annotated class (38.1%; Figures 30 and 31).

6.10.3. Pattern 3 -ECM/Stromal-like Signal Depletion in Hypoxic Cores (MP4 Depleted)

MP4 (ECM/Stromal-like) was depleted in hypoxic spots with the largest absolute effect size in the dataset (mean shift -18.51%, $p = 0.0006$, $d = -1.033$), directionally consistent in 14 of 17 samples (Figure 28). MP4 marker genes include ECM and stroma. MP4 depletion co-localized spatially with MP2 enrichment: PIMO-positive spots with the highest MP2 scores consistently showed the lowest MP4 scores, as confirmed by the tissue-stratified heatmap (Figure 32).

7. Discussion: Tumour Heterogeneity and Spatial OMICS

7.1. Spatial Transcriptomics Resolves Transcriptional Heterogeneity That Bulk Approaches Cannot

A fundamental challenge in translating bulk molecular subtype classifications into spatial tumour biology is that bulk molecular subtyping assigns one label to an entire tumour, collapsing its spatial architecture into a single averaged profile. When I applied reference-based subtype classification to my spatial data, fewer than 5% of Visium spots across all samples reached a confident assignment (Figure 21). This is expected, given the low per-spot library depth and the multi-cell composition of Visium's 55 μm capture areas, but it also shows directly that a classification built on bulk profiles does not transfer to the spatial setting. The same limitation is visible in bulk data itself: in a clinical HNSCC cohort, Linge et al. reported that a substantial proportion of tumours could not be assigned to any Walter et al. subtype and were labelled unclassified/heterogeneous (Linge et al. 2024). This limitation is at the same time an opportunity: because spatial transcriptomics resolves the programmes that bulk subtyping averages away, a spatially defined classification could in future be linked to the molecular profiles that drive a particular outcome or therapy response.

The data-driven NMF and Jaccard-based community integration strategy I used here bypasses these limitations by extracting programmes directly from the spatial data. This approach, pioneered for bulk cancer transcriptomics by Brunet et al. (2004) and subsequently extended to single-cell and spatial contexts, produces biologically interpretable factors without assuming any prior subtype structure (Brunet et al. 2004). Rather than forcing a single subtype label onto each spot, it yielded the five metaprograms I identified in this cohort: MP1 (EMT/Mesenchymal), MP2 (Hypoxia Response), MP3 (Squamous Differentiation), MP4 (ECM/Stromal) and MP5

(Keratinocyte/Squamous-epithelial). These five programmes were stable across all 17 analysed sections and all six cell lines, a level of cross-sample reproducibility that supports their biological reality rather than treating them as computational artefacts.

7.2. The Five Metaprograms Reflect Canonical HNSCC

Biology

The identities assigned to the five consensus MPs are consistent with established transcriptional programmes in HNSCC. Using single-cell RNA-sequencing Puram et al. identified partial EMT (pEMT) as a major axis of intra-tumoural heterogeneity in HNSCC using single-cell RNA-sequencing (Puram et al. 2017). Unlike a complete EMT transition, pEMT is a hybrid state in which malignant cells co-express mesenchymal and extracellular-matrix genes (VIM together with laminins, integrins and matrix metalloproteinases) while retaining epithelial identity, and do so without activating the classical EMT transcription factors (SNAIL1, ZEB1/2, TWIST1/2) (Puram et al. 2017). MP1 overlaps with this axis: VIM and AXL are among its top markers, and HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION is its primary ORA enrichment (Figure 24). My spot-level data cannot distinguish a partial from a complete EMT state, so I describe MP1 as an EMT/mesenchymal programme and note only its overlap with the pEMT markers reported by Puram et al. Consistent with this identity, MP1 was spatially concentrated in the SAS xenografts, the only Mesenchymal-subtype line in the cohort (Figures 25 and 27).

MP2, the Hypoxia Response programme, has the most unambiguous biological identity of the five MPs. Its top marker genes - *DDIT4*, *ADM*, *NDRG1*, *ANGPTL4*, *EGLN3* - are all direct transcriptional targets of HIF-1 α , the master regulator of the cellular hypoxia response (Benita et al. 2009; Inoue et al. 2014; Semenza 2003) and HALLMARK_HYPOXIA is its top over-representation hit (Figure 24), together giving MP2 the clearest, gene-level identity of the five. Because these markers are HIF-1 α targets, MP2 most likely reflects the acute rather than the chronic hypoxic response; under prolonged hypoxia HIF-2 α , which shares only part of the HIF-1 α target-gene

set, becomes the dominant driver (Koh and Powis 2012). Establishing that this is specifically HIF-1 α -dependent would require functional or protein-level experiments beyond the transcriptional snapshot provided here.

MP3 and MP5 together delineate the squamous differentiation axis. In squamous epithelium, commitment to terminal differentiation proceeds from a keratinocyte / squamous-epithelial state, marked by KRT4, KRT13 and KRT10 (MP5), through to a cornified, terminally differentiated state defined by ECM1, CNFN and SPRR2A (MP3) (Moll, Divo, and Langbein 2008; Fuchs and Raghavan 2002). Notably, neither MP expresses canonical basal keratinocyte markers (KRT5, KRT14, TP63) (Fuchs and Raghavan 2002); MP5 therefore reflects a keratinocyte-like, squamous-epithelial differentiation state rather than a basal one. This axis has been characterised extensively in normal skin and mucosal epithelium, and its detection here in HNSCC xenografts confirms that these tumours retain elements of the normal squamous differentiation hierarchy within their spatial transcriptional architecture (Moll, Divo, and Langbein 2008; Fuchs and Raghavan 2002).

MP4, assigned as an ECM/stromal-like programme based on *FSTL1*, *FBN1*, *TPM1*, and *TMSB4X* markers (Sanders, Goldstein, and Wang 1992; Sakai et al. 2016; Voutsadakis 2020; Gunning et al. 2015; Allard and Duan 2018), is by far the most prevalent programme in the cohort (39.2% of all spots; 46.5% of classifiable spots). Its near-ubiquitous dominance is consistent with these genes being broadly expressed by tumour cells across many spatial contexts, particularly at the tumour-stromal interface. In the xenografts, where SpaceRanger alignment to the human reference renders mouse stromal transcripts invisible, MP4 expression reflects tumour-cell-intrinsic activation of ECM and cytoskeletal gene programmes rather than mixture from genuine fibroblasts.

Broader pan-cancer analyses have confirmed that cancer cell states identified by NMF-based approaches recur across tumour types, suggesting that the five programmes identified here are not HNSCC-specific artefacts but reflect general cancer transcriptional states (Barkley et al. 2022; Gavish et al. 2023). By extending this framework to the spatial dimension in a xenograft model and grounding it in direct PIMO oxygen-status measurements, I resolved distinct transcriptional programmes in normoxic versus hypoxic tissue and could begin to relate their composition to a clinically relevant factors, the intrinsic radioresistance of the cell lines (Figure 27).

7.3. Cell-Line Fingerprints Validate and Extend Bulk Molecular Subtyping

Bulk HNSCC is conventionally divided into four molecular subtypes: basal, mesenchymal, atypical and classical, defined from gene expression and recurrent copy-number patterns (Walter et al. 2013). The basal subtype is the least differentiated, dominated by basal keratinocyte markers (TP63, KRT5, KRT14) and EGFR signalling (Walter et al. 2013). The classical subtype is a smoking-associated, tumour-cell-intrinsic programme built on oxidative-stress (KEAP1/NFE2L2) and xenobiotic-detoxification genes together with squamous keratinisation (Walter et al. 2013). The mesenchymal subtype reflects an EMT-like phenotype accompanied by a fibroblast-rich, immune-infiltrated stroma (Walter et al. 2013; Keck et al. 2015), while the atypical subtype is defined largely by the absence of the EGFR (7p) and 3q26 amplifications seen in the other subtypes, together with activating PIK3CA changes, and in patients is enriched for HPV-associated, immune-infiltrated tumours (Walter et al. 2013; “Comprehensive Genomic Characterization of Head and Neck Squamous Cell Carcinomas” 2015). Linge et al. related these molecular subtypes to biological function and radiosensitivity (Linge et al. 2024). An important caveat follows for my cohort. These subtype labels were derived from whole patient tumours, which contain large immune and stromal compartments. The xenografts profiled here do contain substantial mouse-derived stroma and vasculature, but they lack a functional adaptive immune compartment (only NK cells), and, because the sequencing reads were aligned to the human reference genome, mouse stromal and immune transcripts are largely excluded from the human expression matrix. The bulk-subtype signal these cells contribute, particularly for the mesenchymal and atypical labels (Keck et al. 2015), is therefore not represented here. Any comparison between the spatial MPs and these labels is therefore a comparison with the malignant-cell component of each molecular subtype rather than with the subtype as defined in bulk tissue.

The correspondence between bulk molecular subtype and dominant spatial MP provides strong validation that the NMF decomposition captures biologically

meaningful transcriptional variation rather than technical noise. The Mesenchymal-to-MP1 mapping in SAS and the Atypical-to-MP5 mapping in SAT are the clearest examples: in both cases, the transcriptional theme encoded in the bulk subtype label is recapitulated at the spot level, consistent with the spatial approach reproducing bulk biology where a dominant programme exists. These two mappings have clear tumour-cell-intrinsic readings: the mesenchymal label of SAS corresponds to the cancer-cell EMT programme captured by MP1, while for SAT the stromal contribution that co-defines the atypical subtype in patients is not captured here (mouse-derived stroma is present but excluded by human-genome alignment), leaving a residual tumour-intrinsic signal that is dominated by a keratinocyte / squamous-epithelial differentiation identity and is recovered by the spatial decomposition as MP5.

The four tumor models classified as Heterogenous (FaDu, UT5, UT8, Cal33) are, by definition, models in which no single subtype dominates the bulk signal. The spatial data provide the explanation: these lines are MP4-dominant, but with variable secondary programmes that differ by cell line and by section. The Heterogenous bulk label reflects the averaging of these spatially segregated programmes; spatial transcriptomics resolves a longstanding interpretive problem with this subtype by revealing its internal structure.

Cal33 illustrates this clearly. Three sections from the same cell line span MP4-dominant (N154a.37rep, 62.3% MP4), MP2-dominant (N154a.73, 48.3% MP2), and MP5-dominant (N154a.38 Basal, 49.1% MP5) spatial fingerprints. The Basal-to-MP5 mapping in N154a.38 is consistent with the Basal HNSCC subtype's known association with differentiated squamous epithelial identity in bulk transcriptomic studies ("Comprehensive Genomic Characterization of Head and Neck Squamous Cell Carcinomas" 2015). The dominance of the Keratinocyte/Squamous-epithelial programme (MP5) in this section is concordant with that association at the spatial level, suggesting that the Basal label in this cohort maps onto a keratinocyte-like ground state rather than an undifferentiated phenotype - though caution is warranted in generalising from a single section. The same cell line spans MP4-, MP2- and MP5-dominant samples, which fits Cal33's variable radiation response: in fractionated-irradiation xenograft assays it shows a poorly defined dose-response (Koi et al. 2023, 2017), as expected for a transcriptionally heterogeneous line.

7.4. Clinical and Translational Significance of the Metaprogram Framework

Sections 7.2 and 7.3 established that the five consensus MPs reflect canonical HNSCC transcriptional biology. However, I only observed a direct correspondence to a bulk molecular subtype for only one cell line, SAS (Mesenchymal, mapping to MP1); the remaining lines were heterogeneous/unclassified, so no single subtype existed for the MPs to correspond to.

The cross-sample reproducibility of all five programmes across six cell lines and 17 sections under two independent analytical frameworks is consistent with the broader observation that intra-tumoural transcriptional heterogeneity resolves into a small number of recurrent transcriptional programmes (for example EMT, hypoxia, cell cycle and differentiation) that reappear across different tumours. Kinker et al. (2020) demonstrated this across 198 cancer cell lines profiled by single-cell RNA-sequencing, showing that recurrent heterogeneity programmes including EMT, stress, and differentiation recur independently of tissue of origin. My data are consistent with this in the spatially resolved domain, though the xenografts and limited cell line number preclude generalisable conclusions about programme conservation across patient tumours. I now consider what each programme implies for future translational investigation, independently of the experimental conditions under which they were identified.

MP1, the EMT/mesenchymal programme, is of potential interest for invasion and metastasis. It was most abundant in SAS, the only Mesenchymal-subtype line in this study, consistent with its bulk Mesenchymal subtype assignment. SAS is also among the most radioresistant lines in the panel (Koi et al. 2023), so the dominance of MP1 in this line links a mesenchymal transcriptional state to intrinsic radioresistance, matching the graded MP1-to-radioresistance relationship I observed across the cohort (Figure 31).

The significance of partial rather than complete EMT is increasingly recognised: intermediate EMT states, which co-express epithelial and mesenchymal markers, confer collective migratory capacity, tumour-initiating potential, and therapy resistance

that full mesenchymal conversion does not (Nieto et al. 2016; Pastushenko et al. 2018). The pEMT state described by Puram et al. (2017) in primary patient tumours is a useful reference point, but whether the MP1 patterning I observe in xenografts is reproducible in patient tissue, and whether it co-localises with histologically defined invasive fronts, would need to be tested before spatial MP1 quantification could have any translational value.

A further pattern emerged when I arranged the cell lines by their intrinsic radioresistance (Figure 31). MP1 abundance was highest in the most radioresistant lines and declined progressively towards the radiosensitive end of the panel, making it the component that varied most consistently across the models. The squamous-differentiation programmes MP3 and MP5 were higher at the radiosensitive end, but this was concentrated in SAT and, to a lesser extent, UT8 rather than a smooth cohort-wide trend. I consider this relationship biologically coherent: pEMT and mesenchymal transcriptional states, of which MP1 is the spatial correlate here, have been repeatedly associated with radioresistance and broader treatment resistance in HNSCC (Puram et al. 2017; Qiao et al. 2022), whereas commitment to terminal squamous differentiation (MP3 and MP5) reflects a more differentiated phenotype that is generally linked to greater treatment responsiveness (Lee et al. 2026).

