

Spatial transcriptomics profiling of gallbladder adenocarcinoma: a detailed two-case study of progression from precursor lesions to cancer

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Abstract

Background. Most studies on tumour progression from precursor lesion toward gallbladder adenocarcinoma investigate lesions sampled from distinct patients, providing an overarching view of pathogenic cascades. Whether this reflects the tumourigenic process in individual patients remains insufficiently explored. Genomic and epigenomic studies suggest that a subset of gallbladder cancers originate from biliary intraepithelial neoplasia (BillIN) precursor lesions, whereas others form independently from BillINs. Spatial transcriptomic data supporting these conclusions are missing. Moreover, multiple areas with precursor or adenocarcinoma lesions can be detected within the same pathological sample. Yet, knowledge about intra-patient variability of such lesions is lacking.

Methods. To characterise the spatial transcriptomics of gallbladder cancer tumourigenesis in individual patients, we selected two patients with distinct cancer aetiology and whose samples simultaneously displayed multiple areas of normal epithelium, BillINs and adenocarcinoma. Using GeoMx digital spatial profiling, we characterised the whole transcriptome of a high number of regions of interest (ROIs) per sample in the two patients (24 and 32 ROIs respectively), with each ROI covering approximately 200 cells of normal epithelium, low-grade BillIN, high-grade BillIN or adenocarcinoma. Human gallbladder organoids and cell line-derived tumours were used to investigate the tumour-promoting role of genes.

Results. Spatial transcriptomics revealed that each type of lesion displayed limited intra-patient transcriptomic variability. Our data further suggest that adenocarcinoma derived from high-grade BillIN in one patient and from low-grade BillIN in the other patient, with co-existing high-grade BillIN evolving via a distinct process in the latter case. The two patients displayed distinct sequences of signalling pathway activation

during tumour progression, but Semaphorin 4A (*SEMA4A*) expression was repressed in both patients. Using human gallbladder-derived organoids and cell line-derived tumours, we provide evidence that repression of *SEMA4A* promotes pseudostratification of the epithelium and enhances cell migration and survival.

Conclusion. Gallbladder adenocarcinoma can develop according to patient-specific processes, and limited intra-patient variability of precursor and cancer lesions was noticed. Our data suggest that repression of *SEMA4A* can promote tumour progression. They also highlight the need to gain gene expression data in addition to histological information to avoid underestimating the risk of low-grade preneoplastic lesions.

Keywords: biliary intraepithelial neoplasia; gallbladder cancer; semaphorin4A; spatial transcriptomics; tumour progression.

BACKGROUND

Gallbladder cancer accounts for less than 2% of cancer-related deaths and is often fortuitously diagnosed in gallbladder samples following cholecystectomy. The prognosis of the disease remains poor because patients often present at an advanced stage with unresectable tumour. Late diagnosis results from the lack of specific symptoms and of screening strategies, as well as from limited knowledge of the mechanisms driving tumour progression [1, 2]. Several studies investigated the pathology, genomics and epigenomics of tumour progression from precursor to cancer stage. They mostly investigated precursor and cancer lesions from distinct patients,

precluding a good understanding of tumour progression at the individual level. Spatial transcriptomic data on precursor and adenocarcinoma lesions coexisting in a same patient are expected to provide clues on the mechanisms of tumour progression.

Adenocarcinomas account for >90% of gallbladder cancers and are considered to develop according to a metaplasia-dysplasia-adenocarcinoma histogenic sequence, in which the dysplastic stage consists of low-grade and high-grade biliary intraepithelial neoplasia (BillN) [3-8]. BillNs consist of microscopic, flat or micropapillary lesions whose grade depends on the highest degree of cytological and architectural atypia. Low-grade BillNs display moderate cytoarchitectural atypia with pseudostratification of the nuclei, increased nucleo-cytoplasmic ratio and hyperchromasia. High-grade BillNs, formerly called carcinomas *in situ*, are defined by loss of nuclear polarity, marked cytological atypia and complex architectural patterns such as micropapillae [9-11].

Genomic alterations are already found at the BillN stage. *KRAS* and *TP53* mutations were found in BillNs [12, 13] and a progressive increase in TP53 overexpression was proposed to occur during the evolution from low-grade BillN to GBC [14]. A recent exome sequencing study uncovered *CTNNB1*, *TP53*, *ARID2* and *ERBB3* as the most frequently mutated genes in low-grade and high-grade BillNs [15]. When the disease evolves to invasive adenocarcinoma, alterations accumulate, and tumours display significant cell type heterogeneity [16, 17]. At that stage the most frequent mutations affect *KRAS*, *CTNNB1*, *TP53*, *PI3KCA*, *ERBB2*, *CDKN2A* and *CDKN2B* [18-26], indicating that a fraction of the mutations found at the cancer stage can be detected in BillN lesions. At the epigenome level, cancer lesions were split in subtypes with distinct hypermethylation:hypomethylation ratios; progressive and cumulative changes in

promoter methylation were detected during progression from cholecystitis to cancer [26-29]. Increased hypermethylation was observed in adenocarcinomas as compared to BillNs. These epigenomic changes impacted Wnt/ β -catenin signalling, Hedgehog signalling, tumour suppression and cell-microenvironment interactions [30-32]. Further, since gallstone-induced chronic inflammation drives gallbladder carcinogenesis [33], several authors compared the transcriptome of normal gallbladder tissue, gallbladder cancer, and gallbladder tissue exposed for varying lengths of time to gallstones, and identified molecular signatures associated with disease progression [34, 35]. Finally, in line with the genomic and epigenomic studies, single gene analyses revealed aberrant expression levels of TP53, P21, cyclin D1, EZH2, SMAD4 and CDKN2A protein at the BillN stage [11], as well as the ability of a combined activation of KRAS and canonical Wnt/ β -catenin or Notch signalling to induce gallbladder BillNs with malignant potential [36, 37]. Spatial transcriptomic data investigating BillN to adenocarcinoma progression are still lacking.

Considering the genomics of tumour progression, Lin and coworkers provided evidence for patient-specific tumourigenic processes [15]. Their results indicated that precursor and cancer lesions within the same patient bear similar mutations, whereas the mutational signatures significantly vary between patients. Phylogenetic analysis of single nucleotide variants in lesions generated revealed that gallbladder cancer developed either BillN-dependently or BillN-independently [15].

To address the spatial transcriptomics of gallbladder tumour progression in individual patients, we selected samples from two patients displaying simultaneously several areas of gallbladder BillNs and adenocarcinoma, and collected an extensive spatial

transcriptomic data set of each type of lesion per patient. The two patients were selected because of their differing cancer aetiology, offering the possibility to address intra-patient variability and tumour progression in distinct contexts. Our results show that each type of lesion displayed limited variability within the same patient, but significantly differed among patients. This revealed that the two patients have distinct tumourigenic processes, thereby corroborating earlier conclusions at the transcriptomic level. Our molecular investigations using gallbladder organoids also provide evidence that Semaphorin 4A (*SEMA4A*) repression, which was observed in the two patients, can contribute to tumour progression.

METHODS

Spatial transcriptomics

Spatial profiling was performed on formalin-fixed paraffin-embedded (FFPE) tissue sections using GeoMx (NanoString Technologies, Seattle, WA, USA) [38] which was implemented by NanoString. The GeoMx Whole Transcriptome Atlas assay probe cocktail containing 18,677 probes was tested. Regions of interest (ROIs) subjected to spatial transcriptomic profiling encompassed epithelial areas of approximately 200 cells. The 24 ROIs of Patient #1 were all located on the same tissue section. For Patient #2, the ROIs were partitioned over two sections, namely 8 ROIs covering normal epithelium on one section, and 24 ROIs covering lesional tissue on a second section ([Table 1](#)). Additional information is provided in Additional file 1 (Supplementary methods).

Table 1. Number of ROIs subjected to spatial transcriptomic analysis.

	Patient #1	Patient #2
Non-tumoral	4	8
Low-grade BIIIN	8	6
High-grade BIIIN	6	12
Adenocarcinoma	6	6

Histology and staining

Hematoxylin/Eosin (H&E) and Sirius red/fast green stainings were performed on 6 µm sections of FFPE tissues, tumours or organoids. Briefly, tissue sections were deparaffinised 3x 3 min in xylene, 3 min in 99%, 95%, 70% and 30% ethanol and deionised H₂O. Sections were stained 7 sec in 100% hematoxylin, rinsed with H₂O, stained for 7 sec in 100% eosine, and rinsed with deionised H₂O. Dehydration of sections was performed in deionised H₂O, followed by 30%, 70%, 95%, 99% ethanol for 30 sec, and 30 sec in xylene. For collagen staining, slides were incubated into a picric acid solution with Sirius Red (Direct Red 80, Sigma-Aldrich) and Fast Green (SigmaAldrich) following manufacturer's instructions. Coverslips were placed on slides using Depex mounting medium (VWR, Leuven, Belgium). Pictures were taken with panoramic P250 Digital Slide Scanner (Histogenex, Antwerpen, Belgium) using 3DHISTECH Case Viewer software.

Immunofluorescence and immunohistochemistry

Immunofluorescence and immunohistochemistry were performed on 6 µm sections of FFPE tissues. FFPE tissue sections were deparaffinised 3x 3 min in xylene, 2 min in 99%, 95%, 70% and 30% ethanol and deionised water. Antigen retrieval was

performed by the use of Lab Vision PT Module (Thermo Fisher Scientific, Waltham, MA), in 10 mM citrate pH 6. Sections were permeabilised for 10 min in 0.3% Triton X-100 in PBS before blocking for 1 h in 5% HS, 10% BSA, 0.3% Triton X-100 in PBS. Primary antibodies were diluted in blocking solution at 4°C overnight and secondary antibodies were diluted in 10% BSA, 0.3% Triton X-100 in PBS at 37°C for 1 h. Images were taken with panoramic P250 Digital Slide Scanner (Histogenex, Antwerpen, Belgium) using 3DHISTECH Case Viewer software. Primary and secondary antibodies are described in Additional file 1 (Supplementary Table S1).

RNAscope *in situ* hybridisation

RNAscope RNA *in situ* hybridisation was performed on 5 µm sections of FFPE tissues, according to the manufacturer's protocol for manual RNAscope®2.5 HD Assay—RED (#322360, Advanced Cell Diagnostics/Bio-Techne, Abingdon, United Kingdom). The tissue sections were incubated at 60°C for 1h30, deparaffinised 2x 5 min in xylene and dehydrated 2x 2 min in 99% ethanol. Endogenous peroxidase was blocked with hydrogen peroxide for 10 min at room temperature followed by two short washings with deionised water. Slides were heated for 10 sec at 100°C in deionised water, and antigen retrieval was performed for 15 min at 100°C using RNAscope®Target retrieval. Tissue sections were washed in deionised water and 99% ethanol. Slides were dried for 5 min at room temperature and tissues were delineated using an ImmEdge Hydrophobic Barrier Pen (#310018, Advanced Cell Diagnostics/Bio-Techne, Abingdon, United Kingdom). Slides were incubated for 15 min with RNAscope®Protease plus (diluted at 1/5 in deionised water) at 40°C, washed with deionised water and incubated with the Hs-COL1A1-Homo sapiens collagen type I alpha 1 mRNA probe for 2 h at 40°C. The tissue sections were washed with

RNAscope®Wash buffer and six amplifications were performed (using six reagents AMP1-AMP6). The signal detection followed using RNAscope®Fast A and B reagents for 10 min at RT. The slides were kept in phosphate-buffered saline (PBS) overnight and immunostaining was performed: sections were blocked for 45 min at room temperature in 3% milk, 10% bovine serum albumin (BSA), 0.3% Triton in PBS. Primary and secondary antibodies were diluted in 10% BSA, 0.3% Triton in PBS. Primary antibodies were incubated overnight at 4°C and secondary antibodies were incubated 1h30 at 37°C. Pictures were taken with Cell Observer Spinning Disk (Carl Zeiss, Zaventem, Belgium) and analysed with Zen blue software. Primary and secondary antibodies are described in Additional file 1 (Supplementary Table S1).

