Studies on the heterogeneity and function of molecules critical in colorectal cancer progression

Ph.D. Thesis

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1. Introduction

Colorectal cancer (CRC) is a biologically diverse disease driven by complex interactions among epithelial tumor cells, stromal components, and a dynamic microenvironment. Although multiple oncogenic pathways contribute to CRC initiation and progression, recent studies emphasize the significance of molecular heterogeneity and tumor-microenvironmental crosstalk in shaping clinical outcomes. One form of this heterogeneity is the presence of different cell clones within the tumor, such as cancer stem cells characterized by CD44, CD133 and PTK7 expression, that are critical in tumor growth.

One of the most widely used frameworks for capturing this diversity is the Consensus Molecular Subtypes (CMS) classification. CMS2 represents the classical epithelial, WNT/MYC-driven subtype, while CMS4 defines a mesenchymal, stromal-rich phenotype characterized by TGF-β activation, immune exclusion, fibroblast accumulation, extracellular matrix (ECM) remodeling, and aggressive clinical behavior.

The tumor microenvironment (TME) plays a key role in this transition toward invasive behavior. ECM components, especially collagen I, along with cancer-associated fibroblasts

(CAFs), inflammatory cytokines, and nutrient gradients, create selective pressures that influence tumor plasticity. CAFs secrete growth factors that reinforce EMT programs, promote angiogenesis, and modify metabolic dependencies, collectively driving the hallmarks of CMS4 tumors.

Within this microenvironmental framework, serotonin (5-HT) signaling has emerged as an important regulator of CRC biology. Among its receptors, HTR2B is notably relevant: it is significantly upregulated in mesenchymal, CMS4-like tumors and activates pathways including MAPK and mTOR. Serotonin-HTR2B signaling promotes invasive behavior in CRC and influences macrophage polarization toward pro-tumorigenic M2 state. Alongside serotonin-mediated regulation, the NOTCH signaling pathway, particularly NOTCH3, supports the maintenance of undifferentiated cell states, EMT induction, and tumor-stroma communication. NOTCH3 is more prevalent in advanced CRC and linked to poor prognosis, overlapping strongly with CMS4 phenotypes.

Together, these elements—epithelial heterogeneity, stromal interactions, and context-dependent signaling dynamics—compose a coordinated network that influences CRC behavior. Understanding how markers linked to the aggressive behaviour of CRC and the cancer stem cell identity (e.g. CD44, CD133,

PTK7), and HTR2B, NOTCH3 contribute to this heterogeneity and invasiveness is essential for identifying clinically relevant biomarkers and discovering new therapeutic targets.

2. Objectives

The objectives of this thesis are to:

1) Characterize the expression patterns of cancer stem cell markers (CD44, CD133, PTK7) and CMS-related markers (CDX2 for CMS2/3; HTR2B, FRMD6, ZEB1 for CMS4) in patient-derived CRC organoids (PDOs).
2) Determine the extent of co-expression and spatial overlap between CMS2/3 and CMS4 markers within individual CRC organoids, thereby assessing intra-tumoral heterogeneity at the molecular level.
3) Investigate how microenvironmental conditions, including fibroblast co-culture and ECM composition, influence tumor cell phenotype, heterogeneity, and expression of HTR2B.
4) Assess the functional role of HTR2B in CRC proliferation, survival, metabolic adaptation, and invasiveness under physiologically relevant and stress-inducing conditions.

5) Explore whether HTR2B and NOTCH signaling pathways interact, and evaluate their combined influence on tumor growth, survival, and invasion.

3. Methods

3.1 The Organoid model

CRC patient-derived organoids served as the primary experimental model to preserve tumor-intrinsic heterogeneity. Surgical biopsies were enzymatically and mechanically dissociated, and epithelial cells were embedded in growth factor-reduced Matrigel or collagen-I hydrogels for three-dimensional culture. Organoids were propagated in a defined medium enriched with EGF, TGF-ß, and p38 inhibitors, ROCK inhibitor, antioxidants, and essential nutrients. For experiments requiring ECM-specific responses, organoids were maintained in collagen-I matrices, allowing for the assessment of invasive behavior.

3.2 Fibroblast coculturing

Fibroblast co-cultures were established using CCD-18Co colon fibroblasts, mixed with PDO cells at defined ratios prior to embedding in Matrigel. Parallel monocultures served as controls.

3.3 Flow Cytometric methods

Flow cytometry and fluorescence-activated cell sorting (FACS) were used to quantify marker expression and isolate specific epithelial subpopulations. Cells were dissociated using collagenase II or TrypLE, stained with antibodies targeting surface and intracellular markers, and analyzed using a Cytoflex cytometer. Selected subpopulations were sorted for downstream culturing or RNA analysis.

3.4 Immunocyto/histochemistry

Immunocytochemistry and immunohistochemistry employed confocal microscopy to assess spatial distribution of markers within organoid structures. Organoids were stained for stem cell markers, CMS-associated markers, proliferation indicators (Ki67), and EMT/stromal markers such as vimentin and ZEB1.