Hypoxia is itself a recognised driver of EMT and radioresistance (Marie-Egyptienne, Lohse, and Hill 2013), and might therefore be expected to couple the mesenchymal programme to the hypoxic compartment. In my data, however, MP1 behaved in the opposite way to this expectation: rather than being enriched in hypoxic spots, it was significantly depleted there (mean shift -4.59% , $p = 0.0114$, $d = -0.693$; Table 6, Figure 28), while MP5 did not shift significantly (-2.64% , $p = 0.079$) and only MP3 was enriched with oxygenation. This decoupling of the mesenchymal programme from the hypoxic compartment argues that the MP1-radioresistance association reflects an intrinsic transcriptional property of the more resistant lines rather than a secondary consequence of their hypoxic microenvironment, even if hypoxia may reinforce it elsewhere. Consistent with a tumour-cell-intrinsic origin, MP1 was concentrated in pathologist-annotated tumour spots (its second most prevalent MP there, 16.4%) and essentially absent from annotated stroma (Figure 29).

I emphasise that these observations are correlative: they rest on a small panel of cell lines ordered by a radioresistance ranking derived from data external to this spatial cohort, rather than on a formal statistical test, and should be treated as hypothesis-generating. They nonetheless raise the possibility that the balance between the mesenchymal (MP1) and squamous-differentiation (MP3/MP5) programmes contributes to the intrinsic radiosensitivity of HNSCC - a hypothesis that I could address directly by relating spatial metaprogram composition to clonogenic survival across a larger set of models.

The MP3/MP5 differentiation axis raises questions about within-tumour differentiation heterogeneity that bulk grading does not address. The Cal33 data illustrate that within a single cell line, sections can differ substantially in their dominant MP, spanning MP4-dominant, MP2-dominant, and MP5-dominant fingerprints depending on the section profiled. This variability is notable, though it reflects differences between individual xenograft sections rather than providing direct evidence of differentiation-state heterogeneity. In patient tumours, histological grading assigns a single tumour-level score that may not capture the spatial co-existence of transcriptionally distinct differentiation states. Whether a spatially resolved MP3/MP5 balance carries additional information beyond histological grade is a hypothesis that would require testing in patient cohorts with matched Visium and outcome data, and is not supported by the current xenograft dataset.

MP4, the most prevalent programme in the cohort (39.2% of all spots), reflects ECM scaffolding and cytoskeletal gene expression and is interpreted here as a stromal-interface transcriptional state. As discussed in 6.2, MP4 as measured here is tumour-cell-intrinsic: although mouse-derived stroma is present in the xenografts, the human-genome alignment excludes its transcripts, so MP4 is captured within the human malignant cells. In patient tumours, where a genuine CAF-containing stromal compartment is present, the interpretation of MP4-enriched spots would be more complex. CAFs in HNSCC are a functionally heterogeneous population, with myofibroblastic and inflammatory subtypes contributing differently to ECM deposition, immune modulation, and paracrine signalling (Sahai et al. 2020). Whether MP4 as defined in this xenograft dataset would map onto CAF-rich stromal regions in patient tissue, or would instead reflect tumour-cell-intrinsic ECM programmes distinct from

fibroblast contributions, cannot be determined from my data and would require deconvolution in matched patient-derived spatial transcriptomic datasets.

Taken together, the MPs recover transcriptional structure that bulk subtyping cannot. Many tumours in clinical cohorts may receive only a heterogeneous or unclassified bulk label with the traditional bulk molecular subtyping method; The spatial decomposition instead resolves the several MPs that co-exist within each model. It also separates more aggressive MPs (MP1, EMT/mesenchymal) from more differentiated, radiosensitive ones (MP3, MP5). Patient tumours are likely to harbour multiple such programmes too, so this gives a more granular picture than a single bulk subtype. Its broader significance nonetheless remains to be established in patient-derived material: the translational relevance of the individual MPs, whether their spatial composition carries prognostic or predictive information, and whether they recur with the same structure in patient tumours, are open questions that prospective application to clinical Visium cohorts would need to address.

7.5. Three Recurrent Hypoxia-Associated Spatial Patterns

I focused on hypoxia for two reasons. First, it is a leading cause of radioresistance and treatment failure in HNSCC (Section 1.3.3). Second, PIMO provided a direct, tissue-resolved TME marker, allowing me to map hypoxia onto the MPs. This let me test which MPs were spatially associated with hypoxic regions and which were independent of oxygenation, rather than inferring hypoxia from a transcriptional signature. My main observation is that hypoxia, as spatially resolved by PIMO labelling, is associated with not one but three recurrent spatial patterns of transcriptional programme usage in HNSCC tissue. These patterns are descriptive and cross-sectional; while each is biologically interpretable, my design does not establish mechanistic directionality between hypoxia and programme state. Each pattern is discussed below.

7.5.1. Canonical HIF-1 α Response

The enrichment of MP2 in PIMO-positive spots (mean composition shift +16.47%, $p = 0.0012$, Cohen's $d = +0.955$) (Figure 28; Table 6) is the expected finding. It is

consistent with the PIMO classification being transcriptionally valid: spots where pO_2 fell below the PIMO binding threshold of approximately 10 mmHg show elevated expression of canonical HIF-1 α target genes. The large effect size ($d > 0.8$ is conventionally considered large) and directional consistency across 15 of 17 samples confirm that this is a robust, cell-line-independent pattern. This result is consistent with the extensive prior literature demonstrating that HIF-1 α -driven transcription is a universal mammalian response to oxygen deprivation, conserved across cell types and tumour models (Semenza 2003).

The concentration of MP2-dominant spots at necrotic borders is also informative. Necrosis in rapidly growing xenograft tumours arises from progressive oxygen deprivation as tumour bulk outgrows its blood supply. The necrotic border zone represents the spatial region of maximal and most sustained hypoxic stress. The fact that necrotic spots are MP2-dominant (32.4%) is consistent with the interpretation that these cells retain a HIF transcriptional signature despite maximal and sustained hypoxic stress.

7.5.2. Structural, Diffusion-Limited Hypoxia in Keratinised Zones

The enrichment of MP3 in PIMO-positive spots (mean composition shift +9.26%, $p = 0.0146$, $d = +0.662$) (Figure 28; Table 6), together with the co-enrichment of MP5, defines the second recurrent pattern. My observation is that it may be architectural: keratinisation is the most hypoxic annotated tissue class in the cohort (38.5% PIMO-positive), though based on few, largely SAT-derived spots (Table 8) yet keratinised zones are dominated by squamous-differentiation programmes: MP5 (Keratinocyte/Squamous-epithelial) and MP3 (Squamous Differentiation); rather than by the canonical hypoxia programme MP2.

A likely contributor is the tissue architecture itself. Keratin pearls are concentric layers of terminally differentiated, cornified squamous cells forming a dense, avascular matrix that physically excludes vessels, so oxygen reaches their interior only by diffusion from vessels at the periphery; tissue beyond this diffusion distance becomes hypoxic regardless of its metabolic rate (Swartz et al. 2022). This is still genuine hypoxia, since low oxygen availability is the common endpoint whether its origin is metabolic or geometric, and it can in principle still engage HIF signalling. The weaker MP2 signal in these zones therefore need not indicate a fundamentally different form of hypoxia:

a single transcriptional snapshot may miss the window of HIF activation, or capture cells that are already terminally differentiated and transcriptionally quiescent. Hypoxia can also alter keratinocyte differentiation through HIF-independent pathways (Park et al. 2016), so the association between low oxygen and the squamous-differentiation programme need not run through HIF-1 α .

Two cautions remain with this interpretation. First, it rests on relatively few spots (291 keratinisation-annotated spots, ~1% of the cohort, with sizeable, keratinised areas essentially limited to the SAT sections; Table 8), so it is best regarded as hypothesis-generating. Second, transcriptional readouts can under-represent hypoxia in terminally differentiated tissue, and confirmation would require protein-level validation, where hypoxia-related proteins are sometimes more reliable indicators than their transcripts. With these limitations, the co-occurrence of PIMO staining with keratinised, differentiated regions, also reported clinically in head and neck cancer (Kaanders et al. 2002; Janssen et al. 2004), is a useful reminder that PIMO positivity need not always coincide with a canonical HIF transcriptional response.

7.5.3. Depletion of ECM/Stromal-Like Signal in Hypoxic Cores

The depletion of MP4 in PIMO-positive spots is the quantitatively largest effect in the dataset (mean composition shift -18.51%, $p = 0.0006$, $d = -1.033$) (Figure 28; Table 6). This most likely reflects the low stromal cell content of hypoxic, tumour-cell-dense regions rather than active transcriptional downregulation: MP4 is an ECM/stromal-like MP, and these xenografts carry functional vasculature alongside hypoxia but only sparse stroma, so hypoxic tumour-rich areas contain little ECM/stromal signal to begin with. This is also consistent with hypoxia being heterogeneous in origin, comprising not only chronic, diffusion-limited hypoxia distant from vessels but also acute and cycling hypoxia adjacent to functional vasculature, so PIMO-positive spots are not necessarily those furthest from the stromal compartment.

Rather than implying that hypoxic cores are functionally isolated from microenvironmental signals, this pattern is most simply explained by the sparse stroma of the model. Whether a genuine spatial separation of tumour cells from stromal signals occurs, and whether it contributes to immune exclusion or therapy resistance, would need to be tested in patient-derived tissue with paired hypoxia and spatial transcriptomics data, where a proper human stromal compartment is present.

7.6. Clinical Relevance: Hypotheses for Future Investigation

The three-pattern framework raises several hypotheses relevant to clinical HNSCC biology. Current clinical practice uses PIMO IHC or gene expression-based hypoxia signatures as prognostic tools, typically treating hypoxia as a single entity. My data suggest that PIMO positivity in HNSCC may reflect at least two mechanistically distinct phenomena: metabolic HIF-driven hypoxia (Pattern 1, MP2-dominant) and structural avascular hypoxia in keratinised zones (Pattern 2, MP3/MP5-dominant); with different downstream transcriptional consequences. Whether these two forms of PIMO positivity carry different prognostic weight or different sensitivity to hypoxia-modifying treatments is a hypothesis that should be tested in clinical cohorts with matched spatial transcriptomics and outcome data.

7.7. Limitations and Future Directions

Several limitations of my analysis should be noted. The preclinical xenografts, while advantageous for controlled hypoxia labelling with PIMO, lacks the complexity of human HNSCC tumours, which contain a full complement of immune cells and patient-derived stroma. The five MPs identified here reflect the transcriptional repertoire of HNSCC tumour cells in this specific experimental context, and validation in patient-derived spatial transcriptomics data is an important next step.

The 55 μm Visium spot integrates the transcriptome of multiple cells, making it impossible to determine with certainty whether a given MP score reflects a single cell type or a mixture. Single-cell spatial methods with sub-cellular resolution would allow the three spatial patterns to be mapped to individual cells. Additionally, the cross-sectional nature of my data provides a static snapshot of the hypoxia-transcription relationship; longitudinal experiments - for example, re-oxygenation experiments with paired Visium profiles would be required to establish the directionality of programme changes in response to dynamic oxygen shifts.

Finally, the three recurrent spatial patterns described here are observational associations in a xenograft cohort of 17 sections. The design does not establish causal directionality, and the patterns should be interpreted as consistent with rather than proof of the proposed biological mechanisms. Perturbational experiments targeting HIF-1 α , squamous differentiation regulators, and ECM programme genes in spatially resolved models would be required to move from pattern to mechanism.

7.8. Conclusion

In conclusion for the second section, my work demonstrates that transcriptional heterogeneity in HNSCC is not a random or poorly defined feature, but is organised into recurring spatial programmes with distinct biological identities. I identified five metaprograms that captured major tumour states, including EMT/mesenchymal, hypoxia-associated, squamous differentiation, ECM/stromal-like, and keratinocyte-associated programmes. Their recurrence across cell lines and tissue sections suggests that these states represent stable components of HNSCC biology rather than sample-specific variation.

A major finding of my thesis is that spatial organisation can explain patterns that remain unresolved in bulk transcriptomic classification. Tumours classified as “Heterogeneous” at the bulk level were not biologically uninformative. Instead, my results suggest that this label reflects the coexistence of multiple spatially separated transcriptional states within the same tumour. In contrast, tumours with a dominant spatial programme showed clearer agreement with established bulk molecular subtype identities. This indicates that my spatial decomposition approach can refine conventional molecular classification by separating mixed tumour states that are averaged together in bulk data.

I also show that hypoxia in HNSCC should not be interpreted as a single uniform biological condition. PIMO-positive regions displayed distinct transcriptional contexts, including a canonical HIF-associated hypoxia response and a structurally hypoxic state in keratinised tumour regions that was dominated by squamous differentiation and keratinocyte-associated programmes. Notably, this keratinised, structurally hypoxic

state was most prominent in the radiosensitive models with the lowest TCD50, whereas the more radioresistant models were characterised by the mesenchymal MP1 programme. This links the spatial hypoxia phenotype to the intrinsic radioresistance of the cell lines.

Together, my findings provide a spatially resolved concept for understanding intratumour heterogeneity and hypoxia-associated biology in HNSCC. They suggest that separating HIF-driven metabolic hypoxia from structural, avascular hypoxia may be important for interpreting hypoxia biomarkers and for linking spatial tumour states to treatment response. This distinction may also help explain the inconsistent performance of transcriptomic hypoxia signatures in patient stratification: although gene-expression classifiers such as the 15-gene hypoxia signature are prognostic and predictive of benefit from hypoxic modification in some trials (Toustrup et al. 2011, 2012), their validation across independent cohorts and platforms has been variable, motivating alternative approaches such as DNA-methylation-based hypoxia classifiers (Tawk et al. 2023). Although these observations were derived from xenograft models and require validation in patient-derived cohorts, my work establishes testable hypotheses for future studies. In particular, future work should assess whether metaprogram composition, spatial hypoxia patterns, and their interaction with molecular subtype identity can improve patient stratification and support more precise treatment-response prediction in HNSCC.