Gallbladder organoid culture

Human non-tumoral gallbladder tissues were obtained from patients who underwent cholecystectomy at the Cliniques Universitaires Saint-Luc, Brussels, using the method of Rimland and coworkers [39]. The karyotype of the selected organoid line was normal and whole exome sequencing detected an ERBB3^{R675G} missense mutation at an allelic fraction of 0.021. The COSMIC database of somatic mutations in cancer does not report this variant which we considered as non-contributory to our experiments. To analyse the impact of blocking SEMA4A in gallbladder organoids, the latter were split and plated. After 24 h, SEMA4A antibody (IgG-SEMA4A, #14-1002-82 eBioscience/Thermo Fisher scientific, Brussels, Belgium) was added into the medium (10 µg/ml) and organoids were grown for 3 days. Additional information is provided in Additional file 1 (Supplementary Methods).

Cell culture

Human EGI-1 cholangiocarcinoma cells (German Collection of Microorganisms and Cell Cultures, kind gift from L. Fouassier) were grown in Dulbecco's Modified Eagle Medium High Glucose with L-Glutamine (DMEM; Lonza/Westburg Leusden, The Netherlands), 10% foetal bovine serum (#F7524, SigmaAldrich) and 1% Penicillin-Streptomycin (Gibco). Cell cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂.

Clonogenic assay

Colony forming capacity was determined by seeding 200 cells per well of a 6-well plate. Colony formation was allowed to occur over 10 days under 50 ng/ml treatment of recombinant human protein SEM4A4A (rhSEMA4a; Abcam #ab182683, Amsterdam, The Netherlands) or 10 µg/ml of IgG-SEMA4A previously desalted using a Zeba Spin Desalting Column, with the medium replenished every 3 days. Once colonies had formed, plates were washed in PBS and colonies fixed with methanol and stained 0.5 % crystal violet solution (Sigma-Aldrich) for 15 min at room temperature. Following this step, crystal violet was discarded, and plates were washed with water. After drying, plates were scanned, and the number of colonies was analyzed using ImageJ software 1.50 (National Institutes of Health, Bethesda, USA).

Transwell assay

Cell migration was evaluated by using 8.0 µm pore size transwell inserts (Corning) in 24-well plate wells. Prior to migration assessment, cells were pre-treated for 48 h with 50 ng/ml rhSEMA4a or 10 µg/ml of IgG-SEMA4A. Next, cells were seeded into the upper chamber transwell insert (6×10^4 cells) in 200 µl serum-free medium while 750 µl medium with 10% foetal bovine serum was added to the lower chambers. In the

case of cells treated with rhSEMA4A or IgG-SEMA4A, both chambers contained rhSEMA4A (50 ng/ml) or IgG-SEMA4A (10 µg/ml), respectively. After 24 h, cells were fixed with methanol and stained with 0.5 % crystal violet. Non-migrated cells in the upper chamber were removed using a cotton swab. The area covered by migrated cells was analysed using ImageJ software 1.50 (NIH).

***In vivo* assessment of SEMAPHORIN 4A function**

The impact of IgG-SEMA4A and rhSEMA4A on tumour growth was evaluated in NOD scid gamma (NSG) mice carrying EGI-1 subcutaneous xenografts. 10⁶ EGI-1 cells from independent cultures were injected subcutaneously at 4 distinct locations (under the right and left front leg, and under the right and left rear leg) in NSG mice. After 4 weeks, the animals received intraperitoneal injections of PBS, IgG-SEMA4A (5 mg/kg) previously desalted using a Zeba Spin Desalting Column (Thermo Fisher Scientific), or rhSEMA4A (500 µg/kg) in a final volume of 200 µl every other day, receiving a total of 4 doses of treatment. Tumour volume (V) was measured every 48 h using a calliper and calculated by the formula $[V = 0.5 \times (L \times W^2)]$, where L and W represent the longest and the perpendicular tumour axis respectively. Relative tumour volume was defined by normalising to the initial tumour volume at the start of the treatment (V₀).

Data on growth of PBS-treated tumours were obtained with 10 independent EGI-I cell cultures injected in 3 NSG mice (each receiving simultaneous cell injections at 3, 3 and 4 locations, respectively); out of these, 7 tumours were selected for histological analyses and microvascular invasion, and they originated from two mice. Data on growth of rhSEMA4A-treated tumours were obtained with 8 independent EGI-I cell cultures injected in 2 NSG mice (each receiving 4 simultaneous cell injections); out of

these, 7 tumours were selected for histological analyses and microvascular invasion. Data on growth of anti-SEMA4A IgG-treated tumours were obtained with 8 independent EGI-I cell cultures injected in 2 NSG mice (each receiving 4 simultaneous cell injections); the 8 tumours were analysed for histology and microvascular invasion.

Bioinformatic analysis of spatial transcriptomic profiling data

Sequencing quality was assessed for each ROI. Raw number of reads ranged from 1750000 to 21875463. Alignments rates, sequencing saturation and RTSQ30 were respectively higher than 80%, 70%, and 98% in all ROIs. The percent of detected genes (*i.e.* genes with an expression value higher than the LOQ value, defined as the negative probes geometric means + 2 standard deviations) was evaluated per segment, to identify low-performing AOs that should be removed. All ROIs were kept, as values ranged from 13.6% to 51.4%. Raw count normalisation and differential expression analyses were performed using DESeq2 Bioconductor package v1.32.0 [40]. The generalised linear model was fitted using the following design: type of lesion * patient. The lists of differentially expressed genes generated by DESeq2 were ranked on the t-statistic values, and KEGG and HALLMARK gene set enrichment analyses were performed using clusterProfiler v4.0.5 [41].

RESULTS

Selection of normal epithelium, BillIN and adenocarcinoma in samples of human gallbladder

Our goal is to characterise the spatial transcriptome of gallbladder lesions during progression from normal epithelium to adenocarcinoma. This required gallbladder samples that simultaneously contain non-tumoral (*i.e.* histologically normal)

309 epithelium, low-grade BillN, high-grade BillN and adenocarcinoma, from patients with
310 distinct cancer aetiology. Each lesion must be large enough to enable us to analyse
311 the whole transcriptome of several regions of each type of lesion. Samples that met
312 these criteria from two patients were identified in the biobank of the Cliniques
313 Universitaires Saint-Luc: Patient #1 was an 81 year old woman who underwent
314 cholecystectomy to treat cholecystitis; adenocarcinoma was an incidental finding.
315 Patient #2 was a 53 year old man affected with primary sclerosing cholangitis (PSC)
316 whose gallbladder was resected following imaging that revealed a thickening of the
317 gallbladder wall. According to the TNM classification, both patients were staged IIa
318 (pT2a T0 M0). Pathological diagnoses of non-tumoral epithelium, BillNs and
319 adenocarcinoma were made on H&E-stained sections, and were confirmed by two
320 expert pathologists. Patient #1 displayed two small foci of intestinal metaplasia, and
321 no metaplasia was detected in Patient#2. GeoMx Digital Spatial Profiling (NanoString)
322 [38] was implemented on sections adjacent to the H&E-stained sections to collect
323 whole transcriptome data from 56 epithelial ROIs, each covering approximately 200
324 epithelial cells of non-tumoral epithelium, BillN and adenocarcinoma (Table 1).
325 Metaplasia in Patient #1 were too small for spatial profiling. Figure 1 illustrates the
326 spatial distribution of areas in which ROIs were delineated (Fig. 1A), as well as
327 examples of H&E-stained non-tumoral epithelium, BillNs and adenocarcinomas (Fig.
328 1B). All adenocarcinoma ROIs in the two patients were well-differentiated (histological
329 grade 1) and compatible with the definition of pancreatobiliary adenocarcinomas
330 which are characterised by widely spaced tubular structures embedded in a fibrous
331 stroma [42]. Epithelial ROIs were delineated on sections stained with antibodies which
332 detect markers of the epithelium (panCytokeratin), leukocytes (CD45), and
333 mesenchymal cells (α smooth muscle actin). Nuclei were immunolabeled with anti-

Human antigen R antibodies (Additional file 2, Supplementary Fig. S1). The ROIs were subjected to transcriptomic analyses as described in Methods.

Spatial transcriptomic analysis suggests limited intra-patient variability and distinct modes of tumour progression among the two patients

Principal component analysis (PCA) of the 56 transcriptomes revealed a remarkable clustering of the non-tumoral epithelial samples of the two patients (Fig. 2A). ROIs from the same type of lesions clustered together within the same patient, but were separated between patients. In Patient #1, adenocarcinoma ROIs clustered close to high-grade BillN ROIs, whereas adenocarcinomas in Patient #2 appeared closely related to low-grade BillNs. These results were corroborated by the number of differentially expressed genes ($\log_2$ fold change ≥ 1.0 ; $p_{\text{adj}} \leq 0.05$) when cross-comparing all tissue types (Additional file 2, Supplementary Fig. S2A). Together, these data revealed that each lesional type displays limited intra-patient variability, but that distinct mechanisms are driving tumourigenesis in the two patients. Moreover, the PCA plot suggested that adenocarcinoma evolved according to a normal $\rightarrow$ low-grade BillN $\rightarrow$ high-grade BillN $\rightarrow$ adenocarcinoma sequence in Patient #1, and according to a normal $\rightarrow$ low-grade BillN $\rightarrow$ adenocarcinoma sequence in Patient #2, with high-grade BillN emerging separately from adenocarcinoma in this patient.

We next compared the lesions in the two patients and focused on signalling pathways. Using Gene Set Enrichment Analysis (GSEA) [43], we found several enriched signalling pathways when comparing adenocarcinoma and non-tumoral epithelium. Negative or positive enrichment scores reflect enrichment of downregulated or upregulated genes, respectively (Fig. 2B). The use of KEGG or HALLMARKS gene

sets revealed several pathways that were enriched in both patients, and other pathways that were enriched in only one patient. Heatmaps illustrate genes from the HALLMARKS and KEGG pathway gene sets that are differentially expressed between adenocarcinoma and non-tumoral epithelium in the two patients (Fig. 2C; Additional file 2, Supplementary Fig. S2B).