3.5 Gene expression analysis

Gene expression analysis was performed using qRT-PCR. RNA was isolated from whole organoids or sorted subpopulations, reverse transcribed, and analyzed using SYBR-Green chemistry. Relative expression was calculated via the ΔCt method normalized to housekeeping genes.

3.6 Functional assays

Functinoal assays included serotonin ELISA to quantify 5-HT production, and CellTiter-Glo 3D viability assays to assess metabolic activity under varying nutrient conditions (glucose-free, amino acid-free). Organoids were treated with serotonin, HTR2B agonists, antagonists (RS-127445, SB-204741), NOTCH inhibitors (DAPT, DBZ), or chemotherapeutic agents (5-FU). Experiments were conducted both in Matrigel and collagen to evaluate microenvironment-dependent responses.

Combined, these approaches provided a multidimensional assessment of tumor heterogeneity, signaling dynamics, and microenvironmental interactions.

3.7 Statistics

Statistical analyses were performed using SPSS 29.0.1.0, GraphPad, and Microsoft Excel. Survival analyses were conducted using Kaplan-Meier curves with log-rank tests, with expression values z-score transformed prior to evaluation. Experimental data were evaluated using paired or unpaired Student's t-tests, or one-way ANOVA with Tukey post hoc correction. Unless indicated otherwise, results represent three or four PDO lines.

Results

4.1 Heterogeneity of CRC stem cell markers

Expression of the cancer stem cell markers CD44, CD133, and PTK7 varied substantially both across patient-derived organoid (PDO) lines and within individual organoids, underscoring the pronounced phenotypic heterogeneity characteristic of colorectal cancer. Confocal imaging demonstrated PTK7 and CD133 were frequently enriched in the apical surface, while CD44 had more variable distribution. Additionally, the expression of CD44, CD133, and PTK7 was heterogenous in the same and surrounding organoids from the same patient.

4.2 Stem cell markers and proliferation

Cell sorting, followed up by flow cytometric confirmation, demonstrated that CD44, CD133, and PTK7 can be separated into distinct high and low expressing populations. After a week of culturing, in high expressing populations for CD44 and CD133 the number of organoids, size of organoids, and their proliferative potential (KI67 positivity) was higher compared to organoids derived from low expressing populations. PTK7 expression levels on the other hand did not impact these qualities.

4.3 CMS heterogeneity in PDOs

Higher expression of CMS2/3 markers (CDX2) predicted better patient survival, while higher expression of CMS4

markers (FRMD6, HTR2B, and ZEB1) predicted significantly worse patient survival. Analysis of CMS-associated markers revealed that organoids commonly contain epithelial clusters with features of both CMS2/3 and CMS4 subtypes. CDX2, representing epithelial differentiation and CMS2/3 characteristics, displayed heterogenous expression patterns in PDOs. In contrast, FRMD6 and ZEB1 had low to absent positivity, while HTR2B appeared with notable heterogeneity in PDOs.

Confocal imaging confirmed the coexistence of CMS2/3-like and CMS4-like populations within single PDO lines. Double-positive cells (CDX2+/HTR2B+) were readily observed, demonstrating that CMS states do not represent mutually exclusive categories but instead reflect plastic transcriptional programs that shift in response to local signals. This intra-organoid CMS heterogeneity provides an explanatory framework for the diverse behavior of CRC even within a single tumor.

4.4 Serotonin metabolism in CRC

Expression of serotonin transport and metabolism components (SERT, TPH1, MAO-A) was detectable across PDO lines, indicating active serotonergic processing. Interestingly,

although PDOs expressed these metabolic enzymes, ELISA measurements revealed no significant serotonin production by epithelial tumor cells or fibroblasts under baseline conditions. Instead, the data suggest that CRC cells prioritize serotonin uptake and breakdown over synthesis. This metabolic orientation has functional relevance: increasing extracellular serotonin availability elevated HTR2B-mediated responses, demonstrating a dependency on exogenous 5-HT.

4.5 HTR2B survival signaling under metabolic stress

To examine the physiological relevance of HTR2B signaling, PDOs were subjected to metabolic stress through glucose- or amino acid-free media or treatment with 5-FU. In states of nutrient deprivation, mTORC1 activation is critical for CRC survival. Concomitantly, HTR2B expression was upregulated in media deprived of glucose or amino acids or treated with 5-FU, demonstrating that metabolic stress induces this receptor. Rapamycin treatment decreased HTR2B protein and RNA expression levels, demonstrating that HTR2B expression is regulated by the mTORC1 axis. Unexpectedly, serotonin and HTR2B agonists sensitized organoids to stress-induced cell death, contrasting with the classical survival-promoting roles described in other cancers. This effect was abolished by HTR2B agonists, confirming the receptor's direct involvement.