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Abbreviations

Abbreviation	Definition
AJCC	American Joint Committee on Cancer
AOI	area of interest
BAM	Binary Alignment Map
BSA	bovine serum albumin
CAIX	carbonic anhydrase IX (gene CA9)
cDNA	complementary DNA
CT	cancer-testis
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA damage response
DEG	differentially expressed gene(s)
DESeq2	differential expression analysis R package (software name)
DGE	differential gene expression
DKFZ	Deutsches Krebsforschungszentrum (German Cancer Research Center)
DKTK	Deutsches Konsortium für Translationale Krebsforschung (German Cancer Consortium)
DKTK-ROG	DKTK Radiation Oncology Group
DM	distant metastasis
DMA	differential methylation analysis
DMFS	distant-metastasis-free survival
DMP	differentially methylated position
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EMT	epithelial-to-mesenchymal transition
FDR	false discovery rate
FFPE	formalin-fixed paraffin-embedded
GO	Gene Ontology
GPCF	Genomics and Proteomics Core Facility
GSEA	Gene Set Enrichment Analysis
GTE _x	Genotype-Tissue Expression (portal)
H&E	haematoxylin and eosin
HGNC	HUGO Gene Nomenclature Committee
HIF	hypoxia-inducible factor (HIF-1 α)
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
IHC	immunohistochemistry
KEGG	Kyoto Encyclopedia of Genes and Genomes
LOLA	Locus Overlap Analysis
LRC	loco-regional control
LRT	likelihood ratio test
MACH-NC	Meta-Analysis of Chemotherapy in Head and Neck Cancer
MP	Metaprogram
MSigDB	Molecular Signatures Database

NMF	non-negative matrix factorisation
ODCF	Omics IT and Data Management Core Facility
ODP	overlapping DEG panel
ORA	over-representation analysis
OS	overall survival
PBS	phosphate-buffered saline
PCA	principal component analysis
PCR	polymerase chain reaction
pEMT	partial epithelial-mesenchymal transition
PFS	progression-free survival
PIMO	pimonidazole
RCT	Radiochemotherapy
RMA	Robust Multi-chip Average
RNA	ribonucleic acid
RNA-seq	RNA sequencing
ROI	region of interest
RT	radiotherapy
RT-qPCR	reverse-transcription quantitative PCR
SOP	standard operating procedure
STAR	Spliced Transcripts Alignment to a Reference (aligner)
TCGA	The Cancer Genome Atlas
TCGA-HNSC	TCGA Head and Neck Squamous Cell Carcinoma cohort
TCGAbiolinks	Bioconductor/R package for TCGA data access (software)
TME	tumour microenvironment
TNM	Tumour-Node-Metastasis (staging system)
UICC	Union for International Cancer Control
UMAP	Uniform Manifold Approximation and Projection
UMI	unique molecular identifier
VST	variance-stabilising transformation
WT	wild-type
UT5	UTSCC5
UT45	UTSCC45

Acknowledgement

Firstly, I thank the Almighty Allah, for we can only try but He is the one who gives. I came a long way learning that hard work and humbleness are the only two things you can control in life on that path to success, everything else is just sheer luck, timing and lots of external factors you can never control. Therefore, every day I just try to be a little bit more hardworking and humbler and let the universe decide what it has for me.

It's hard to acknowledge people in writing, I always find it really challenging. So many people for so many things to be grateful for. A journey of almost 4 years, but what about the journey before that? It started way long ago. I thank my parents for allowing me to have the best education possible. In a country where lots of children die even before reaching teens, a majority of them don't even have access to basic rights, they managed to provide me with education in one of the best schools of the country. Today the freedom, privilege that I enjoy every day is the result of their sacrifice; things they could never have but tried their best to make it possible for us. Specially, my mother who only went out of her own city only a handful of times, but today I get to travel to so many countries because of her choice to sacrifice her life for us. I am not so proud of it, no human being should ever sacrifice their whole life, happiness and moments for the sake of the others, there should be a balance of everything. I just hope day by day I get more opportunities to make it up to her and my father. So, they get to have the life they deserve.

I thank Angshu bhaiya. Not a relative by blood but a gift from Allah in a period where I needed him the most. From being a motivated arrogant kid, he turned me into a composed and confident person. I can write a whole book on this, but maybe later when the time is right. I thank my brothers Marshal bhaiya and Raien for trying their best as much as they can to make my life easier with complete support.

I thank my PI Ina Kurth from the bottom of my heart. Coming directly from Bangladesh to Germany wasn't easy, the first 6 months were quite difficult. But life became a lot easier - having such a helpful group with a supportive environment. Ina

has helped me in every moment I needed help; starting with visa applications to trying to learn a language in my working hour, she never once discouraged me, rather tried her best to accommodate what I needed for a better career and personal life. The growth I had both in my scientific thinking and career perspective, big credit goes to Ina for helping all the way through. From day one, she let me have an independent way of guiding my project, which was quite challenging at the beginning, but slowly it helped me turning into a better researcher and person.

I thank my colleagues from E220- Verena, Mareike, Wahyu, Julian, Rose, Tom, Cristina, Maria, Olga, Manuela, and Mahnaz from E220. Wahyu and Mareike have always been super kind to me. I can always discuss with Wahyu (when he is awake and responding) with any matter, he has always been a support center excluding his jokes of course. I can discuss freely anything with him regarding anything without having any doubt. Mareike has been like an elder sister to me, being the person always remembering even little things to make my day a whole lot better. I never expected someone caring for me so much in such a short period of time. It doesn't matter in which part of the world I am in the future; I will always remember her and try to bear the act of kindness she showed to me, all my life.

Julian has been one of my closest friends + colleague during the whole period. Since his joining in the group, life has certainly become better. I wish the world has more people like him; it will be a better and nicer place. I can't say enough about Rose, what a blissful person! I always joke saying "if there comes a day, I have issues with Rose, then the problem is with me". She has also been my technical support in this short German life. Any daily life help needed starting from fixing something to suggestion about buying anything she is there of course. Maria is technically my supervisor also in my project, she has always been supportive to me. It always felt good to have someone always there when you need any help, she always tried her best to help, for which I am always grateful. Same goes for Cristina. She sits next to my desk, whenever I need any kinds of help, or face any sudden issues (don't want to say ODCF/Cluster) I always go to her. She has been always helpful in any kinds of things. Tom has always been kind to me from the start. We don't have much shared work, but he is always there asking me how I was, how everything is going, which is sometimes all you need in a distant land far from home. Olga and I also don't share much work, but I have always appreciated her for her gentle, kind, and

supportive personality. Mahnaz has helped me a lot at the end part of my PhD project, but apart from these times, whenever I needed any help, she was always there to help. I thank Manuela for her support. Finally, I can't appreciate enough Verena. I always look up to her. Her approach to doing things is something I really try to follow. The improvement I had throughout my journey in PhD as a researcher, a lot of the credit goes to her.

I want to thank my Bangladeshi community in Heidelberg. Utsho bhai and Saddam bhai welcomed me in Germany in my very first day. Being in a new country with no German language experience, things would not be so easy without their help. I want to thank Nayeem bhai and Arman bhai for supporting me throughout the whole time with all kinds of help possible. I am grateful to Shahid bhai, Saiful bhai, Rizvana apu, Jeba apu, Nitu apu and Ruwayfi and so many Bangladeshi people from the community who helped me with different things in different times. I want to thank my Vettters friends - Lena, Stefan, Jessica, Simona, Katherine, Shuang, Aisha, and Elena. Getting friends from different countries, knowing about these countries and cultures, having some moments to share, trying to support each other during this tough PhD journey have been an incredible experience for me. Specially Lena, Stefan, and Jessica were my go-to person every time I had an emergency, or just trying to brag about things randomly. I was always confident in my mind knowing that someone like them is always there to help me with any kinds of situations.

I thank my friends Tonmoy, Lincon, and Maisha for making my regular days, occasional weekend adda and vacation days way nicer. The more you age in life, the more you understand that it's the people around you who are the most important. I work hard the whole year so I can enjoy great vacation times with you.

Finally, I want to thank my wife, Anne. It has been quite a journey of 10 years. We had to remain apart for almost two years after our marriage, hardest period of my life. I will never let that phase return in my life even if I have to sacrifice everything else. It has come to a point so many years ago that life without you is impossible. I can't think of a life without you, that's just plain simple. I always make plans, but none of those plans will make any sense if you are not there. Thanks for always supporting me and giving me a reason to go out there every day even when things are not working. I hope life gets more easier for us in the upcoming days in sha

Allah. Even if it doesn't get easier, I would still welcome it as long as you are with me, may Allah bless us both into being better versions of us every tomorrow.

I would like to acknowledge the use of Anthropic's Claude as a writing assistant throughout the preparation of this thesis. This tool was employed exclusively to enhance the clarity, structure, and readability of the text. All scientific content, data analysis, and interpretation were conducted independently. Figures 1 & 2 were created with Claude and figures 17 & 18 was created using BioRender (<https://BioRender.com>), which were used to generate schematic illustrations for improved visual communication of concepts.

Supplementary Materials

Supplementary Table 1. Clinical, pathological and molecular annotation of the DKTK-ROG cohort.

Sample ID	P53	K-means cluster	Subtype	TP53.status	TP53.variant	Mutation Type	IHC positivity	Score	LR C	LRCtime	OS	OSTime	Gender	Age	Tumor localisation	Tumor stage	N status	R status	Grading	UICC status	ECE status	P16	HPV16 DNA	HPV16 RNA
1	Positive	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type	50	4	0	59,1	0	59,1	Male	43	Oropharynx	T2	N2b	R0	G3	IV	Yes	Positive	Positive	Positive
2	Negative	Low CT Panel Expression	Basal	altered	p.Q167*	FS/Nonsense	0	0	0	52,1	0	52,1	Male	55	Oral Cavity	T2	N1	R0	G2	III	Yes	Negative	Positive	Negative
3	Negative	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type			0	51,2	0	51,2	Male	52	Oropharynx	T1	N2b	R0	G1	IV	Yes	Positive	Positive	Positive
4	Negative	High CT Panel Expression	Heterogeneous Subtypes	NA	NA	Unknown			0	49,3	0	49,3	Male	61	Oropharynx	T3	N2b	R1	G2	IV	No	Positive	NA	Positive
5	Negative	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type			0	58	0	58	Male	59	Oropharynx	T2	N2b	R0	G2	IV	No	Positive	Positive	Positive
6	Negative	High CT Panel Expression	Mesenchymal	altered	p.Q192*	FS/Nonsense	5	1	0	15,9	1	15,9	Male	50	Oropharynx	T3	N2	R0	G3	IV	Yes	Positive	Positive	Positive
7	Negative	High CT Panel Expression	Atypical	wt	wt	Wild Type			0	39,5	0	39,5	Male	50	Oropharynx	T1	N2b	R1	G2	IV	Yes	Positive	Positive	Positive
8	Negative	Low CT Panel Expression	Heterogeneous Subtypes	wt	wt	Wild Type			0	34,3	0	34,3	Male	50	Oropharynx	T2	N2c	R1	G3	IV	No	Positive	Positive	Positive
9	Positive	Low CT Panel Expression	Classical	altered	p.Y220C	Missense	95	4	0	24,9	1	24,9	Male	58	Oral Cavity	T1	N1	R0	G2	III	Yes	Positive	Negative	Negative
10	Negative	Intermediate CT Panel Expression	Mesenchymal	wt	wt	Wild Type			0	37,3	1	37,3	Male	52	Oropharynx	T2	N0	R1	G3	II	No	Positive	Positive	Positive
11	Positive	Low CT Panel Expression	Heterogeneous Subtypes	NA	NA	Unknown			0	72,2	0	72,2	Male	55	Oropharynx	T1	N2	R1	G2	IV	Yes	Negative	Positive	Negative
12	Positive	Low CT Panel Expression	Atypical	NA	NA	Unknown			0	15,1	1	15,1	Male	60	Oropharynx	T3	N0	R1	G3	III	No	Negative	Positive	Negative

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13	Negative	Low CT Panel Expression	Mesenchymal	wt	wt	Wild Type			0	5,2	1	5,2	Female	67	Oral Cavity	T4	N2	R1	G3	IV	Yes	Positive	Positive	Negative
14	Positive	Low CT Panel Expression	Heterogeneous Subtypes	NA	NA	Unknown			0	63,6	0	63,6	Female	62	Oropharynx	T2	N2	R0	G3	IV	Yes	Positive	Negative	Negative
15	Negative	High CT Panel Expression	Atypical	wt	wt	Wild Type	20	2	0	73,4	0	73,4	Male	68	Oropharynx	T2	N1	R1	G2	III	No	Positive	Positive	Positive
16	Negative	Intermediate CT Panel Expression	Heterogeneous Subtypes	wt	wt	Wild Type			0	61,3	0	61,3	Male	66	Oropharynx	T4	N1	R0	G3	IV	No	Positive	Positive	Positive
17	Negative	Low CT Panel Expression	Heterogeneous Subtypes	wt	wt	Wild Type	0	0	0	30	0	30	Male	24	Oral Cavity	T2	N2b	R0	G2	IV	No	Positive	Positive	Negative
18	Negative	Low CT Panel Expression	Mesenchymal	wt	wt	Wild Type			0	37,9	0	37,9	Male	54	Oral Cavity	T2	N2b	R0	G3	IV	Yes	Positive	Positive	Positive
19	Negative	Low CT Panel Expression	Low CT Panel Expression	wt	wt	Wild Type			0	42,3	1	48	Female	45	Oropharynx	T2	N2b	R0	G3	IV	Yes	NA	Positive	Negative
20	Negative	High CT Panel Expression	Heterogeneous Subtypes	wt	wt	Wild Type	5	1	0	46,8	0	46,8	Male	61	Oropharynx	T3	N2b	R1	G3	IV	Yes	Positive	Positive	Positive
21	Negative	Intermediate CT Panel Expression	Intermediate CT Panel Expression	wt	wt	Wild Type			0	48,3	0	48,3	Female	65	Oropharynx	T2	N2b	R0	G3	IV	No	NA	Positive	Positive
22	Negative	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type			0	59,5	0	59,5	Male	62	Oropharynx	T2	N1	R1	G3	III	No	Positive	Positive	Positive
23	Negative	Low CT Panel Expression	Heterogeneous Subtypes	wt	wt	Wild Type			0	64,2	1	64,2	Male	68	Oropharynx	T2	N2c	R1	G2	IV	No	Positive	Positive	Positive
24	Negative	Low CT Panel Expression	Low CT Panel Expression	altered	p.T284fs	FS/Nonsense			0	62,6	0	62,6	Male	54	Oropharynx	T4	N0	R1	G3	IV	No	NA	Positive	Negative
25	Negative	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type			0	45,6	1	51,4	Male	71	Oropharynx	T3	N2b	R0	G3	IV	Yes	Positive	Positive	Positive
26	Negative	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type			0	47,3	0	47,3	Female	56	Oropharynx	T2	N2b	R0	G2	IV	Yes	Positive	Positive	Positive
27	Negative	Intermediate CT Panel Expression	Heterogeneous Subtypes	wt	wt	Wild Type			0	34,3	0	34,3	Male	62	Oropharynx	T2	N2b	R0	G2	IV	Yes	Positive	Positive	Positive
28	Negative	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type			1	26,3	1	36,5	Male	55	Oropharynx	T2	N2b	R0	G3	IV	Yes	Positive	Positive	Positive
29	Negative	High CT Panel Expression	Heterogeneous Subtypes	wt	wt	Wild Type			0	34,2	0	34,2	Male	58	Oropharynx	T2	N2b	R0	G3	IV	Yes	Positive	Positive	Positive