Galbladder cancer is often associated with mutations in *PI3KCA*, *CTNNB1*, *KRAS*, *TP53*, and *ERBB2* [18-26]. GSEA revealed that PI3K-AKT-mTOR signalling (HALLMARK) is enriched in adenocarcinoma of both patients (Fig. 2C), and out of the 38 leading edge genes in Patient #2, 23 overlapped with the leading edge genes in Patient #1. HALLMARK gene sets are based on coordinately expressed and biologically relevant genes, and identify pathway activation phenotypes [44]. Therefore, the positive enrichment of PI3K-AKT-mTOR signalling reflects activation of the pathway. Further, GSEA suggested enrichment of Wnt signalling in both patients, when considering the KEGG Wnt signalling gene set in Patient #1 and the HALLMARK Wnt- β -catenin gene set in Patient #2 (Fig. 2C). However, the two gene sets differ in their composition, leading to different conclusions in the two patients. In Patient #1, Wnt ligands (*WNT7B*, *WNT8A*, *WNT10A*, *WNT11*), receptors (*FZD2*, *FZD5*) and effector (*TCF7L2*) were upregulated in adenocarcinoma as compared to non-tumoral epithelium. Genes induced by Wnt signalling and reflecting activation of a negative feedback loop (*AXIN2*, *GSK3B*) further reveal dynamic activity of the Wnt pathway in this patient (Additional file 2, Supplementary Fig. S2C). In contrast, in Patient #2, only 13 genes from the HALLMARK Wnt- β -catenin gene set were significantly enriched. Among these, most genes are not typical for Wnt signalling and belong to pathways with which Wnt signalling crossreacts. *CTNNB1* is upregulated in adenocarcinoma of

Patient #2 ($\log_2$ fold change=1.10; $p_{\text{adj}}=8.65 \times 10^{-10}$), in parallel with upregulation of Wnt signalling inhibitors *DKK4* ($\log_2$ fold change=0.86; $p_{\text{adj}}=84.76 \times 10^{-4}$) and *CSNK1E* ($\log_2$ fold change=0.51; $p_{\text{adj}}=1.66 \times 10^{-3}$). Therefore, the analysis of genes of the HALLMARK Wnt- β -catenin gene set does not strongly support that Wnt signalling is active in Patient #2. KRAS signalling differs between the two patients, as evidenced by enrichment of RAS signalling (KEGG) in Patient #1, but downregulation of several KRAS targets within the KRAS signalling up gene set (HALLMARK) in Patient #2 (Fig. 2C). Similar to KRAS signalling, the p53 pathway differed between patients. Finally, GSEA did not highlight ERBB signalling. However, we found significant overexpression of EGFR, ERBB2 and ERBB3 in Patient #2, but only overexpression of ERBB2 in Patient #1 (Fig. 3A).

Although both patients can display enrichment of the same pathway, we noticed that the sequence of enrichment during tumourigenesis may differ among the patients. Indeed, PI3K-AKT-mTOR signalling became enriched in precursor lesions of Patient #1, namely at the low-grade BillN $\rightarrow$ high-grade BillN transition, whereas it became enriched only at the adenocarcinoma stage in Patient #2 (Fig. 3B). Other pathways whose enrichment is shared between the patients may in contrast display a similar sequence of enrichment. Indeed, androgen response and estrogen signalling became enriched at the precursor-to-adenocarcinoma transition (Fig. 3B). Notch signalling was also enriched in adenocarcinoma of both patients, and the enrichment was only significant when comparing non-tumoral epithelium and adenocarcinoma, not when comparing the precursor to adenocarcinoma transitions. This likely reflected a progressive activation throughout the tumourigenic process, without significant jumps between lesional states. Moreover, comparing the expression of leading edge genes

in the Notch pathway also revealed interesting differences such as the strong upregulation of *NOTCH3* in Patient #1 ($\log_2$ fold change=2.05; $\text{padj}= 5.2 \times 10^{-11}$) and more modest upregulation of this gene in Patient #2 ($\log_2$ fold change=0.77; $\text{padj}= 1.5 \times 10^{-3}$) (Fig. 3C).

Finally, the proposed sequence of tumor progression in patient #2, namely normal → low-grade BillN → adenocarcinoma, suggests that low-grade BillNs in this patient display upregulated pathways that may be indicative of aggressiveness. In that context, GSEA analyses comparing non-tumoral tissues and low-grade BillNs showed enrichment of the MYC oncogenic pathway in patient #2, unlike in patient #1 (Fig. 3D). We also noticed that low-grade BillNs of patient #2, unlike those of patient #1, displayed increased expression of Midkine as compared to non-tumoral tissue ($\log_2$ fold change = 2.89; $\text{padj}: 7.684 \times 10^{-15}$). Midkine is known to promote immunosuppressive macrophage differentiation in gallbladder cancer [17]. Also, EGFR, ERBB2 and ERBB3 expression was upregulated in the low-grade BillNs of patient #2 (Fig. 3A).

Spatial transcriptomic analysis reveals induction of collagen gene expression in tumoral epithelia

Nepal and coworkers considered the hallmark "epithelial-mesenchymal transition (EMT)" as indicative of poor prognosis [26]. In Patient #1, the corresponding HALLMARK gene set has the highest enrichment score when comparing adenocarcinoma with non-tumoral epithelium (Fig. 2B-C). The sequence of EMT enrichment is shown in Fig. 4A. No similar enrichment was found in Patient #2. Importantly, transcription factors typical for EMT and *CADHERINS* showed no

significant differential expression during tumour progression in either patient (Fig. 4B). In contrast, extracellular matrix-coding genes were significantly upregulated and contributed significantly to the enrichment of the EMT pathway in the GSEA analyses (Fig. 4C). To support the latter data at the histological level, we resorted to RNAscope *in situ* hybridisation. We detected rare mRNAs coding for *COL1A1* in non-tumoral epithelia of the two patients. Strong induction of *COL1A1* was detected in high-grade BillIN of Patient #1, but also in low-grade BillNs of Patient #2 (Fig. 4D).

SEMAPHORIN4A downregulation promotes tumour progression

Our GSEA data uncovered axon guidance signalling as a potential driver of tumour progression in Patient #1 (Fig. 2B). Axon guidance genes, including *SEMAPHORIN/PLEXIN* ligand-receptor pairs, were enriched in Patient #1 adenocarcinomas, but not in Patient #2 (Additional file 2, Supplementary Fig. S3A). *SEMA4A* was downregulated in the adenocarcinomas of both patients, and this was noticed already at the precursor stages (Fig. 5A). The involvement of *SEMA4A* in gallbladder cancer is unexplored, but *SEMA4A* loss-of-function mutation in familial colorectal cancer type X was found to promote cancer development, thereby revealing a tumour suppressor role for *SEMA4A* [45, 46]. Furthermore, we analysed a public RNAseq dataset from 10 patients for which paired non-tumoral tissue and adenocarcinoma had been collected [47]. The results showed that *SEMA4A* was significantly reduced in gallbladder tissue (Additional file 2, Supplementary Fig. S3B), in line with our findings in patients #1 and #2. This prompted us to investigate the role of *SEMA4A* in gallbladder cancer development.

We first generated organoids from gallbladder epithelium and selected a line which displayed no karyotypic anomalies. It expressed biliary-specific markers and exhibited biliary transport functions (Additional file 2, Supplementary Fig. S4). It also expressed the genes coding for SEMA4A and its receptor Plexin B1 (PLXNB1) (Additional file 2, Supplementary Fig. S3C). To mimick the downregulation of *SEMA4A* observed in our transcriptomic analyses, we incubated the organoids for 3 days with a blocking anti-SEMA4A IgG antibody. We found no change in cell proliferation, but observed local areas of pseudostratification of the epithelium in a subset of organoids (Fig. 5B). The histology of those areas was reminiscent of BillN, indicating that inhibiting SEMA4A impacts cell polarisation.

We next determined if SEMA4A had additional tumour suppressor properties. Since the organoid lines were not able to induce tumour formation after subcutaneous injection in immunodeficient NSG mice, we used the human extrahepatic cholangiocarcinoma cell line EGI-1. *In vitro*, clonogenic and transwell migration assays demonstrated that adding rhSEMA4A to cultured EGI-1 cells reduced their clonogenicity and migration (Fig. 5C). Blocking anti-SEMA4A IgG antibody slightly but not significantly increased colony formation, and did not impact cell migration (Fig. 5C). *In vivo*, subcutaneous injection of EGI-1 cells in immunodeficient NSG mice resulted in the formation of tumours. We did not observe any significant histological differences between EGI-1 xenografts treated with intraperitoneal administration of rhSEMA4A, anti-SEMA4A IgG, or the control condition, except for microvascular invasions (Fig. 5D; Additional file 2, Supplementary Fig. S3D). Indeed, consistent with the decreased migration induced *in vitro* by rhSEMA4A, administration of rhSEMA4A resulted in a significant reduction of microvascular invasion in EGI-1 cell-derived tumours (Fig. 5D).

Anti-SEMA4A IgG antibody had no effect on microvascular invasion in the tumours. Recombinant SEMA4A did not impact tumour growth. In contrast, blocking IgG anti-SEMA4A antibody accelerated growth at the earliest stages of tumour growth to progressively reach a plateau (Fig. 5E). We conclude that SEMA4A can control tumour progression by impacting polarity, clonogenicity and migration of cells.

DISCUSSION

Earlier mutational profiling of precursor and cancer lesions coexisting in a same patient provided evidence that adenocarcinoma development may be BillIN-dependent or -independent [15]. Here, using GeoMx technology we extended these findings at the transcriptional level in two patients. We showed that lesions exhibited low intra-patient variability, but exhibited patient-specific sequences of signalling pathway activation.

In Patient #1, ROIs from a same type of lesion were often located at a short distance from each other, except for adenocarcinoma ROIs which were more scattered throughout the tissue sample. In Patient #2, high-grade BillIN ROIs were close to each other, but low-grade BillIN, adenocarcinoma and non-tumoral epithelium ROIs were significantly dispersed (Fig. 1A). Still, in spite of the scattering within the tissue, the transcriptomic profile of lesions belonging to the same histological type showed low intra-patient variability. Such transcriptomic homogeneity likely reflects that cells from a same type of lesion proliferated in a similar environment and with limited accumulation of new mutations. Clonal analysis of gallbladder cancers revealed subclonal diversification [48], in line with significant epithelial cell heterogeneity in the adenocarcinoma lesions noticed in single cell RNA sequencing studies [16, 17]. However, our patient samples contained all lesional types on the same tissue sections,

suggesting that cancer lesions had not enough time to accumulate genomic lesions, invade the tissue and produce subclones.

The neighbourhood of low-grade BillN, high-grade BillN and adenocarcinoma which may occur in pathological samples, leads us to surmise that the epithelium undergoes a normal epithelium → low-grade BillN → high-grade BillN → adenocarcinoma histogenic sequence. *A contrario*, the transcriptomic profile of Patient #2 strongly suggests that adenocarcinoma derived from low-grade BillN, not from adjacent high-grade BillNs. This contrasted with Patient #1 whose adenocarcinoma ROIs were closely related to high-grade BillNs. We excluded that adenocarcinoma in Patient #2 corresponded to low-grade BillNs extending in Rockitansky-Aschoff sinuses. In Patient #2, only 58 genes were 2-fold up- or downregulated when comparing low-grade BillN and adenocarcinoma, revealing that low-grade BillN may be at high risk for evolution towards invasive cancer.

Many signalling pathways were activated during tumour progression and several were common between the two patients. However, the sequence of pathway activation differed between patients, some of the common pathways being activated at the BillN stage in one patient, but only in the adenocarcinoma cells in the other patient. Therefore, our work suggests that various combinations of pathway activations may end up yielding cancer, no specific pathway or combination of pathways being responsible for transition from one stage to the other.