4.6 Fibroblast-mediated ECM remodeling and HTR2B regulation

Co-culturing PDOs with fibroblasts induced profound morphological and transcriptional changes consistent with CMS4-like reprogramming. Organoids developed elongated, branching structures and showed enhanced ECM remodeling, accompanied by increased fibrillar collagen deposition. Fibroblasts promoted increased expression of HTR2B and the mesenchymal marker lumican, and decreased expression of the epithelial marker EpCAM, thus corresponding to a partial EMT state (pEMT). This demonstrates that stromal cues can shift epithelial cells toward mesenchymal and invasive states.

These findings highlight a strong fibroblast/collagen-HTR2B axis in CRC. The amplification of HTR2B expression in the presence of fibroblasts suggests that stromal signals and mechanical cues potentiate serotonin responsiveness and integrate into the broader EMT network. This may explain the enrichment of HTR2B in CMS4 tumors, which are characterized by stromal activation and immune exclusion.

4.7 HTR2B and invasion in permissive microenvironments

When PDOS were embedded in collagen I matrices that mimic the stromal architecture of invasive CRC, they exhibited

extensive protrusive growth and collective invasion. Serotonin or HTR2B agonists further enhanced invasion depth and branching complexity.

This demonstrates that HTR2B's invasive role is highly dependent on the ECM context. In collagen and with HTR2B agonist or serotonin treatment, amongst those PDOs from HTR2B-high sorted CRC cells, invasion and organoid area were substantially higher. Thus, HTR2B acts as a microenvironment-responsive regulator of invasion, linking serotonergic input with ECM remodeling and mesenchymal transition.

These findings reveal a dual, context-dependent role for HTR2B: while it can promote invasive behavior and stromal responsiveness, its activation under extreme metabolic deprivation may instead render cells more vulnerable. This duality may reflect the divergent signaling outputs of HTR2B in epithelial versus stromal-dominated environments. These results highlight the importance of evaluating HTR2B's function in microenvironmentally relevant contexts.

4.8 Interaction between HTR2B and NOTCH3

NOTCH3, like HTR2B is most highly expressed in CMS4 CRC tumors and similarly is correlated with poorer patient survival

outcomes. Contrary to HTR2B, collagen enrichment did not modify NOTCH3 expression. Furthermore, NOTCH ligands were presented by both PDOs and cancer-associated fibroblasts (CAFs), however, with significantly different expression patterns. NOTCH3 expression displayed considerable overlap with HTR2B-high cell populations. Inhibition of NOTCH signaling (via DAPT or DBZ) reduced HTR2B-driven invasion. These results suggest that HTR2B-induced invasiveness requires NOTCH pathway activity and that the two pathways cooperate to sustain mesenchymal behavior.

4. Conclusions

This thesis demonstrates that patient-derived colorectal cancer organoids exhibit substantial intra-tumoral heterogeneity across stemness, CMS-related, and invasive phenotypes. Cancer stem cell markers CD44, CD133, PTK7 are broadly distributed and dynamically regulated, underscoring the functional plasticity of CRC cells. The coexistence of CMS2/3-like and CMS4-like cells within the same organoid underscores the plasticity of CRC and challenges the notion that CMS classifications represent stable, uniform tumor subtypes. Instead, CMS states emerge dynamically from local environmental pressures, stromal interactions, and metabolic conditions.

A central insight of this work is the discovery that HTR2B exerts a distinctly dual, microenvironment-dependent role in CRC. Under metabolic stress, HTR2B expression was strongly induced, and this upregulation was dependent on mTORC1 activity. Unexpectedly, activation of HTR2B through serotonin or agonists sensitized organoids to cell death, revealing a vulnerability rather than a survival advantage under metabolic stress. In contrast, when PDOs were placed in collagen-rich environments, HTR2B acted as a potent enhancer of invasion, particularly in HTR2B-high sorted populations. In these ECM-permissive conditions, serotonergic activation amplified mesenchymal transition and stromal responsiveness. Taken together, these findings demonstrate that HTR2B integrates metabolic cues and extracellular matrix signals to drive starkly different outcomes, underscoring the receptor's context-specific function and the importance of evaluating its biology within physiologically relevant microenvironments.

Importantly, this study uncovers a link between HTR2B and NOTCH3 in orchestrating invasive behavior. Their overlapping expression patterns and functional interdependence suggest that serotonin and NOTCH signaling form a coordinated axis that governs invasion and adaptation to stromal cues. This provides a mechanistic explanation for their shared association

with poor prognosis and highlights their potential as combinatorial therapeutic targets.

Collectively, this work identifies HTR2B as a key microenvironment-responsive regulator of CRC progression, connecting serotonin signaling with EMT, cellular stress, stromal remodeling, and NOTCH signaling. These findings support HTR2B as a promising biomarker of aggressive CRC states and a potential therapeutic target, especially in tumors exhibiting CMS4-like features.

5. Bibliography of the candidate's publications:

Publications used for the dissertation:

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