30	Negative	High CT Panel Expression	Atypical	wt	wt	Wild Type			0	38,9	0	38,9	Male	45	Oropharynx	T4	N2b	R0	G3	IV	Yes	Positive	Positive	Positive
31	Positive	Low CT Panel Expression	Mesenchymal	altered	p.Q331*	FS/Nonsense	100	4	1	10,5	0	11,3	Female	49	Oropharynx	T4	N0	R1	G1	IV	No	Positive	Negative	Negative
32	Positive	Low CT Panel Expression	Mesenchymal	altered	p.R196*	FS/Nonsense	100	4	0	54,7	0	54,7	Male	49	Oral Cavity	T1	N1	R1	G2	III	Yes	Positive	Negative	Negative
33	Positive	Intermediate CT Panel Expression	Heterogeneous Subtypes	NA	NA	Unknown			0	53	0	53	Male	70	Oropharynx	T3	N3	R0	G2	IV	Yes	Positive	Positive	Positive
34	Negative	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type			0	57,3	0	57,3	Male	53	Oropharynx	T2	N3	R1	G2	IV	Yes	Positive	Positive	Positive
35	Negative	Intermediate CT Panel Expression	Heterogeneous Subtypes	wt	wt	Wild Type			0	70,2	0	70,2	Female	57	Oropharynx	T2	N3	R0	G3	IV	Yes	Positive	Positive	Positive
36	Positive	Low CT Panel Expression	Heterogeneous Subtypes	NA	NA	Unknown			0	44,5	1	44,5	Female	65	Oropharynx	T1	N3	R0	G2	IV	Yes	Positive	Negative	Negative
37	Negative	Low CT Panel Expression	Classical	altered	p.W53*	FS/Nonsense	0	0	0	44,2	0	44,2	Male	43	Oropharynx	T2	N3	R1	G2	IV	Yes	Negative	Positive	Negative
38	Negative	High CT Panel Expression	Heterogeneous Subtypes	wt	wt	Wild Type			0	40,8	0	40,8	Male	69	Oropharynx	T3	N3	R0	G2	IV	Yes	Positive	Positive	Positive
39	Negative	Intermediate CT Panel Expression	Atypical	NA	NA	Unknown			0	73,5	0	73,5	Male	60	Oropharynx	T2	N3	R0	G3	IV	Yes	Positive	Positive	Positive
40	Negative	Low CT Panel Expression	Atypical	wt	wt	Wild Type			0	67,1	0	67,1	Male	46	Oropharynx	T2	N1	R0	G2	III	Yes	Positive	Positive	Negative
41	Negative	Intermediate CT Panel Expression	Heterogeneous Subtypes	wt	wt	Wild Type			0	63,7	0	63,7	Female	57	Oropharynx	T2	N3	R0	G2	IV	Yes	Positive	Positive	Positive
42	Positive	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type	80	4	0	96,6	0	96,6	Male	70	Oropharynx	T2	N3	R0	G2	IV	Yes	Positive	Positive	Positive
43	Negative	Low CT Panel Expression	Basal	altered	p.F54fs	FS/Nonsense			0	46,9	0	46,9	Male	57	Oral Cavity	T2	N3	R0	G2	IV	Yes	Positive	Negative	Negative
44	Positive	Low CT Panel Expression	Classical	altered	p.R280G	Missense	100	4	0	98,6	0	98,6	Male	59	Oral Cavity	T4	N3	R0	G2	IV	No	Positive	Negative	Negative
45	Negative	Low CT Panel Expression	Heterogeneous Subtypes	altered	p.D184fs	FS/Nonsense			0	5,7	0	5,7	Male	56	Oral Cavity	T4	N3	R1	G3	IV	Yes	Positive	Positive	Negative
46	Negative	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type			0	55,7	0	55,7	Male	63	Oropharynx	T2	N2b	R0	G3	IV	Yes	Positive	Positive	Positive

47	Negative	Intermediate CT Panel Expression	Heterogeneous Subtypes	wt	wt	Wild Type			0	41,6	0	41,6	Female	65	Oropharynx	T2	N1	R1	G3	III	No	Positive	Negative	Positive
48	Negative	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type			0	43,6	1	43,6	Male	62	Oropharynx	T4	N0	R1	G3	IV	No	Positive	Positive	Positive
49	Negative	Low CT Panel Expression	Heterogeneous Subtypes	wt	wt	Wild Type			0	43,2	0	43,2	Female	51	Oropharynx	T3	N2	R1	G1	IV	No	Positive	Negative	Negative
50	Negative	Low CT Panel Expression	Heterogeneous Subtypes	wt	wt	Wild Type	55	4	0	38,8	0	38,8	Male	59	Hypopharynx	T2	N2	R1	G2	IV	No	Positive	Positive	Positive
51	Negative	Intermediate CT Panel Expression	Heterogeneous Subtypes	wt	wt	Wild Type			0	43,9	0	43,9	Female	58	Oropharynx	T3	N2	R1	G2	IV	No	Positive	Negative	Negative
52	Negative	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type			0	75,7	0	75,7	Male	54	Oropharynx	T3	N3	R1	G2	IV	No	Positive	Positive	Positive
53	Negative	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type			0	41,2	0	41,2	Female	52	Oropharynx	T2	N2	R1	G2	IV	No	Positive	Positive	Positive
54	Positive	Low CT Panel Expression	Heterogeneous Subtypes	altered	p.P152L	Missense	95	4	0	62,4	0	62,4	Male	68	Oropharynx	T2	N2	R0	G3	IV	No	Positive	Negative	Negative
55	Negative	Low CT Panel Expression	Classical	altered	p.E204*	FS/Nonsense	0	0	0	58,4	0	58,4	Male	55	Oropharynx	T3	N2c	R1	G2	IV	No	Positive	Negative	Negative
56	Negative	Intermediate CT Panel Expression	Mesenchymal	wt	wt	Wild Type			0	40,3	0	40,3	Male	67	Oropharynx	T2	N0	R1	G3	II	No	Positive	Positive	Positive
57	Positive	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type	95	4	0	65,4	0	65,4	Male	51	Hypopharynx	T1	N2	R1	G2	IV	No	Positive	Positive	Positive
58	Negative	Low CT Panel Expression	Mesenchymal	NA	NA	Unknown			0	71,8	0	71,8	Male	59	Hypopharynx	T2	N2b	R1	G2	IV	No	Negative	Positive	Negative
59	Negative	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type			0	50,4	0	50,4	Female	68	Oropharynx	T1	N1	R0	G3	III	No	Positive	Positive	Positive
60	Positive	Low CT Panel Expression	Heterogeneous Subtypes	wt	wt	Wild Type	95	4	0	59,6	1	59,6	Female	60	Oropharynx	T2	N2	R1	G2	IV	No	Positive	Negative	Negative
61	Negative	Low CT Panel Expression	Atypical	wt	wt	Wild Type			1	20,6	1	32,1	Male	54	Oropharynx	T2	N2	R1	G2	IV	No	Positive	Negative	Negative
62	Negative	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type			0	52,5	0	52,5	Female	67	Oropharynx	T2	N2	R1	G2	IV	No	Positive	Positive	Positive
63	Negative	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type	20	2	0	52,7	0	52,7	Male	56	Oropharynx	T2	N2	R1	G3	IV	Yes	Positive	Positive	Positive

64	Negative	High CT Panel Expression	Atypical	NA	NA	Wild Type			0	67	0	67	Female	57	Oropharynx	T2	N2	R0	G3	IV	No	Positive	Positive	Positive
65	Positive	Low CT Panel Expression	Mesenchymal	altered	p.R175H	Missense	85	4	0	64	0	64	Male	55	Oropharynx	T1	N2	R1	G2	III	Yes	Negative	Positive	Negative
66	Negative	High CT Panel Expression	Heterogeneous Subtypes	wt	wt	Wild Type			0	45,7	0	45,7	Male	53	Oral Cavity	T2	N2	R0	G3	IV	No	Positive	Negative	Positive
67	Negative	Low CT Panel Expression	Heterogeneous Subtypes	altered	p.P75fs	FS/Nonsense	0	0	0	56,8	0	56,8	Male	48	Oral Cavity	T2	N0	R1	G2	II	No	Negative	Positive	Negative
68	Negative	High CT Panel Expression	Atypical	wt	wt	Wild Type			0	43,5	0	43,5	Male	66	Oropharynx	T1	N2	R0	G2	IV	Yes	Positive	Positive	Positive
69	Negative	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type			0	44,8	0	44,8	Male	51	Oropharynx	T2	N2b	R0	G3	IV	No	Positive	Positive	Positive
70	Negative	Low CT Panel Expression	Atypical	wt	wt	Wild Type			0	27,4	0	27,4	Male	40	Oropharynx	T2	N2b	NA	NA	IV	Yes	Positive	Positive	Positive
71	Positive	Low CT Panel Expression	Classical	altered	p.R273C	Missense	90	4	0	9,2	1	11,1	Male	61	Oral Cavity	T3	N2a	R0	G3	IV	Yes	Positive	Negative	Negative
72	Positive	Low CT Panel Expression	Classical	wt	wt	Wild Type	95	4	0	6,7	1	7,2	Male	49	Hypopharynx	T2	N2b	R0	G3	IV	Yes	Negative	Positive	Negative
73	Negative	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type			0	58,7	0	58,7	Male	69	Oropharynx	T3	N2b	R0	G3	IV	Yes	Positive	Positive	Positive
74	Positive	Low CT Panel Expression	Heterogeneous Subtypes	NA	NA	Unknown			1	33,3	1	36,5	Female	63	Oral Cavity	T2	N1	R0	G2	III	Yes	Positive	Negative	Negative
75	Positive	Intermediate CT Panel Expression	Heterogeneous Subtypes	wt	wt	Wild Type	95	4	1	23,1	0	45,4	Male	42	Oral Cavity	T2	N2b	R0	G2	IV	Yes	Negative	Positive	Positive
76	Negative	Low CT Panel Expression	Heterogeneous Subtypes	NA	NA	Unknown			0	62,3	0	62,3	Male	68	Oropharynx	T3	N2a	R0	G3	IV	Yes	Positive	Negative	Negative
77	Negative	Intermediate CT Panel Expression	Mesenchymal	wt	wt	Wild Type			0	59,6	0	59,6	Male	69	Oropharynx	T2	N2b	R0	G3	IV	No	Positive	Positive	Positive
78	Negative	High CT Panel Expression	Atypical	wt	wt	Wild Type			0	59,8	0	59,8	Male	66	Oropharynx	T1	N2c	R0	G3	IV	Yes	Positive	Positive	Positive
79	Negative	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type			0	54,5	0	54,5	Male	29	Oropharynx	T1	N1	R0	G3	III	Yes	Positive	Positive	Positive
80	Negative	High CT Panel Expression	Heterogeneous Subtypes	wt	wt	Wild Type			0	57,8	0	57,8	Male	70	Oropharynx	T3	N2a	R1	G3	IV	No	Positive	Positive	Positive

81	Positive	Intermediate CT Panel Expression	Atypical	altered	p.R342*	FS/Nonsense	70	4	0	66,9	0	66,9	Male	52	Oropharynx	T2	N2b	R0	G3	IV	Yes	Negative	Negative	Positive
82	Negative	Intermediate CT Panel Expression	Classical	wt	wt	Wild Type			0	58,2	0	58,2	Male	73	Oropharynx	T3	N2b	R0	G3	IV	Yes	Positive	Positive	Positive
83	Negative	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type			0	49	0	49	Male	75	Oropharynx	T2	N2b	R1	NA	IV	Yes	Positive	Positive	Positive
84	Negative	Intermediate CT Panel Expression	Atypical	wt	wt	Wild Type			0	34,7	0	34,7	Female	61	Oropharynx	T2	N2b	R0	G2	IV	No	Positive	Negative	Positive
85	Negative	Intermediate CT Panel Expression	Heterogeneous Subtypes	NA	NA	Unknown			0	73,1	0	73,1	Male	61	Oropharynx	T2	N2b	R1	G2	IV	No	Positive	Positive	Positive
86	Negative	Intermediate CT Panel Expression	Atypical	altered	p.R290H	Missense	5	1	0	70,2	0	70,2	Male	60	Oropharynx	T2	N2a	R1	G3	IV	No	Positive	Positive	NA
87	Negative	Low CT Panel Expression	Heterogeneous Subtypes	altered	p.G245D	Missense	40	3	0	33	0	33	Male	52	Oropharynx	T4	N2b	R0	G3	IV	Yes	Negative	Positive	Negative

Supplementary Table 2. Spot classifiability across the cohort. Visium spots receiving a confident dominant-metaprogram (MP) assignment versus those excluded (all five MP scores undefined). Pooled across 17 hypoxia-annotated sections.

Category	N
Total spots	42184
Classifiable (dominant MP assigned)	35511
Excluded (all MP scores NA)	6673
% classifiable	84.2
% excluded	15.8

Supplementary Table 3. Pooled per-spot bulk molecular-subtype classification.

Centroid-correlation subtype calls applied to individual Visium spots, pooled across all sections; the majority remain unclassifiable (“Heterogenous”) at single-spot resolution.

Subtype	n	Total	%
Heterogenous	37756	39160	96.4
Basal	1361	39160	3.5
Atypical	15	39160	0.0
Mesenchymal	15	39160	0.0
Classical	13	39160	0.0

Supplementary Table 4. Per-sample spot-subtype composition. Per-section breakdown of centroid-based spot subtype calls. n, spots; total, classifiable spots; %, percentage.