The HALLMARK gene set "Inflammatory response" was enriched in adenocarcinomas of both patients (not shown), reflecting their common chronic inflammatory

background. Still, the tumour aetiology differed in Patients #1 and #2 , with Patient #2 being affected with PSC, a disease with high incidence of adenocarcinoma [49]. The adenocarcinoma in Patient#2 was mucosecreting (Fig. 1B), unlike the carcinoma in Patient #1. The mutational profile of cholangiocarcinoma in PSC is heterogeneous and affects genes similar to those in non-PSC associated cholangiocarcinoma, the most frequently mutated being *TP53*, *KRAS*, *PI3KCA* and *GNAS*. In low-grade and high-grade dysplastic lesions, loss or amplifications of several genes, as well as mutations in *ERBB2* and *TP53*, can already occur [50, 51]. Our work extend these data at the transcriptomic level and highlight that low-grade BillIN can be very closely related to adenocarcinoma.

EMT is a phenotypic continuum during which epithelial cells evolve to a mesenchymal state via transitional or hybrid states [52]. It involves disruption of polarity and intercellular adhesion, changes in the interaction between cells and extracellular matrix, and increased migration [53, 54]. Interestingly, both patients display increased expression of *COL1A1* and *COL1A2*. This differs from pancreatic cancer in which *COL1A2* is no longer expressed [55], leading to the production of collagen $\alpha1/\alpha1/\alpha1$ trimers which promote tumor progression.

SEMA4A is a tumour suppressor in colorectal cancer [45, 46]. Here we found that it is downregulated in both patients during gallbladder tumour progression, starting at the BillIN stage. Downregulation of *SEMA4A* in gallbladder cancer was also found in other patients (Additional file 2, Supplementary Fig. S3B). Gallbladder organoids expressed *SEMA4A* and its receptor *PLXNB1* and the levels of *SEMA4A* expression varied considerably (Additional file 2, Supplementary Fig. S3B), likely explaining the variable

pseudostratification of the gallbladder organoids when treated with blocking IgG antibody (Fig. 5B). Also, the low levels of *SEMA4A* and *PLXNB1* in cholangiocarcinoma EGI-1 cells, as compared to organoids derived from normal gallbladder epithelium, fit with the notion that *SEMA4A* is repressed in biliary cancer cells and with our observation that anti-*SEMA4* blocking antibodies have limited or no effect on clonogenicity and migration of EGI-1 cells *in vitro*. *In vivo*, we detected a higher level of *SEMA4A* in EGI-1 cell-driven tumours than in *in vitro* cultured EGI-1 cells (Additional file 2, Supplementary Fig. S3C). We excluded that this results from *SEMA4A* production by tumour-invading mouse cells, as our PCR primers were designed to specifically detect human *SEMA4A*. Inhibiting this *in vivo* production of *SEMA4A* enabled us to monitor growth-promoting properties of anti-*SEMA4* blocking antibodies. How these anti-*SEMA4A* antibodies promote EGI-1 cell-derived tumour growth remains unclear. Indeed, our data show that inhibiting *SEMA4A* accelerates tumour growth during 4 days. This effect slows down to reach a plateau (Fig. 5E), and at the plateau stage we noticed a slight but not significant increase in proliferation rate, as evidenced by immunostaining for phospho-Histone H3 (Additional file 2, Supplementary Fig. S3C). We hypothesise that anti-*SEMA4A* antibodies promoted proliferation mainly during the first 4 days of treatment. Interestingly, rh*SEMA4* did not impact tumour growth, but decreased microvascular invasion, suggesting that reduction of *SEMA4* promotes metastasis. The signalling pathways mediating the effects of *SEMA4A* on migration, polarity and potentially proliferation deserve further investigation. Further studies will determine how frequently *SEMA4A* is repressed at early stages of gallbladder cancer and whether understanding its pathway may lead to identify biomarkers of early diagnosis of gallbladder tumours.

CONCLUSION

Our spatial transcriptomic analysis reveals that precursor and cancer lesions can display limited intra-patient variability during gallbladder cancer progression and supports that tumourigenic mechanisms are patient-specific. Repression of *SEMA4A* may contribute to tumour progression. Our work also underscores that low-grade BillNs may be at high risk for developing to cancer and should ideally be characterised by gene expression profiling.

ABBREVIATIONS

BillN, biliary intraepithelial neoplasia; EMT, epithelial-mesenchymal transition; FFPE, formalin-fixed paraffin-embedded; H&E, haematoxylin and eosin; PSC, primary sclerosing cholangitis; ROI, region of interest.

DECLARATIONS

Ethics approval. The study on human samples was conducted in compliance with the ethical guidelines of the 2013 Declaration of Helsinki and was approved by the Comité d'Ethique Hospitalo-Facultaire (UCLouvain and Cliniques Universitaires Saint-Luc) with numbers 2018/06Jul/281 and 2021/26OCT/444. In accordance with article 8 of the internal rules of the Cliniques universitaires Saint-Luc, the need for informed consent was waived to the present retrospective study. The study is based solely on the analysis of residual human body material and on the collection of data existing in the medical files of patients who have not expressed their opposition to the use of their medical file for scientific research purposes. An informed consent exemption request was thus presented to the Ethics Committee, which was accepted. Mice received

humane care and the research protocol was approved by the Animal Welfare Committee of the Université Catholique de Louvain with number 2022/UCL/MD/17.

Consent for publication. Not applicable.

Data availability. The dataset supporting the conclusions of this article is available in Gene Expression Omnibus, [GSE259311](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE259311).

Competing interests. The authors declare no competing interests.

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Authors' contribution. S.P., F.M.-N., A.L., M.K., L.G. and F.L. designed the study; S.P., F.M.-N., A.L., S.C., S.A., C.H., S.T., acquired data; S.P., F.M.-N., A.L., L.D., N.L., C.S., M.K., L.G. and F.L. analysed and interpreted data. S.P., F.M.-N., A.L., L.D., N.L., L.G. performed statistical analyses. S.P., F.M.-N. and F.L. drafted the manuscript. All authors read and approved the final paper.

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ADDITIONAL FILES

Additional file 1

pdf

Title: Supplementary Methods

- Spatial transcriptomics

- Immunofluorescence and immunohistochemistry

- Gallbladder organoid culture

- RNA extraction and Reverse Transcription-quantitative PCR

Additional file 2

pdf

Title: Supplementary figures

- Supplementary Fig. S1. Delineation of ROIs for spatial transcriptomic analysis.

- Supplementary Fig. S2: Differential gene expression in spatial transcriptomics

- Supplementary Fig. S3: Expression of axon guidance genes and function of SEMA4A

- Supplementary Fig. S4. Characterisation of gallbladder organoids.

FIGURE LEGENDS

Fig. 1 Selection of non-tumoral (histologically normal) epithelium, BillIN and adenocarcinoma in samples of human gallbladder. (A) Low magnification view of gallbladder sections. Squares indicate tissue areas in which several epithelial ROIs were delineated as shown in Additional file 2, Supplementary Fig. S1. (B) Illustrative examples of non-tumoral epithelium, low-grade BillIN, high-grade BillIN and adenocarcinoma. ADC, area containing adenocarcinomas; H&E, haematoxylin-eosin; HG, area containing high-grade BillINs; LG, area containing low-grade BillINs; NT, area containing non-tumoral epithelium.

Fig. 2 Distinct modes of tumour progression in two patients revealed by spatial transcriptomic analysis. (A) PCA plot of the whole transcriptome of 56 ROIs comprising non-tumoral (histologically normal) epithelia, low-grade biliary BillINs, high-grade BillINs and adenocarcinomas. (B) Heatmaps of GSEA enrichment scores comparing adenocarcinoma and non-tumoral epithelium using the KEGG pathway and HALLMARK gene sets ($p_{\text{adj}} \leq 0.05$). (C) Heatmaps of genes from the HALLMARK gene sets that are differentially expressed between adenocarcinoma and normal epithelium ROIs ($p_{\text{adj}} \leq 0.05$). ADC, adenocarcinoma; HG, high-grade BillIN; LG, low-grade BillIN; NES, normalised enrichment score; NT, non-tumoral epithelium.

Fig. 3 Distinct modes of signalling pathway activation in two patients revealed by spatial transcriptomic analysis. (A) Expression of ERBB receptors and ERBB signalling pathway genes during tumour progression. Tables mention the fold change inductions between lesions in the two patients. The corresponding volcano plots are shown, with

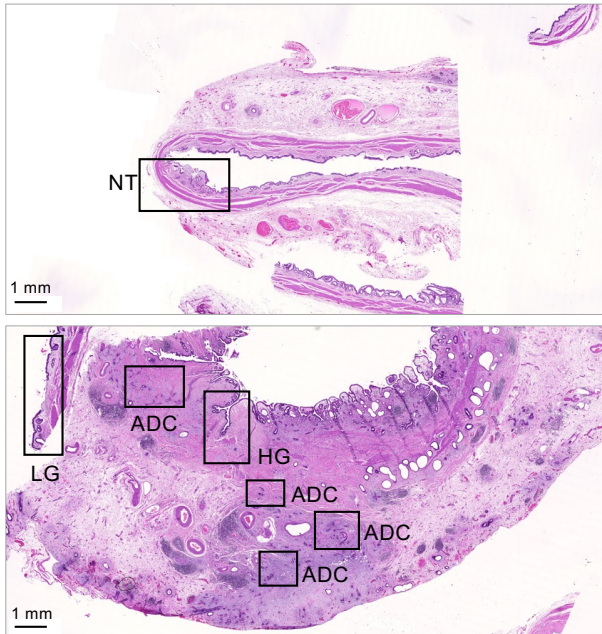
blue dots highlighting EGFR/ERBB receptors. (B) Sequence of enrichment of signalling pathways during tumour progression as determined by GSEA using KEGG pathway and HALLMARK gene sets. Significant enrichments are indicated with p_{adj} values. Red boxes, lesions showing enrichment of the pathway. ns, not significant. (C) Differential expression of genes between adenocarcinoma and non-tumoral epithelium in the KEGG pathway Notch. (D) Heatmaps of GSEA enrichment scores comparing low grade BillNs and non-tumoral tissues in the two patients. ADC, adenocarcinoma; HG, high-grade BillN; LG, low-grade BillN; NES, normalised enrichment score; ns, non-specific; NT-non-tumoral epithelium.

Fig. 4 EMT and collagen gene expression during tumour progression. (A) Enrichment sequence of EMT (HALLMARK) in Patient #1 demonstrates enrichment throughout tumourigenesis. Significant enrichments are indicated with p_{adj} values. Red boxes, lesions showing enrichment of the pathway. (B) Gene expression heatmaps of EMT-promoting transcription factors, and of *VIMENTIN* and *CADHERINS* show little or no variation during tumourigenesis. (C) Heatmap and volcano plots showing *COLLAGEN* and *LAMININ* gene expression in the two patients. Blue dots in volcano plots indicate *LAMININ* genes. (D) RNAscope *in situ* hybridisation demonstrates induction of *COL1A1* mRNA (red dots) starting in high-grade BillNs in Patient #1 and in low-grade BillN of Patient #2. Tissue sections were immunostained to mark epithelial cells (E-CADHERIN; E-CAD), nuclei (Hoechst), and mesenchymal cells (Smooth muscle protein 22 α ; SM22 α). ADC, adenocarcinoma; HG, high-grade BillN; LG, low-grade BillN; NES, normalised enrichment score; NT, non-tumoral epithelium.

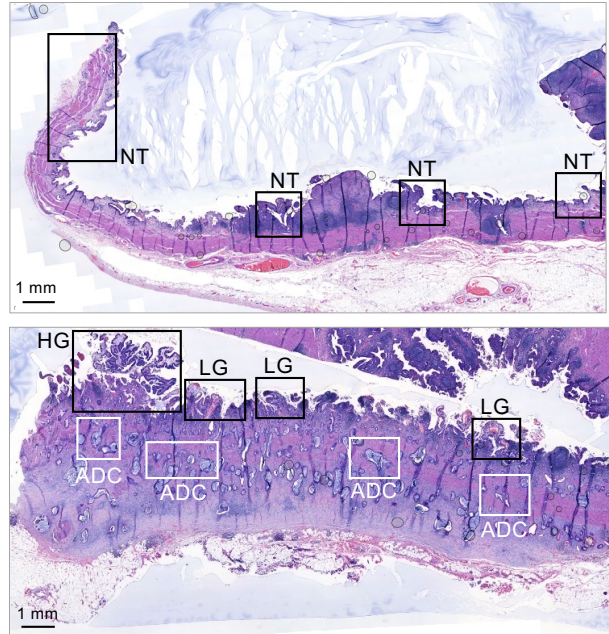
877 **Fig. 5** SEMA4A displays tumour suppressor properties. (A) *SEMA4A* gene expression
878 is reduced during gallbladder cancer progression. ADC, adenocarcinoma; HG, high-
879 grade BillIN; LG, low-grade BillIN; NES, normalised enrichment score; NT, non-tumoral
880 epithelium; ns, non-specific. (B) The epithelium of gallbladder organoids treated with
881 blocking anti-SEMA4A IgG antibody displays focal areas of pseudostratification. This
882 effect was monitored in two experiments out of four. (C) rhSEMA4A reduces
883 clonogenicity and transwell migration of cultured EGI-1 cells, whereas anti-SEMA4A
884 IgG antibody had little or no effect. Data show means $\pm$ SEM; $n=3$ or 4 ; statistical
885 significance was calculated by applying a paired t -test (*, $p<0.05$). (D) The histology
886 (Sirius red/fast green staining) and microvascular invasion (MVI) are illustrated in
887 subcutaneous EGI-1 cell tumours following intraperitoneal injection of rhSEMA4A or of
888 blocking IgG SEMA4A antibodies, according to the timing shown in panel E. There is
889 no significant histological difference between the tested conditions, except that
890 rhSEMA4A reduces the number of MVI events in EGI-1 cell tumours, as quantified in
891 the graph. One-way ANOVA was used to compare means (*, $p<0.05$). $n=7$ (control), 7
892 (IgG SEMA4A) and 8 (rhSEMA4A). (E) Growth of subcutaneous EGI-1 cell tumours
893 following intraperitoneal injection of PBS (control), blocking anti-SEMA4A IgG
894 antibody, or rhSEMA4A. $n=10$ (control), 8 (IgG SEMA4A) and 7 (rhSEMA4A). Relative
895 tumour volume and SEM are plotted. Differences between groups were evaluated by
896 performing a two-way Analysis of Variance (two-way repeated measures ANOVA) with
897 Bonferroni correction (*, $p<0.05$; **, $p<0.01$). For further statistical validation, a random
898 intercept-random slope model with continuous time was fitted. This showed a
899 significant interaction between the time and group effect ($p=0.03$), in particular, the
900 contrast between SEMA4A IgG and control is significant ($p=0.048$) but not that
901 between control and rhSEMA4A ($p=0.95$).

A

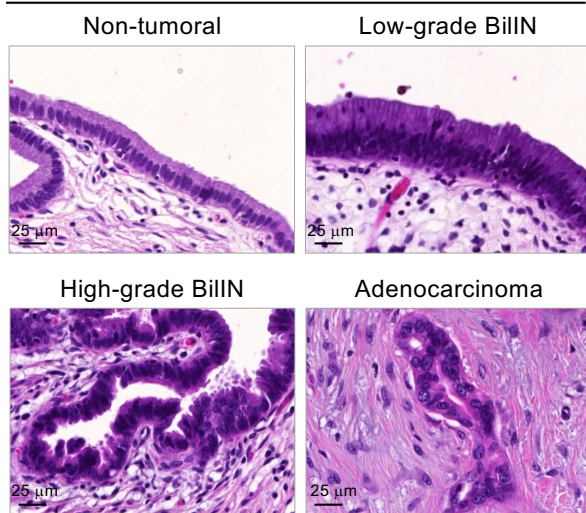
Patient #1 (H&E)



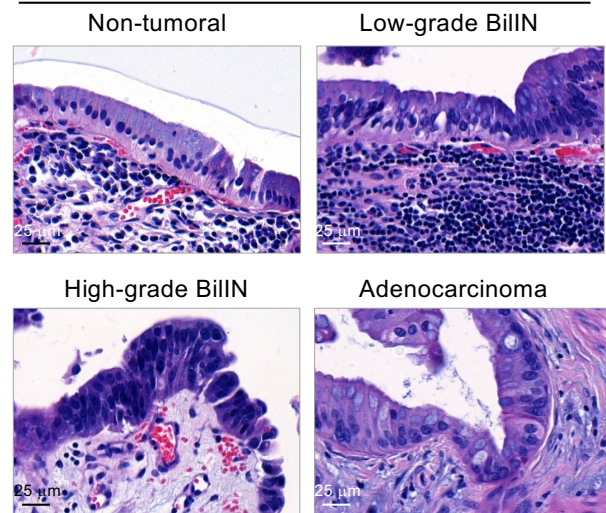
Patient #2 (H&E)

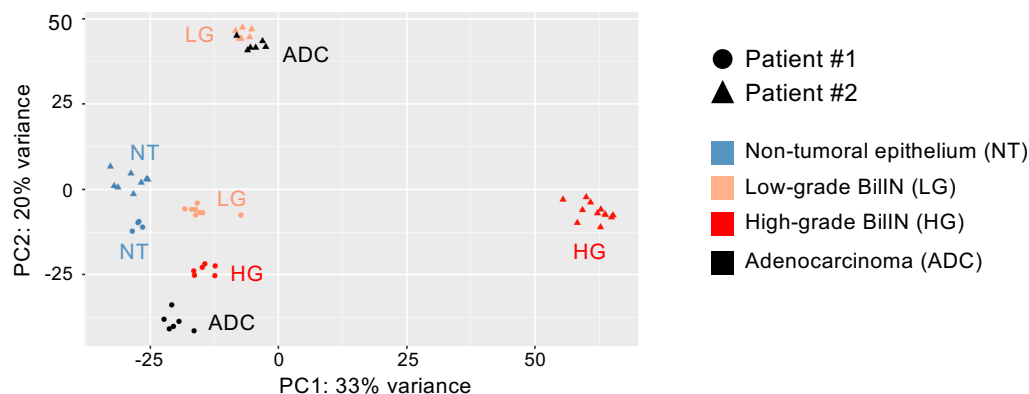
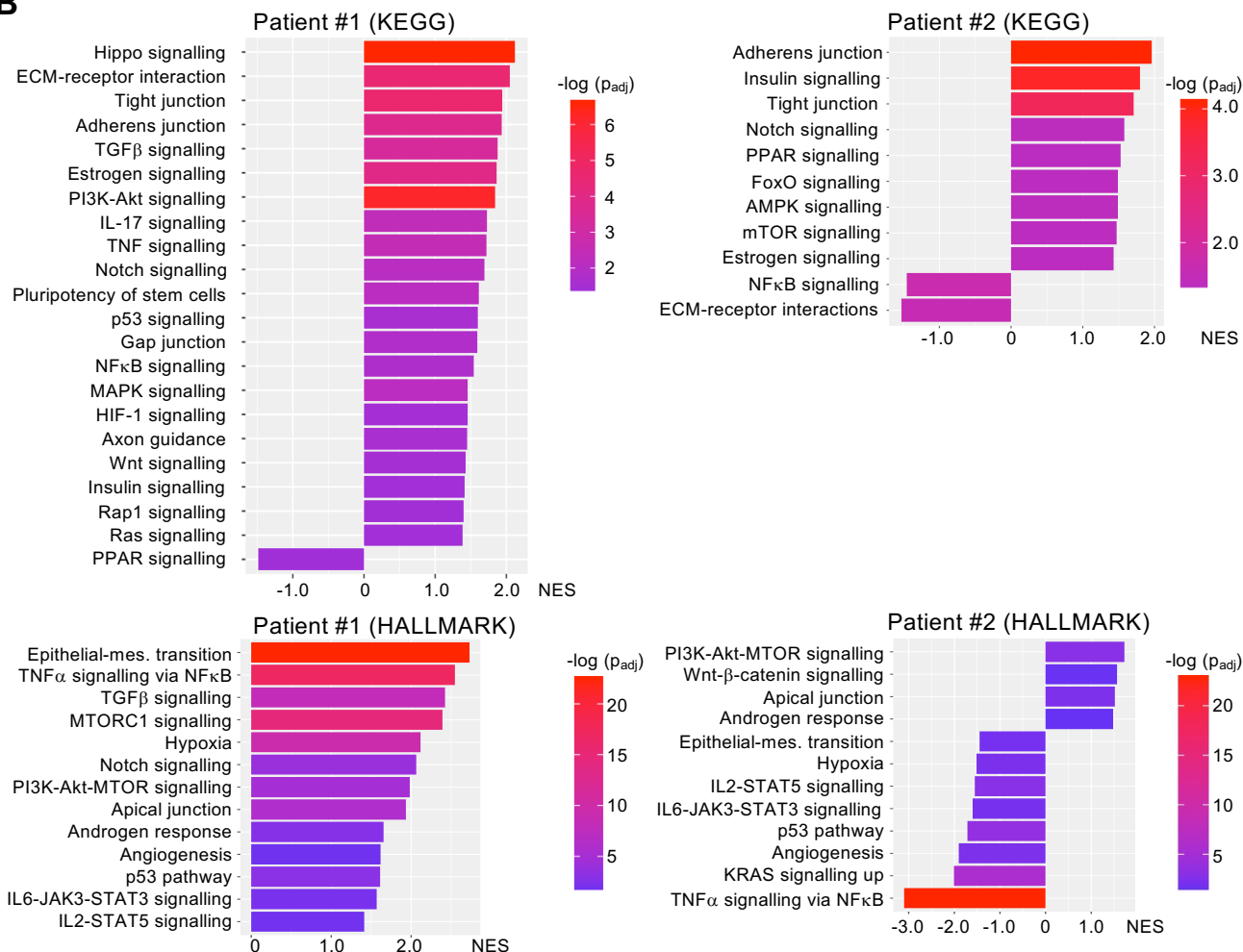
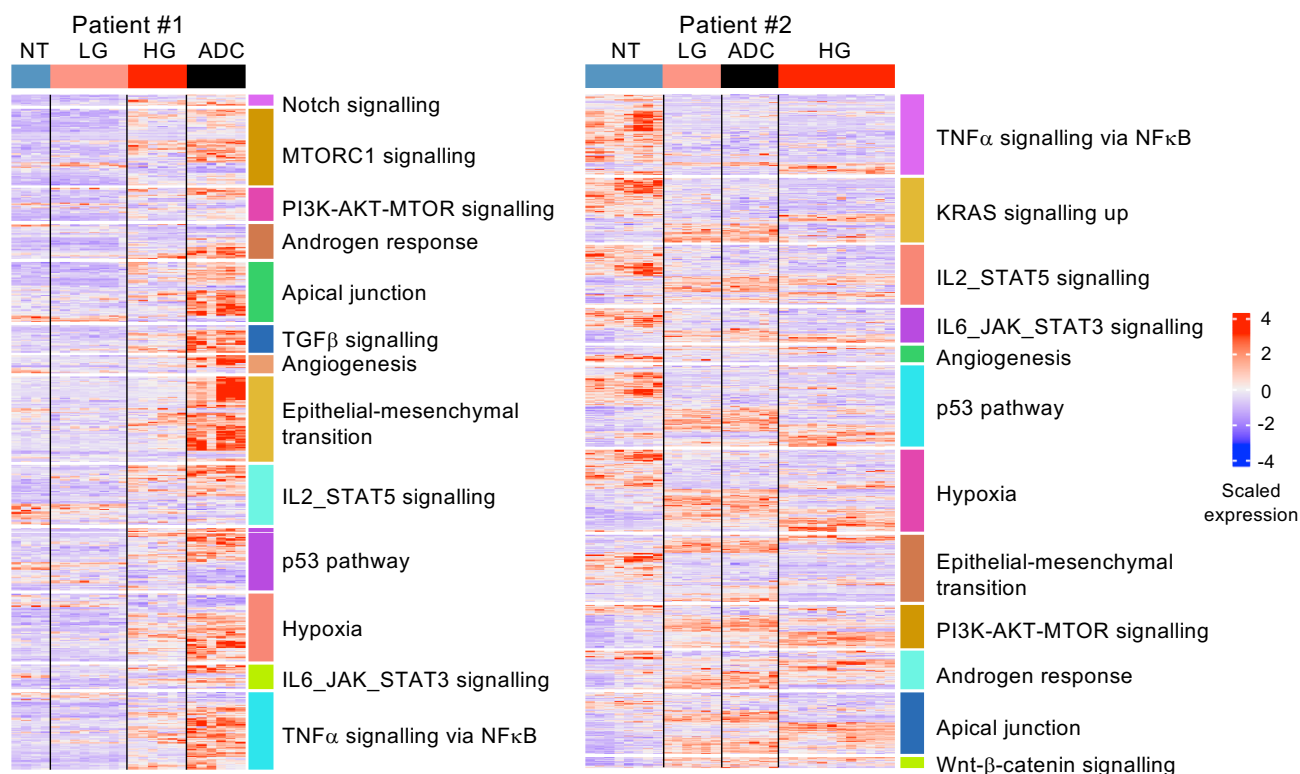
**B**