Sample	Subtype	n	Total	%
N150a014rep_FaDu_SPT_A1	Heterogenous	1417	1417	100.0
N150a020_FaDu_SPT_A1	Heterogenous	1470	1470	100.0
N150d320_FaDu_SPT_B1	Heterogenous	3419	3419	100.0
N153a083_UT5_C1	Basal	412	1725	23.9
N153a083_UT5_C1	Heterogenous	1313	1725	76.1
N153a084_UT5_SPT_A1	Basal	92	1756	5.2
N153a084_UT5_SPT_A1	Heterogenous	1664	1756	94.8
N153b219_UT5_SPT_D1	Basal	164	2369	6.9
N153b219_UT5_SPT_D1	Heterogenous	2205	2369	93.1
N154a037rep_Cal33_SPT_D1	Basal	74	1876	3.9
N154a037rep_Cal33_SPT_D1	Heterogenous	1802	1876	96.1
N154a038_Cal33_SPT_B1	Basal	131	3119	4.2
N154a038_Cal33_SPT_B1	Heterogenous	2988	3119	95.8
N154a073_Cal33_SPT_A1	Basal	164	2847	5.8
N154a073_Cal33_SPT_A1	Heterogenous	2683	2847	94.2
N155a131_UT8_SPT_B1	Basal	85	2947	2.9
N155a131_UT8_SPT_B1	Heterogenous	2862	2947	97.1
N155b199_UT8_SPT_C1	Basal	5	1702	0.3
N155b199_UT8_SPT_C1	Heterogenous	1697	1702	99.7
N155b200_UT8_SPT_D1	Basal	168	2745	6.1
N155b200_UT8_SPT_D1	Heterogenous	2577	2745	93.9
N156a074_SAS_SPT_D1	Basal	1	2865	0.0
N156a074_SAS_SPT_D1	Heterogenous	2864	2865	100.0
N156b181_SAS_SPT_B1	Heterogenous	2292	2292	100.0
N165a002_SAT_SPT_C1	Atypical	11	2969	0.4
N165a002_SAT_SPT_C1	Basal	20	2969	0.7
N165a002_SAT_SPT_C1	Classical	13	2969	0.4
N165a002_SAT_SPT_C1	Heterogenous	2925	2969	98.5

N165a067rep_SAT_SPT_C1	Atypical	4	1907	0.2
N165a067rep_SAT_SPT_C1	Basal	1	1907	0.1
N165a067rep_SAT_SPT_C1	Heterogenous	1887	1907	99.0
N165a067rep_SAT_SPT_C1	Mesenchymal	15	1907	0.8
N165b149rep_SAT_SPT_A1	Basal	44	1735	2.5
N165b149rep_SAT_SPT_A1	Heterogenous	1691	1735	97.5

Supplementary Table 5. Per-sample dominant-metaprogram composition shifts

between hypoxic and normoxic regions. For each section and MP: percentage of dominant-MP spots in normoxic and hypoxic tissue and their difference (Shift = % hypoxic – % normoxic). Positive = enriched under hypoxia. n = 17 sections.

Sample	Cell line	MP	% Normoxic	% Hypoxic	Shift (pp)
N150a014rep_FaDu_SPT_A1	FaDu	MP1	13.0	18.8	5.8
N150a014rep_FaDu_SPT_A1	FaDu	MP2	16.8	22.9	6.1
N150a014rep_FaDu_SPT_A1	FaDu	MP4	70.3	58.3	-11.9
N150a020_FaDu_SPT_A1	FaDu	MP1	17.7	4.0	-13.8
N150a020_FaDu_SPT_A1	FaDu	MP2	5.1	22.2	17.1
N150a020_FaDu_SPT_A1	FaDu	MP4	77.2	73.8	-3.4
N150d320_FaDu_SPT_B1	FaDu	MP1	23.2	10.2	-13.0
N150d320_FaDu_SPT_B1	FaDu	MP2	14.2	65.2	51.0
N150d320_FaDu_SPT_B1	FaDu	MP4	62.6	24.6	-38.0
N153a083_UT5_C1	UT5	MP1	34.6	35.5	0.9
N153a083_UT5_C1	UT5	MP2	32.0	26.2	-5.8
N153a083_UT5_C1	UT5	MP3	2.5	1.7	-0.8
N153a083_UT5_C1	UT5	MP4	29.8	36.2	6.5
N153a083_UT5_C1	UT5	MP5	1.1	0.4	-0.7
N153a084_UT5_SPT_A1	UT5	MP1	7.9	7.5	-0.4
N153a084_UT5_SPT_A1	UT5	MP2	13.5	36.0	22.5
N153a084_UT5_SPT_A1	UT5	MP3	1.2	4.7	3.5
N153a084_UT5_SPT_A1	UT5	MP4	74.1	48.8	-25.3
N153a084_UT5_SPT_A1	UT5	MP5	3.2	3.0	-0.2
N153b219_UT5_SPT_D1	UT5	MP1	26.4	12.1	-14.3
N153b219_UT5_SPT_D1	UT5	MP2	2.9	21.2	18.3
N153b219_UT5_SPT_D1	UT5	MP3	2.0	18.1	16.1
N153b219_UT5_SPT_D1	UT5	MP4	67.2	44.5	-22.7
N153b219_UT5_SPT_D1	UT5	MP5	1.5	4.0	2.6
N154a037rep_Cal33_SPT_D1	Cal33	MP1	2.6	2.2	-0.4
N154a037rep_Cal33_SPT_D1	Cal33	MP2	20.5	65.5	45.0
N154a037rep_Cal33_SPT_D1	Cal33	MP3	0.1	0.7	0.7
N154a037rep_Cal33_SPT_D1	Cal33	MP4	75.1	30.2	-44.9
N154a037rep_Cal33_SPT_D1	Cal33	MP5	1.8	1.5	-0.3
N154a038_Cal33_SPT_B1	Cal33	MP1	0.1	0.1	0.0
N154a038_Cal33_SPT_B1	Cal33	MP2	12.3	35.4	23.1
N154a038_Cal33_SPT_B1	Cal33	MP3	1.5	11.3	9.7
N154a038_Cal33_SPT_B1	Cal33	MP4	34.6	4.3	-30.2
N154a038_Cal33_SPT_B1	Cal33	MP5	51.5	48.9	-2.6
N154a073_Cal33_SPT_A1	Cal33	MP1	2.7	4.1	1.4
N154a073_Cal33_SPT_A1	Cal33	MP2	41.2	73.9	32.7
N154a073_Cal33_SPT_A1	Cal33	MP3	0.4	1.9	1.5
N154a073_Cal33_SPT_A1	Cal33	MP4	55.1	18.1	-37.0
N154a073_Cal33_SPT_A1	Cal33	MP5	0.7	2.0	1.4
N155a131_UT8_SPT_B1	UT8	MP1	10.6	3.1	-7.5
N155a131_UT8_SPT_B1	UT8	MP2	18.6	59.7	41.0
N155a131_UT8_SPT_B1	UT8	MP3	6.2	20.0	13.8
N155a131_UT8_SPT_B1	UT8	MP4	57.1	11.9	-45.2
N155a131_UT8_SPT_B1	UT8	MP5	7.4	5.3	-2.2
N155b199_UT8_SPT_C1	UT8	MP1	37.1	24.8	-12.3
N155b199_UT8_SPT_C1	UT8	MP2	0.2	1.4	1.2
N155b199_UT8_SPT_C1	UT8	MP4	62.8	73.5	10.8
N155b199_UT8_SPT_C1	UT8	MP5	0.0	0.3	0.3

N155b200_UT8_SPT_D1	UT8	MP1	5.9	4.5	-1.4
N155b200_UT8_SPT_D1	UT8	MP2	26.9	31.4	4.5
N155b200_UT8_SPT_D1	UT8	MP3	24.4	30.2	5.8
N155b200_UT8_SPT_D1	UT8	MP4	39.5	30.5	-9.0
N155b200_UT8_SPT_D1	UT8	MP5	3.4	3.4	0.0
N156a074_SAS_SPT_D1	SAS	MP1	51.2	39.6	-11.6
N156a074_SAS_SPT_D1	SAS	MP2	5.0	13.3	8.4
N156a074_SAS_SPT_D1	SAS	MP4	43.9	47.1	3.2
N156b181_SAS_SPT_B1	SAS	MP1	51.4	40.6	-10.7
N156b181_SAS_SPT_B1	SAS	MP2	2.3	14.6	12.3
N156b181_SAS_SPT_B1	SAS	MP4	46.3	44.7	-1.5
N165a002_SAT_SPT_C1	SAT	MP1	1.4	0.6	-0.8
N165a002_SAT_SPT_C1	SAT	MP2	1.7	4.4	2.7
N165a002_SAT_SPT_C1	SAT	MP3	14.7	49.2	34.5
N165a002_SAT_SPT_C1	SAT	MP4	36.6	11.4	-25.2
N165a002_SAT_SPT_C1	SAT	MP5	45.7	34.4	-11.2
N165a067rep_SAT_SPT_C1	SAT	MP1	0.5	0.3	-0.2
N165a067rep_SAT_SPT_C1	SAT	MP2	0.2	0.0	-0.2
N165a067rep_SAT_SPT_C1	SAT	MP3	23.2	50.9	27.6
N165a067rep_SAT_SPT_C1	SAT	MP4	14.1	2.4	-11.7
N165a067rep_SAT_SPT_C1	SAT	MP5	61.9	46.4	-15.6
N165b149rep_SAT_SPT_A1	SAT	MP2	1.6	1.9	0.3
N165b149rep_SAT_SPT_A1	SAT	MP3	11.8	56.7	45.0
N165b149rep_SAT_SPT_A1	SAT	MP4	35.3	6.2	-29.1
N165b149rep_SAT_SPT_A1	SAT	MP5	51.3	34.9	-16.4
N165b149rep_SAT_SPT_A1	SAT	MP1	0.0	0.3	0.3

Supplementary Table 6. Tissue-class microarchitecture summary. Pathologist-annotated H&E tissue classes with PIMO-positive fraction, spot count, and dominant/secondary MP across 16 annotated sections.

Tissue class	N spots	% PIMO ⁺	Dominant MP	Dom. %	Second MP	Sec. %
Bacteria	6	66.7	MP4	83.3	MP1	16.7
Keratinization	291	38.5	MP5	39.9	MP3	30.2
Ambiguous	2076	30.0	MP4	45.0	MP2	18.7
Epidermis	66	51.5	MP4	97.0	MP2	1.5
Blood vessels	20	25.0	MP4	75.0	MP2	15.0
Inflammatory infiltrate	627	24.2	MP4	75.8	MP2	10.4
Muscle	186	23.7	MP4	87.1	MP1	10.2
Necrosis	7607	25.4	MP2	32.4	MP4	27.1
Tumor	20376	16.6	MP4	54.8	MP1	16.4
Immune cells	14	14.3	MP4	100.0	NA	NA
Edema	160	8.8	MP4	83.1	MP5	11.2
Unannotated	846	8.5	MP4	62.8	MP1	13.7
Stroma	421	3.6	MP4	55.1	MP5	35.9

Supplementary Table 7. Consensus metaprogram signature genes. Genes defining each consensus MP, ordered by NMF loading rank (1 = highest). MP1, EMT/mesenchymal; MP2, hypoxia response; MP3, squamous differentiation; MP4, ECM/stromal-like; MP5, keratinocyte/basal.

Rank	MP1	MP2	MP3	MP4	MP5
1	VIM	DDIT4	ECM1	FSTL1	KRT4
2	AXL	ADM	CNFN	FBN1	KRTDAP
3	ANO1	SERPINB2	LCN2	TPM1	KRT13
4	F3	NDRG1	SPRR2A	TMSB4X	KRT10
5	EMP3	ANGPTL4	LCE3D	NFIX	SERPINB13
6	LAMC2	ERO1A	SPRR2D	IGFBP4	CRNN
7	FSTL1	IGFBP3	LCE3E	RNF130	S100A8

8	LAMB3	ERRF1	SPRR3	HSPA5	SPINK5
9	WNT7B	EGLN3	KRTDAP	EMP1	SPRR3
10	TPM1	FOS	SBSN	HNRNPF	DSG1
11	CAVIN1	SERPINE1	SPRR2G	CAVIN1	FLG
12	CAV2	PFKFB3	C15orf48	TPM2	PI3
13	FOSL1	ITGA5	CRCT1	RHOA	S100A9
14	COL6A2	PPP1R15A	CALML5	SERF2	LYPD2
15	HMGA2	SLC2A1	SPNS2	REEP5	GLTP
16	DCBLD2	BMP2	LMO7	HNRNPA3	SERPINB3
17	CORO6	MT1X	NDRG2	ASAP2	ERBB2
18	PHLDA1	TIMP3	ERO1A	ARPC5	SLURP2
19	IL24	HK2	FLG	PITPNA	CSTA
20	FST	CASP14	IVL	PPP2CB	CLIC3
21	ANXA2	VEGFA	HEPHL1	TNPO1	CYSRT1
22	PRSS22	ATF3	PI3	CXCL14	HOPX
23	TRIM47	GPRC5A	IL36G	CTDSP1	ERV3-1
24		MXD1	SLURP2	HIF1A	AQP3
25		PGF	S100A7	QKI	KRT80
26		HBEGF	CSTA	LASP1	KLK13
27		BHLHE40	TMPRSS11E	LUC7L2	IVL
28		BNIP3L	DSG1	RTN4	ZNF117
29			ADGRF1	MLF2	LGALSL
30			MXD1	ARIH1	SPINK7
31			SPINK7	UBE2B	GJB2
32			ELF3	TRIM8	ASPG
33			HOPX	ATP2A2	TMPRSS11D
34			KRT78	EFHD2	KLK10
35			TGM1	CAPZA2	SULT2B1
36			S100A9	BICD2	
37			SULT2B1		

Supplementary Table 8. Hallmark pathway over-representation for MP1

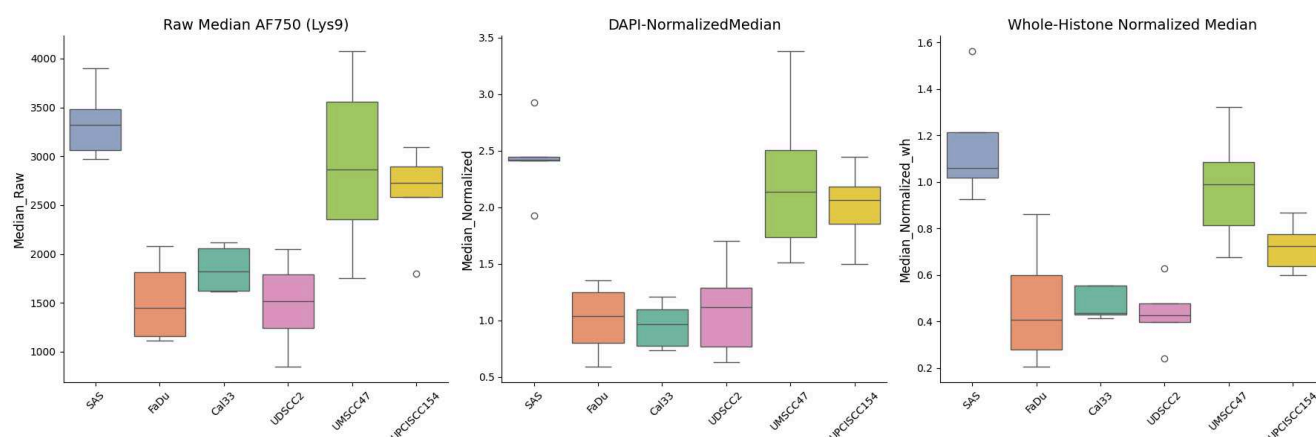
(EMT/mesenchymal). Over-representation analysis against MSigDB Hallmark gene sets.