Patient #1 (H&E)

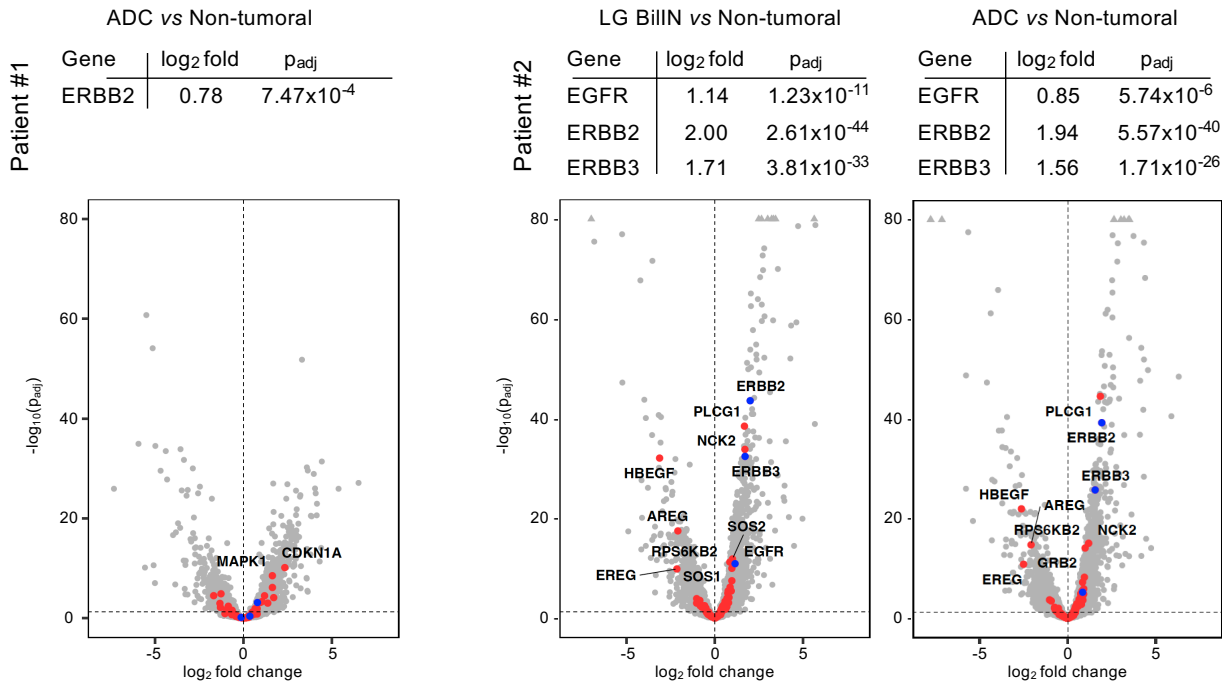


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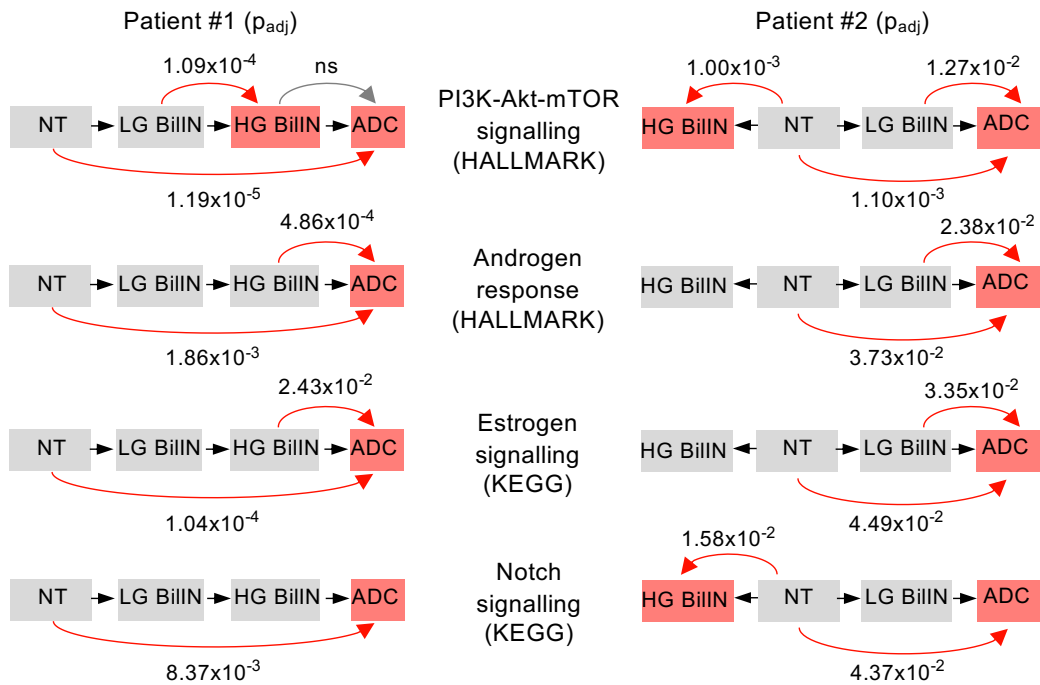


A**B****C**

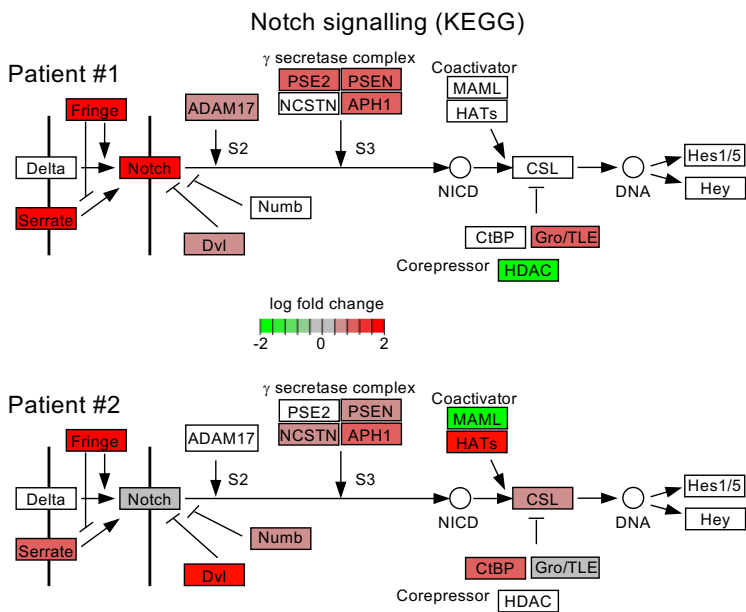
A ERBB signalling



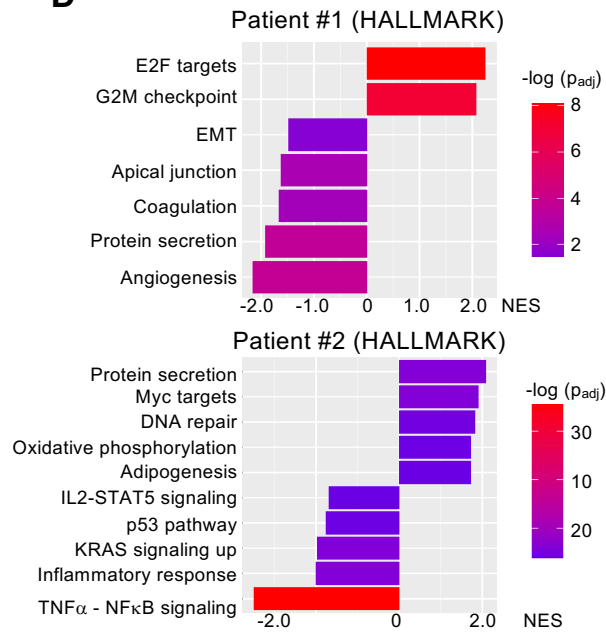
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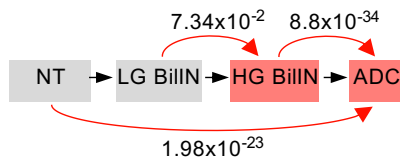
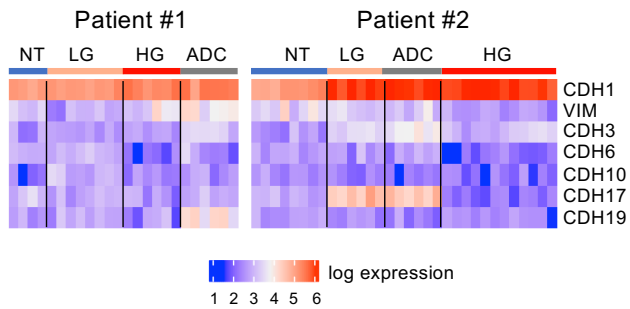
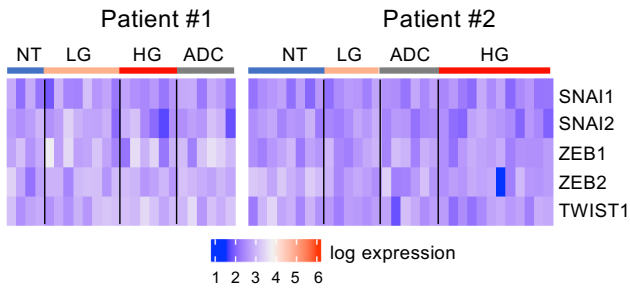
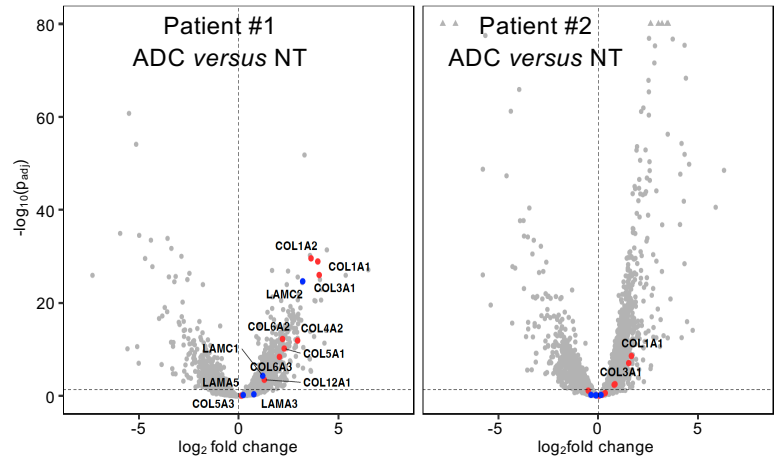
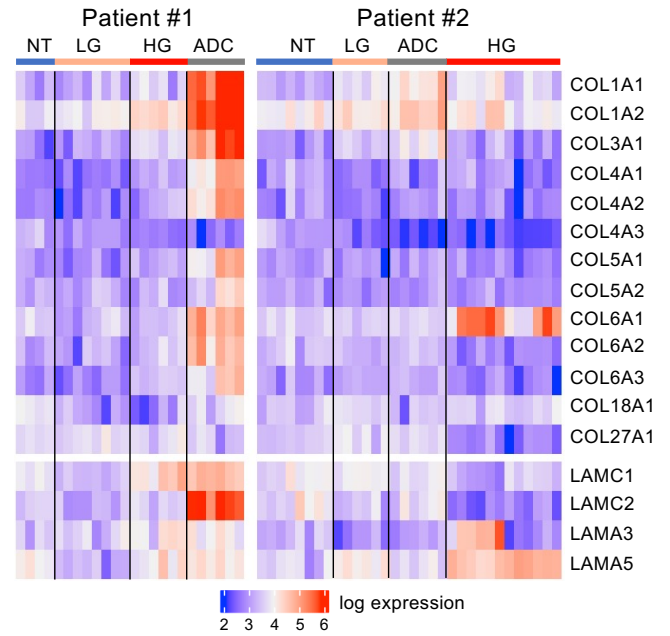
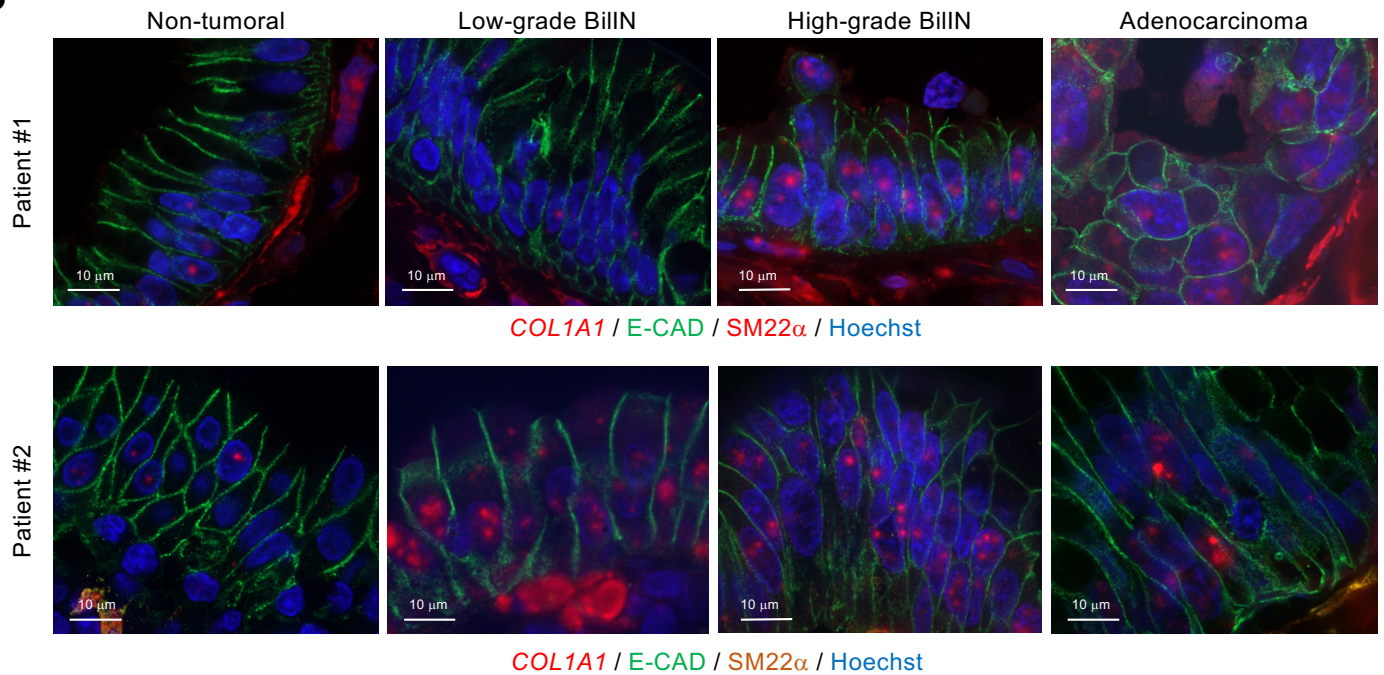


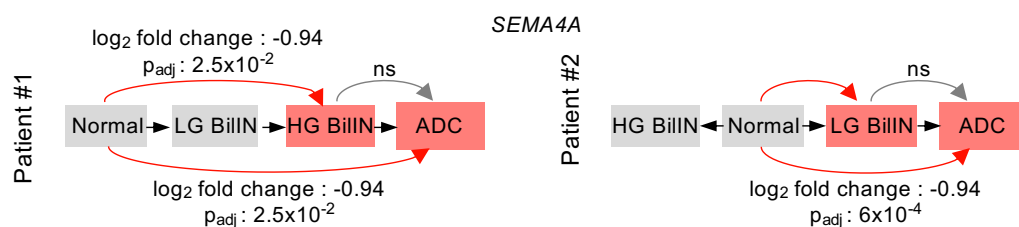
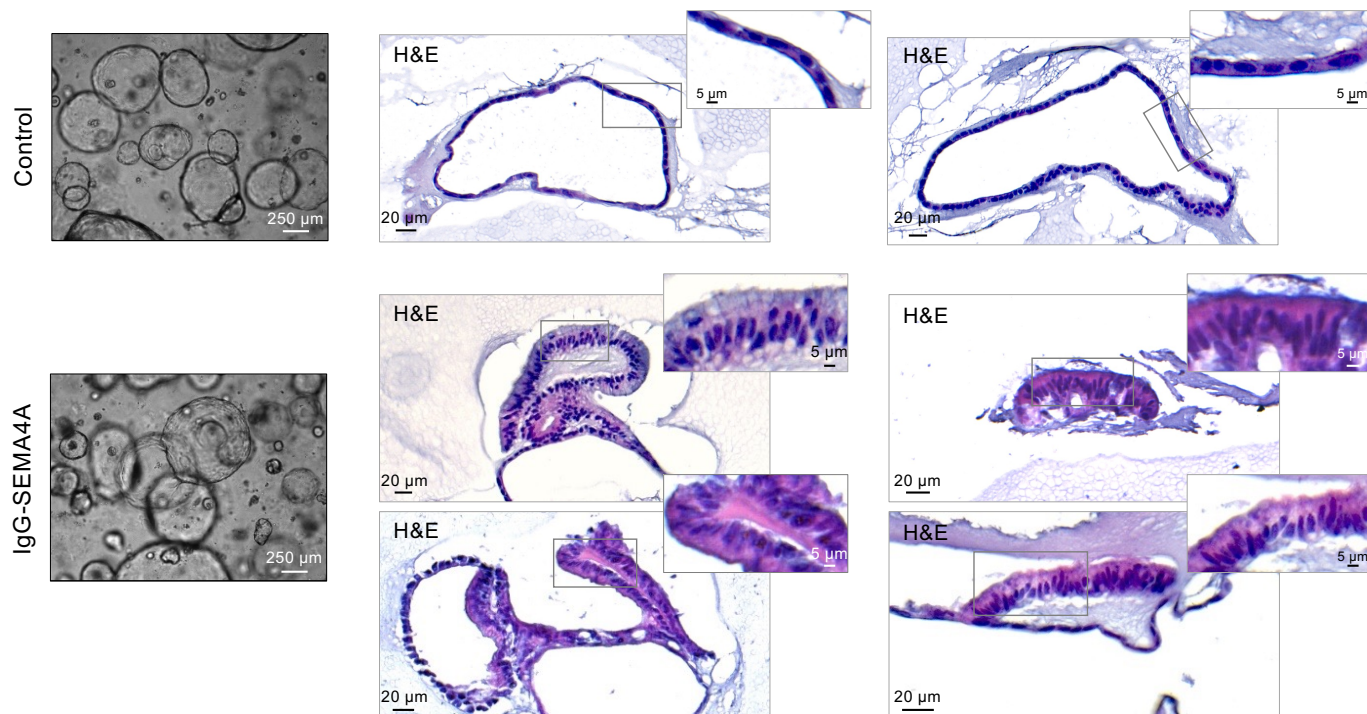
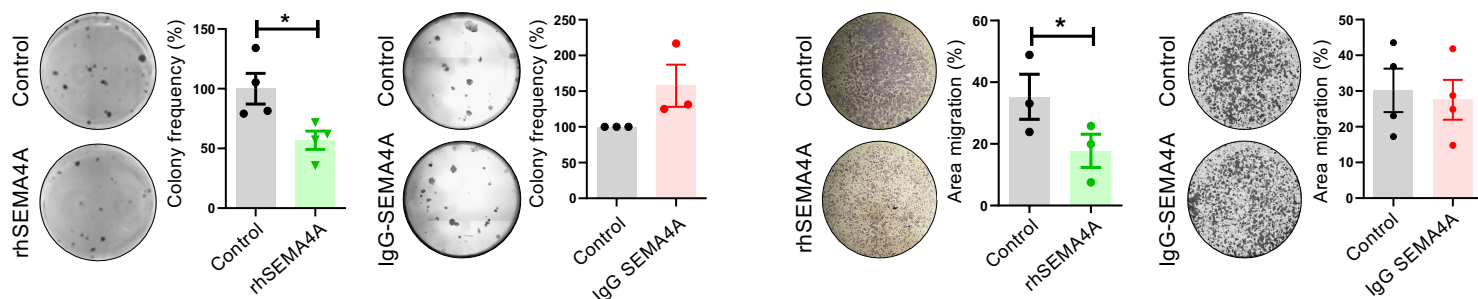
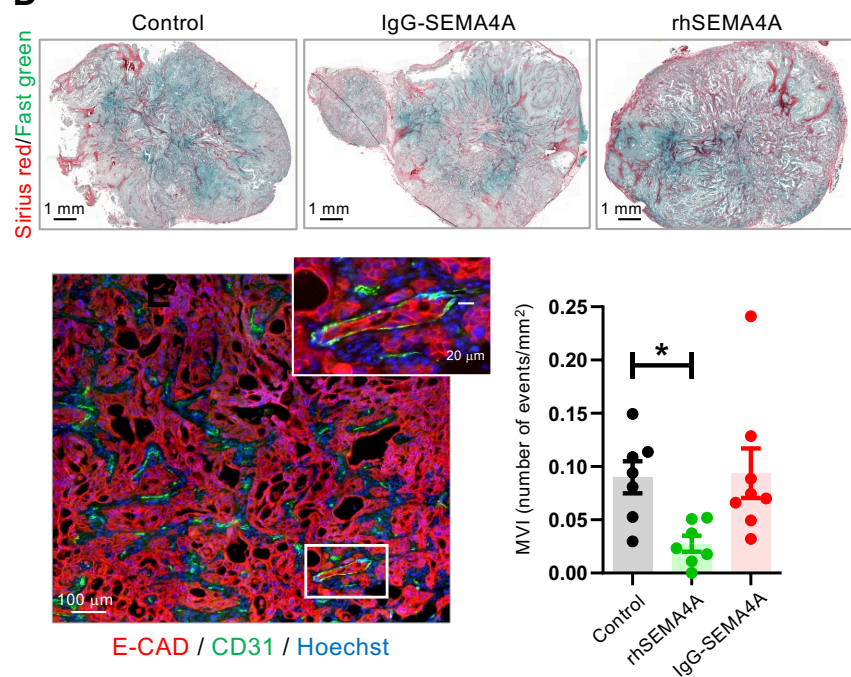
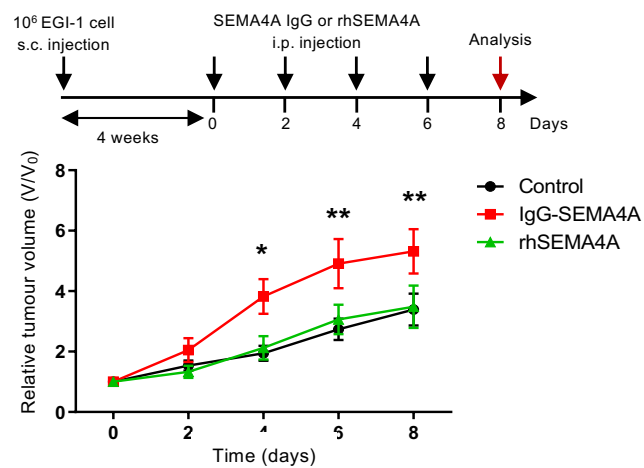
C