Gene ratio, signature genes in set / signature size; Bg ratio, set size / background; Fold enr., fold enrichment; p.adj, Benjamini-Hochberg-adjusted p-value; Member genes, signature genes in the set.

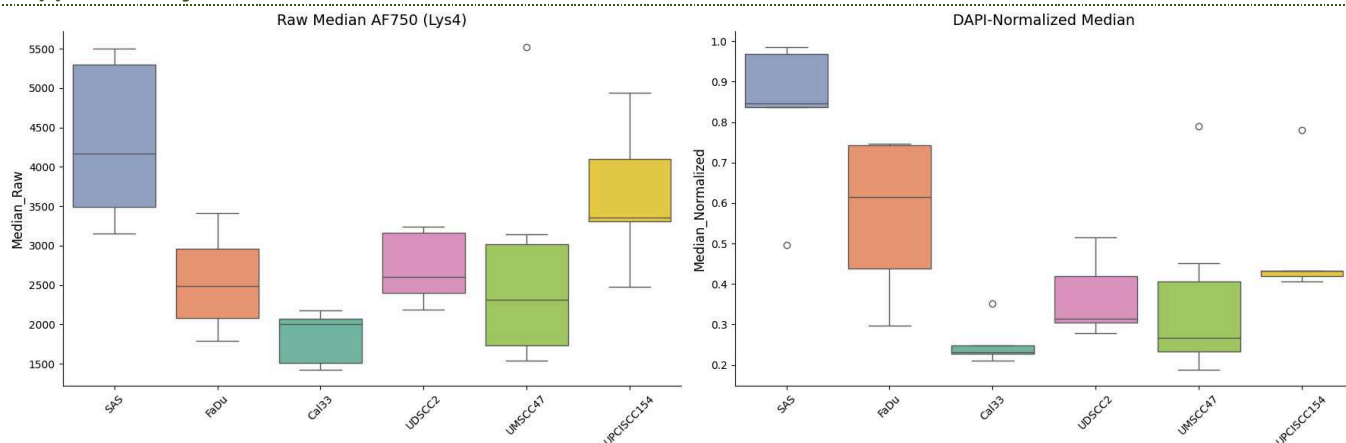
Hallmark gene set	Gene ratio	Bg ratio	Fold enr.	p-value	p.adj (BH)	Count	Member genes
Epithelial mesenchymal transition	6/17	200/4384	7.74	6.8e-05	1.1e-03	6	VIM / EMP3 / LAMC2 / FSTL1 / TPM1 / COL6A2
Inflammatory response	4/17	200/4384	5.16	6.3e-03	3.3e-02	4	AXL / F3 / EMP3 / DCBLD2
Tnfa signaling via nfkb	4/17	200/4384	5.16	6.3e-03	3.3e-02	4	F3 / LAMB3 / FOSL1 / PHLDA1
Hypoxia	3/17	200/4384	3.87	4.0e-02	1.6e-01	3	F3 / CAVIN1 / ANXA2
Uv response dn	2/17	144/4384	3.58	1.1e-01	2.9e-01	2	F3 / ANXA2
Angiogenesis	1/17	36/4384	7.16	1.3e-01	2.9e-01	1	FSTL1
Apical surface	1/17	44/4384	5.86	1.6e-01	2.9e-01	1	DCBLD2
Apical junction	2/17	200/4384	2.58	1.8e-01	2.9e-01	2	LAMC2 / LAMB3
Kras signaling up	2/17	200/4384	2.58	1.8e-01	2.9e-01	2	ANO1 / DCBLD2
Myogenesis	2/17	200/4384	2.58	1.8e-01	2.9e-01	2	COL6A2 / FST
Protein secretion	1/17	96/4384	2.69	3.1e-01	4.6e-01	1	CAV2
Coagulation	1/17	138/4384	1.87	4.2e-01	5.5e-01	1	F3
Il2 stat5 signaling	1/17	199/4384	1.30	5.5e-01	5.5e-01	1	PHLDA1
Adipogenesis	1/17	200/4384	1.29	5.5e-01	5.5e-01	1	CAVIN1
Complement	1/17	200/4384	1.29	5.5e-01	5.5e-01	1	F3
Estrogen response late	1/17	200/4384	1.29	5.5e-01	5.5e-01	1	LAMC2

Supplementary figures 1-6 is done by Cylia Ouadah. This part comprises the IF analysis comparing the HPV-positive and HPV-negative cell lines. Supplementary figures 1-3 show boxplots of the median values per sample for: (a) raw mean intensity per cell, (b) DAPI-normalized integrated intensity, and (c) whole-histone normalized integrated intensity for H3K9me3. The results show no evidence of differences in histone modification levels, when comparing HPV-negative cell lines to HPV-positive cell lines.

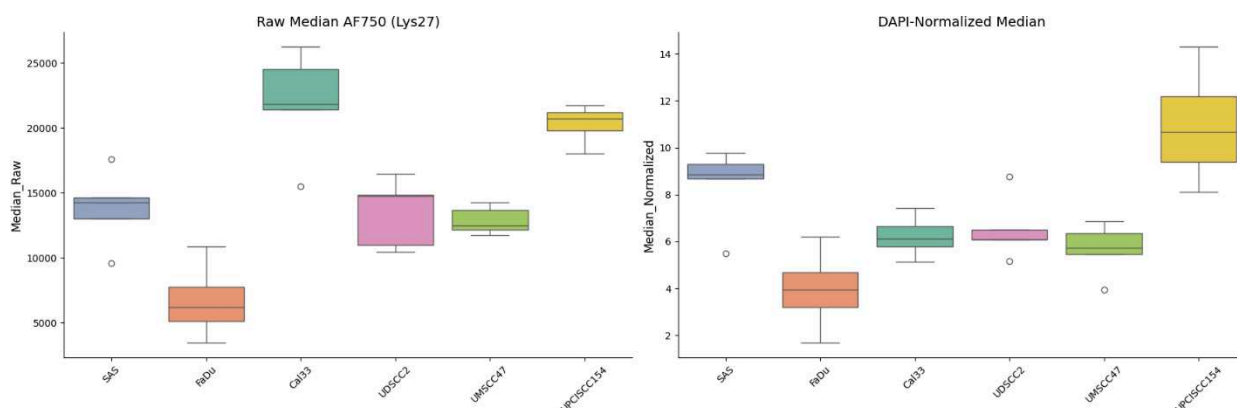
Supplementary figures 4-6 show violin plots of the distribution of (a), (b) and (c), without calculating a median per sample, grouped by HPV-negative and HPV-positive groups. Similarly, no conclusions can be drawn on the histone modification levels between the two groups.



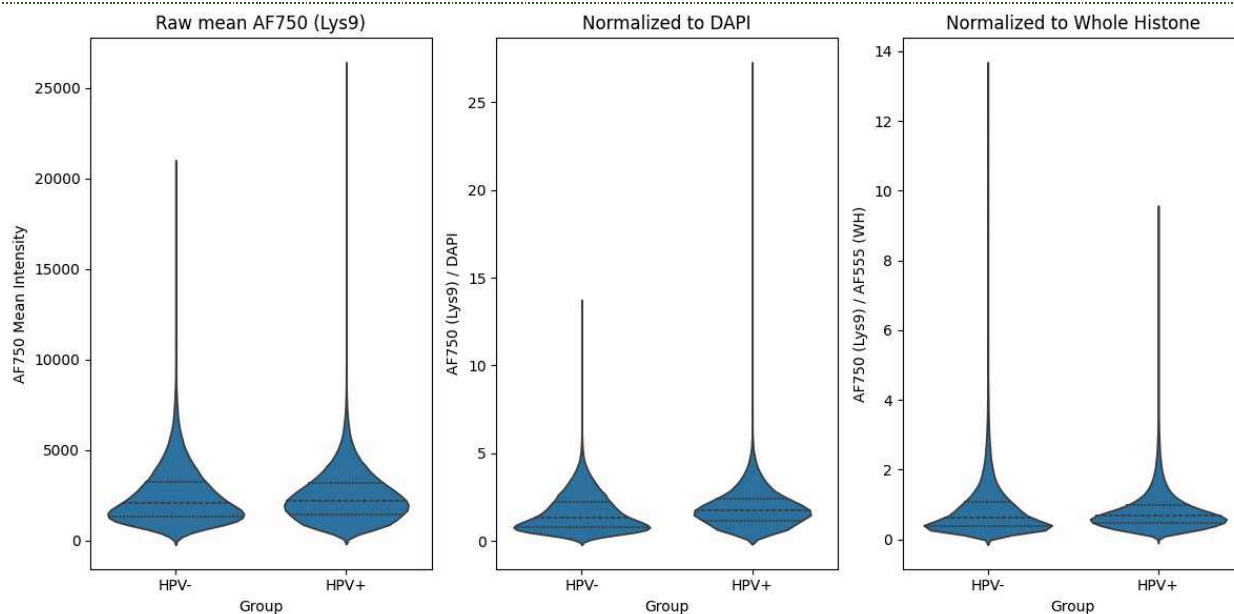
Supplementary Figure 1: Boxplot of the median integrated intensity per sample, grouped by cell line, for H3K9me3 – From left to right: medians of (i) raw mean nuclei intensity, (ii) integrated intensity normalized by DAPI integrated intensity, (iii) integrated intensity normalized by whole histone integrated intensity



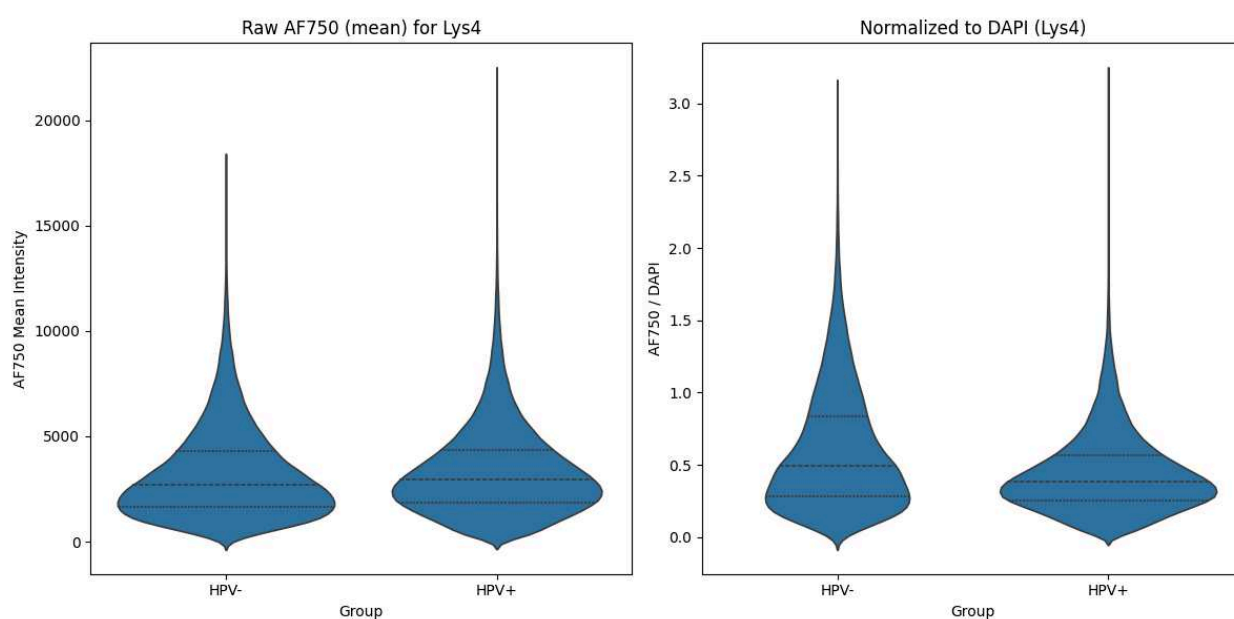
Supplementary Figure 2: Boxplot of the median integrated intensity per sample, grouped by cell line, for H3K4me3 – From left to right: medians of (i) raw mean nuclei intensity, (ii) integrated intensity normalized by DAPI integrated intensity



Supplementary Figure 3: Boxplot of the median integrated intensity per sample, grouped by cell line, for H3K27me3 – From left to right: medians of (i) raw mean nuclei intensity, (ii) integrated intensity normalized by DAPI integrated intensity

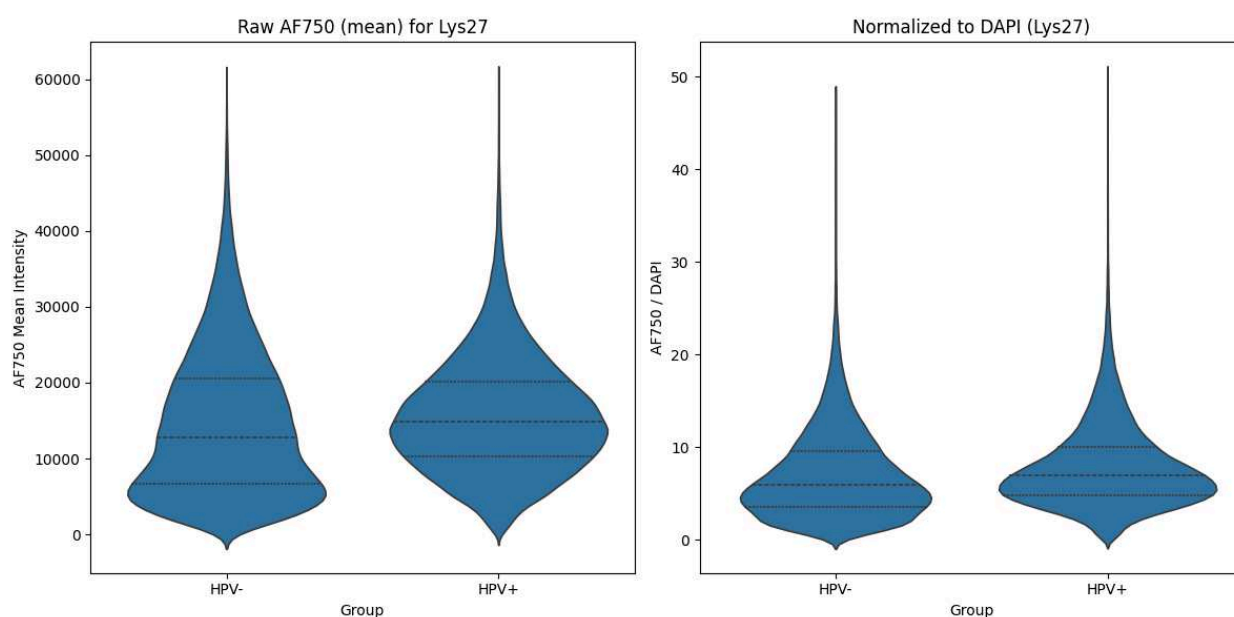


Supplementary Figure 4: Violin plot for all analyzed cells for H3K9me3, grouped by HPV status. From left to right: distributions of (i) raw mean intensity, (ii) integrated intensity normalized by DAPI integrated intensity, (iii) integrated intensity normalized by whole histone integrated intensity

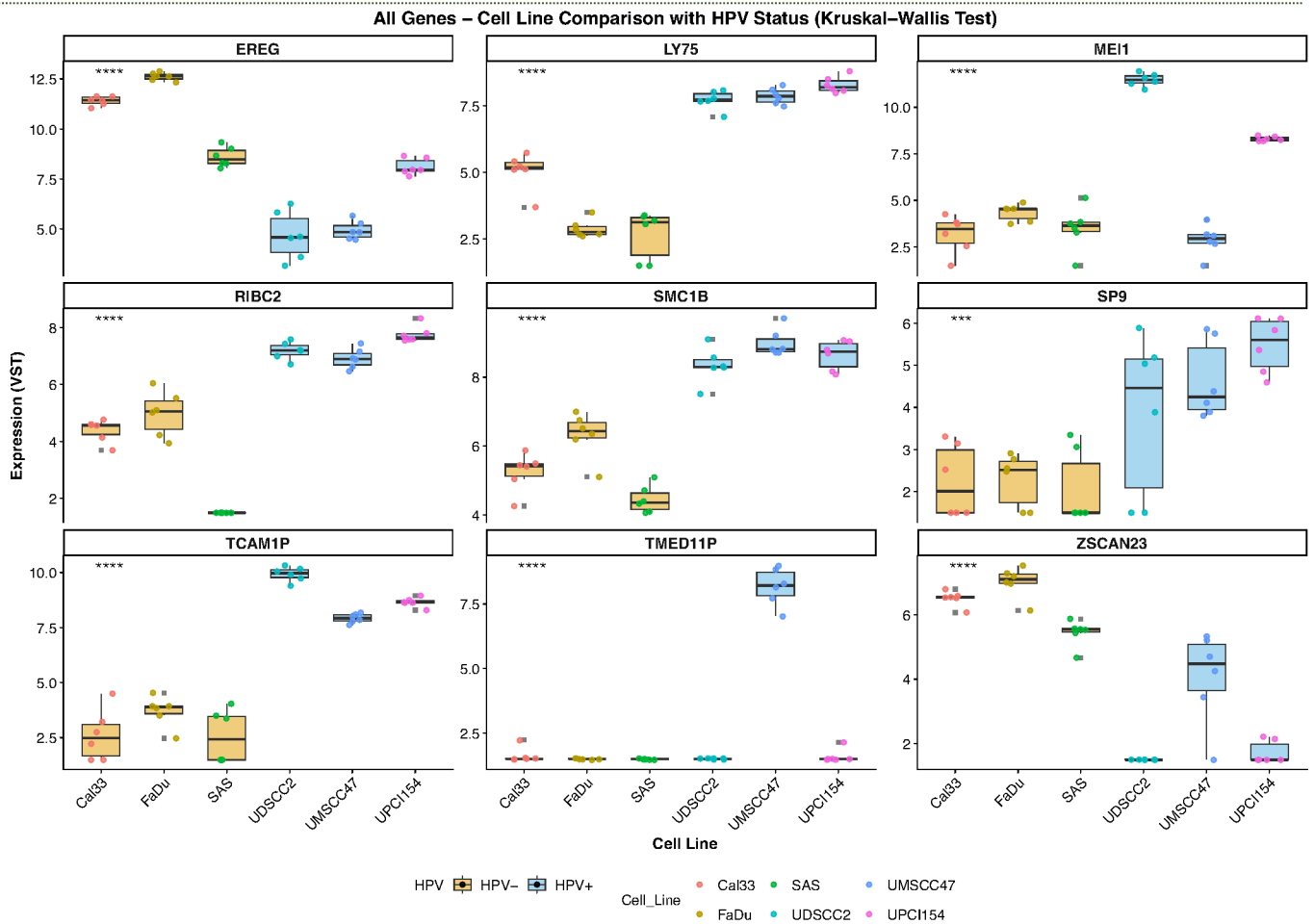


Supplementary Figure 5: Violin plot for all analyzed cells or H3K4me3, grouped by HPV status.

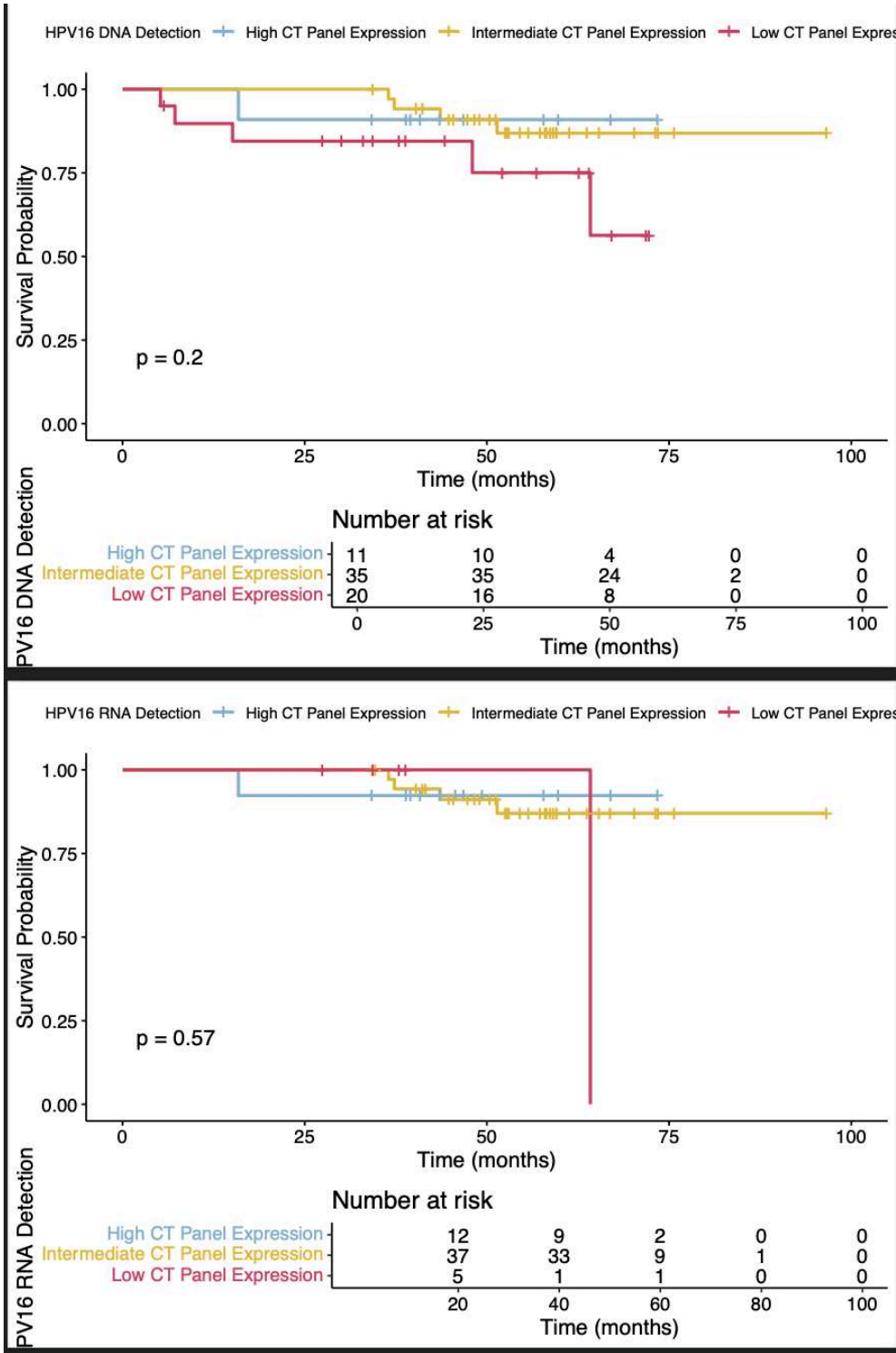
From left to right: distributions of (i) raw mean intensity, (ii) integrated intensity normalized by DAPI integrated intensity



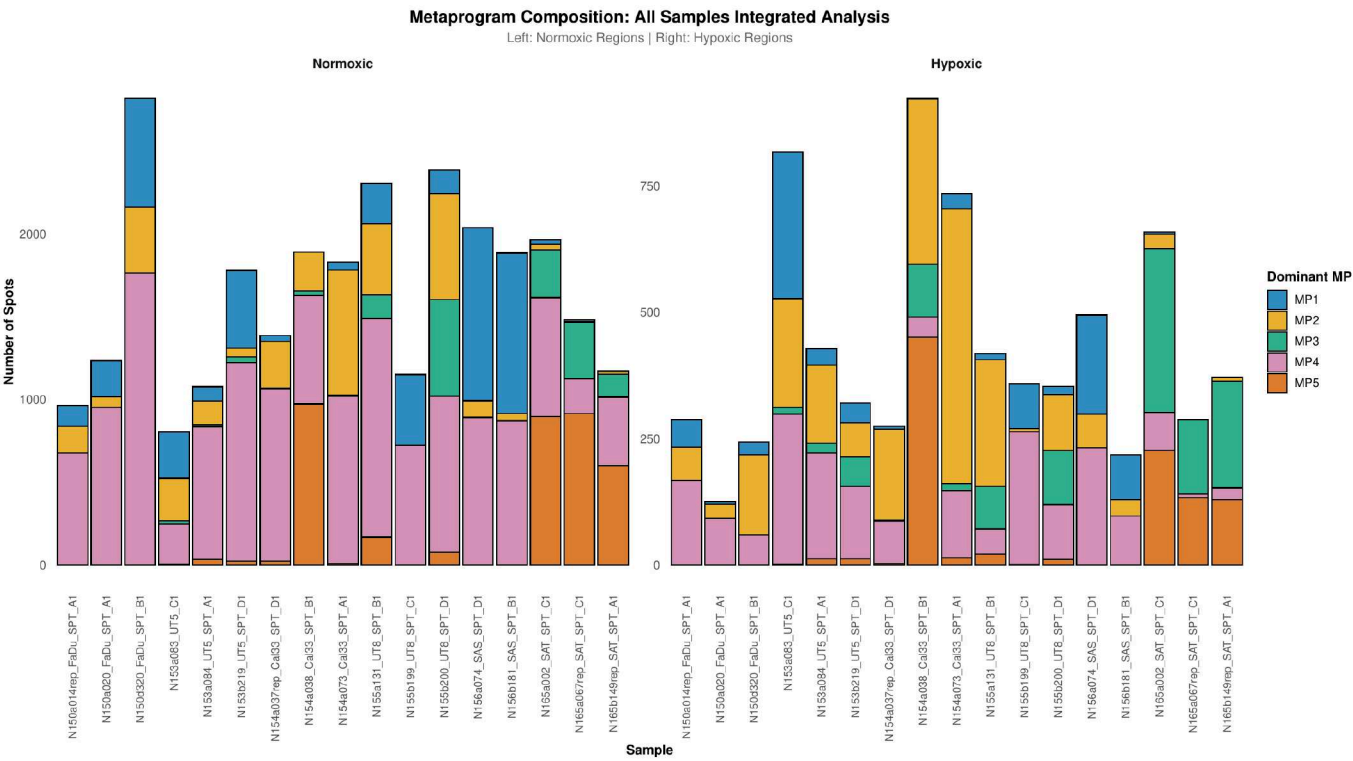
Supplementary Figure 6: Violin plot for all analyzed cells for H3K27me3, grouped by HPV status. From left to right: distributions of (i) raw mean intensity, (ii) integrated intensity normalized by DAPI integrated intensity



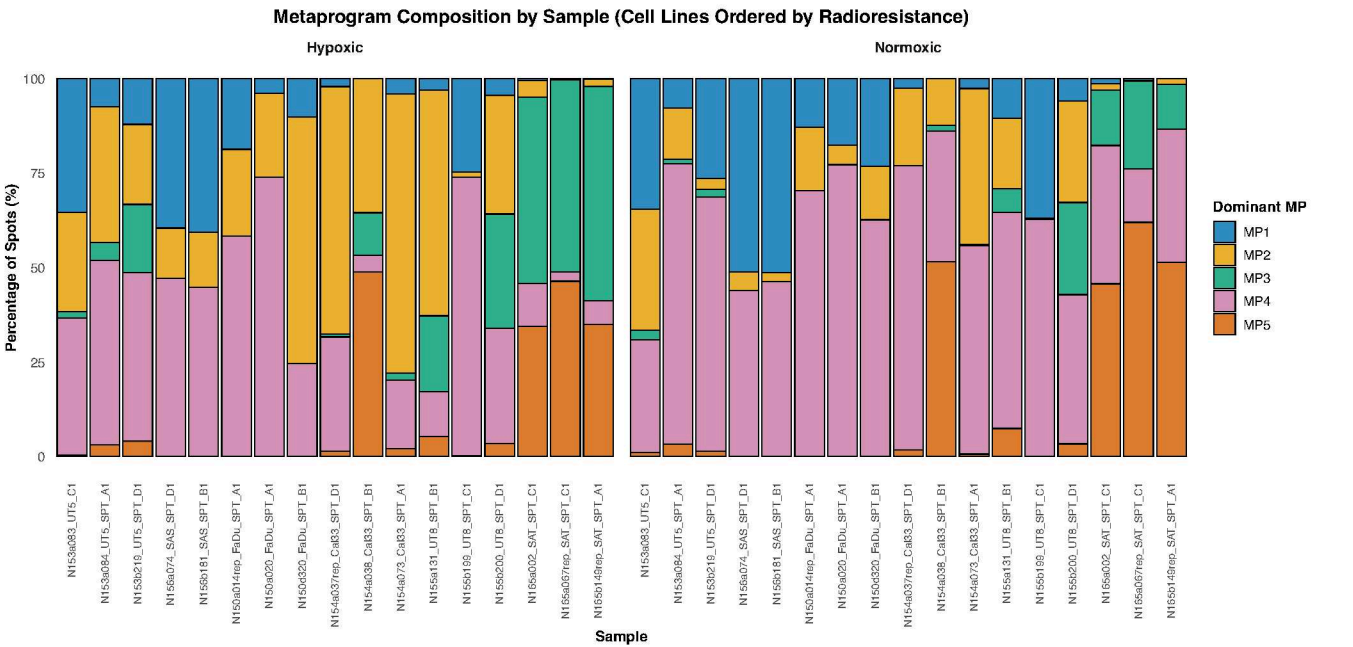
Supplementary Figure 7. *Expression of selected genes across HPV-negative and HPV-positive HNSCC cell lines. Boxplots showing variance-stabilized transformed (VST) expression levels of selected genes across HPV-negative cell lines CAL33, FaDu, and SAS and HPV-positive cell lines UDSCC2, UMSCC47, and UPC1154. Each panel represents one gene, with individual data points overlaid on the boxplots. Differences in expression across cell lines were assessed using the Kruskal-Wallis test, with significance levels indicated by asterisks.*



Supplementary Figure 8. Kaplan-Meier overall survival by CT panel expression within HPV detection-method subgroups. Overall survival of DTK-ROG patients stratified into high, intermediate and low CT panel expression groups, shown separately for HPV16 DNA-positive (top) and HPV16 RNA-positive (bottom) tumours, with number-at-risk tables below each plot; p-values from log-rank tests.

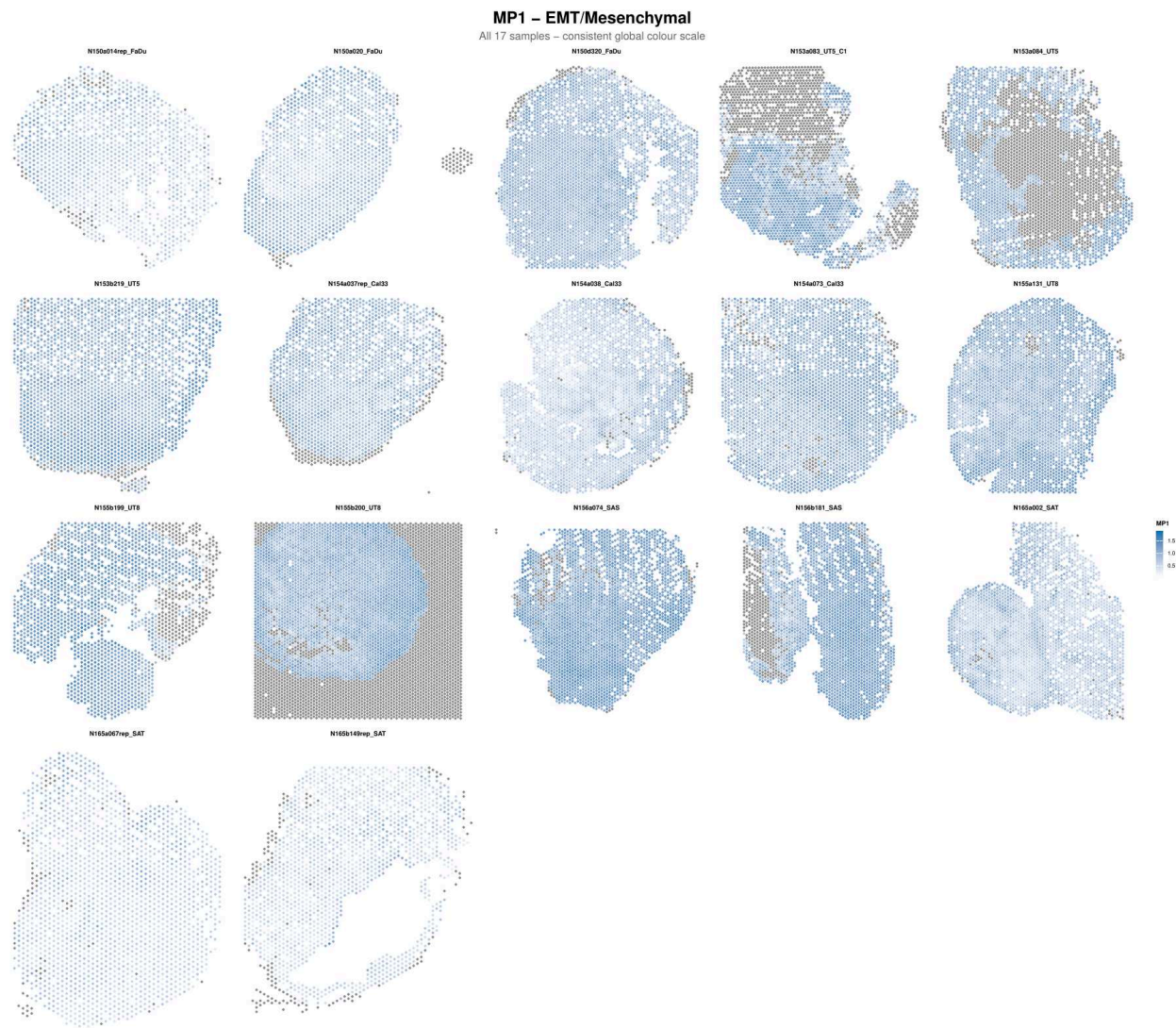


Supplementary Figure 9. Dominant-metaprogram composition in normoxic versus hypoxic regions across all sections. Stacked-bar counts of spots assigned to each dominant MP (MP1-MP5), shown separately for normoxic (left) and hypoxic (right) regions of every section.



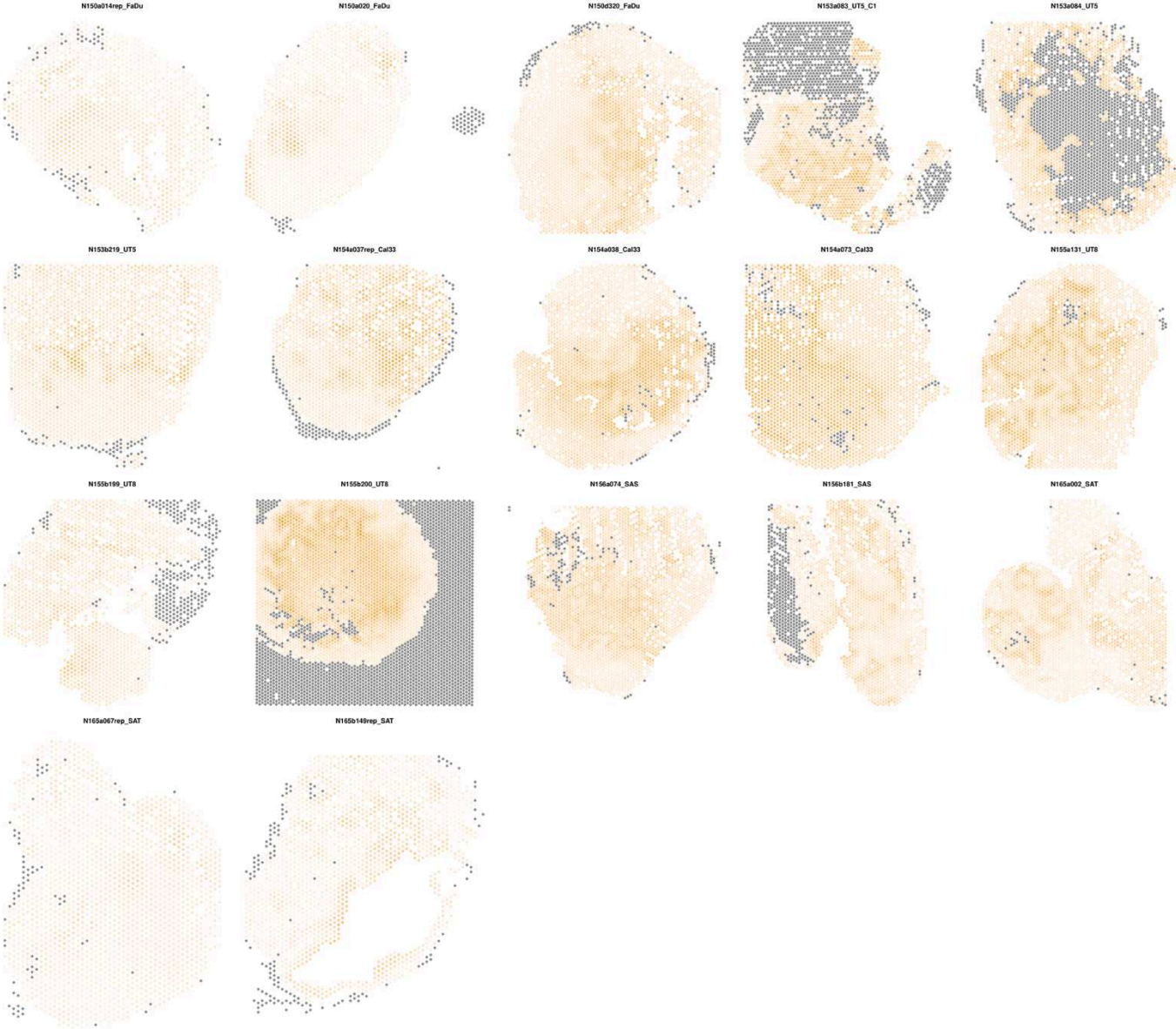
Supplementary Figure 10. Metaprogram composition spots ordered by radioresistance with PIMO annotation. A comparison figure is shown for Normoxic vs Hypoxic areas.

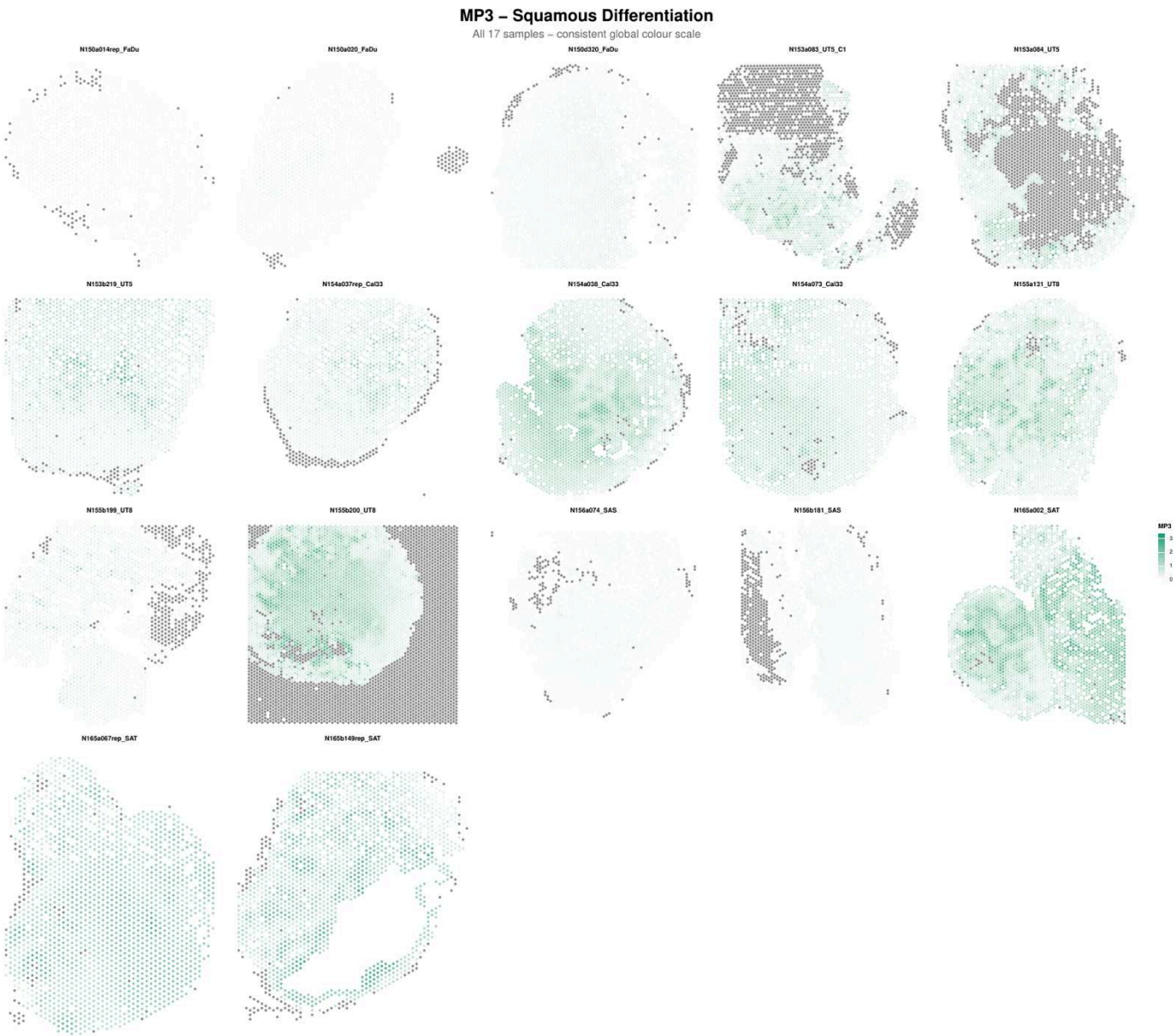
Supplementary Figure 11. MP scoring visual representation for all 17 samples (Global Scoring).



MP2 – Hypoxia Response

All 17 samples – consistent global colour scale





MP4 – ECM/Stromal
All 17 samples – consistent global colour scale