D



AEMT: Patient #1 (p_{adj})**B****C****D**

A**B****C****D****E**

ADDITIONAL FILE 1

SUPPLEMENTARY METHODS

Spatial transcriptomics. Spatial profiling was performed on 5 μ m-thick formalin-fixed paraffin-embedded (FFPE) tissue sections using GeoMx technology (NanoString Technologies, Seattle, WA, USA) ¹ which was implemented by NanoString. FFPE human gallbladder slides were baked for 1 h at 65°C, and subsequently processed on Leica automation platform with a protocol that includes three steps: slide baking, antigen retrieval for 20 min at 100°C, and 1.0 μ g/ml proteinase K treatment for 15 min. After taking the slides off the Leica automation platform, each slide was incubated overnight with the GeoMx Whole Transcriptome Atlas assay probe cocktail which contains 18,677 probes. The following day, the slides were washed and immunostained for markers of epithelium (anti-panCytokeratin), leukocytes (CD45), α smooth muscle actin (α SMA)-expressing mesenchymal cells, and cell nuclei (HuR), using the following antibodies: anti-CD45 (CST, #13917); anti- α SMA (AbCam, #ab202368); anti-HuR (Santa Cruz, #SC5261); anti-panCytokeratin (Novus, NBP2-33200AF488). Fluorescently-labeled slides were loaded onto a GeoMx machine and were scanned for selection of regions of interest (ROIs). Barcodes were collected and subsequent barcoding reading was performed on Illumina NGS platform.

Immunofluorescence and immunohistochemistry.

Supplementary Table S1. List of primary and secondary antibodies

Primary antibody	Host	Company and reference	Dilution
CK7	Mouse	Leica Biosystems #CK7-OVTL-L-CE	1:100
E-CADHERIN	Mouse IgG2	BD #610182	1:300
SM22 α	Rabbit	Proteintech #10493-1-AP	1:50
SOX9	Rabbit	Abcam #ab5535	1:100

Secondary antibody	Host	Company and reference	Dilution
Alexa Fluor 488 anti-rat	Donkey	ThermoFisher Scientific #A-21208	1:500
Alexa Fluor 488 mouse igG2a	Goat	ThermoFisher Scientific A-21131	1:500
Alexa Fluor 594 anti-mouse	Donkey	ThermoFisher Scientific #A-21203	1:500
Alexa Fluor 594 anti-rabbit	Donkey	ThermoFisher Scientific A-21207	1:500

Gallbladder organoid culture. Human non-tumoral gallbladder tissues were obtained from patients who underwent cholecystectomy at the Cliniques Universitaires Saint-Luc, Brussels, Belgium. We adapted the method of Rimland and coworkers ². Gallbladders were drained of bile and a small piece of the gallbladder body was excised, washed with Hanks Balanced Salt Solution (HBSS; Gibco/Thermo Fisher Scientific, Brussels, Belgium) and scraped along the luminal mucosal surface to mechanically dissociate the epithelium in Advanced DMEM/F-12 medium (Gibco). The cell suspension was washed twice through centrifugation at 300 g for 5 min and the cell pellet was resuspended in growth factor-reduced Matrigel (Corning/Merck, Hoeilaart, Belgium) before plating in droplets of 50 μ l in a 24-well plate. After Matrigel solidification, media was added in the presence of 10 μ M ROCK inhibitor Y-27632 (SigmaAldrich/Merck, Hoeilaart, Belgium) for the first 48 h after isolation or passage. The

organoid culture medium consisted of 500 μ l Advanced DMEM/F-12 containing 1x N2-serum free supplement (Gibco), 1x B27-serum free supplement (Gibco), 2 mM L-glutamine (Gibco), 1% Penicillin-Streptomycin (Gibco), 20% R-Spondin-conditioned media, 3 μ M CHIR 99021 (Sigma), 100 ng/ml recombinant human noggin (SigmaAldrich), 2.5 μ M PGE2 (R&D Systems/Bio-Techne, Abingdon, United Kingdom), 100 ng/ml recombinant human Epidermal Growth Factor (EGF; R&D Systems), 5 μ M A 83-01 (PreproTech/Thermo Fisher Scientific, Brussels, Belgium) and 10 μ M forskolin (MedChem Express/Bio-Connect, Huissen, The Netherlands). Media was changed every 2-3 days.

Four organoid lines were developed. The first line displayed trisomy 20 and 16q deletion, the second and third lines failed to expand. The fourth line grew as expected and was selected for further studies. Karyotyping (GTG banding) of this line revealed no anomaly and was performed as described ³ with the following adaptations: Cells were centrifuged at 405 g, and slides were prepared in a temperature- and humidity-controlled environment chamber. HBSS was replaced with PBS, and a 2.5% trypsin solution (Thermo Fisher Scientific) was used. Slides were dried from 16 to 24 h in a dry oven at 70°C before staining with a 1.5% Giemsa solution (CellaVision - RAL Diagnostics, Martillac France). The Permount mounting medium was replaced with DPX (SigmaAldrich), and slides were briefly immersed in toluene (VWR Chemicals/Avantor, Leuven, Belgium) before the embedding step. Metaphases were selected with the Metafer scanning platform (v4.3; MetaSystems/Amplitech Corneilles-en-Parisis, France) and analysed with Ikaros software (v6.3; MetaSystems). The coding-region of the genome of the organoid was analysed by whole exome sequencing (WES). Briefly, DNA was extracted using a DNeasy blood and tissue kit (ID 69504, Qiagen, Antwerp, Belgium). Quality controls and WES were performed by MacroGen Europe (Amsterdam, The Netherlands). Briefly, two (TWIST Core Exome plus RefSeq) libraries from the same organoid line were sequenced. Raw data (.fasta files) were aligned to the reference human genome assembly (GRCh38) using BWA 0.7.15 (Li and Durbin, Bioinformatics 2009), with the two libraries submitted together as different read

groups. Aligned sequences (.bam file) were processed using Samtools 1.12 "MarkDup" (for marking duplicates) and GATK 4.2 "BQSR" (for base quality scores recalibration). This yielded a coverage depth of 332X (with duplicates removed). Germline single-nucleotide variants and small indels were identified using GATK 4.2 "Haplotype Caller" while somatic single-nucleotide variants (SNV) and small indels were identified using Mutect2 (both following Broad Institute best practices). The variant calls (.vcf) files generated were annotated, imported and further analyzed on Highlander 17.18 (<https://sites.uclouvain.be/highlander/>), the in-house bioinformatics framework of the Genomics and Bioinformatics platform of UCLouvain (PGEN: <https://www.deduveinstitute.be/pgen-bioinformatics>) for variant annotation, filtering and visualization, with a local database currently containing more than 4300 WES categorized by pathology. We filtered for variants in genes described as frequently mutated in gallbladder cancer according to Kuipers *et al.* ⁴. An ERBB3^{R675G} missense mutation was detected, at an allelic fraction of 0.021.

To analyse the impact of blocking SEMA4A in gallbladder organoids, the latter were split and plated. After 24 h, SEMA4A antibody (IgG-SEMA4A, #14-1002-82 eBioscience/Thermo Fisher scientific, Brussels, Belgium) was added into the medium (10 µg/ml) and organoids were grown for 3 days. Prior incubation, SEMA4A antibody was desalted using a Zeba Spin Desalting Column (Thermo Fisher Scientific).

RNA extraction and Reverse Transcription-quantitative PCR. Human gallbladder organoids were purified from the Matrigel dome using Cell Recovery Solution (Corning) and RNA was extracted using RNAqueous™-Micro Total RNA Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. cDNA synthesis was performed with MMLV reverse transcriptase (Thermo Fisher Scientific) according to manufacturer's protocol. Gene expression was quantified by qPCR using Kappa Syberfast qPCR kit (Sopachem, Nazareth, Belgium) using the following conditions: 3 min at 95°C, 40 cycles of denaturation (3 sec at 95°C), annealing and extension (30 sec at 60°C). *RPL19* was used as internal control.

Oligonucleotides used in qPCR are described in the table below. Quantification of gene expression was performed using the 2^{(-Delta Delta C(T))} method and was expressed as a ratio of target to *RPL19* levels. Each amplification was performed in duplicate.

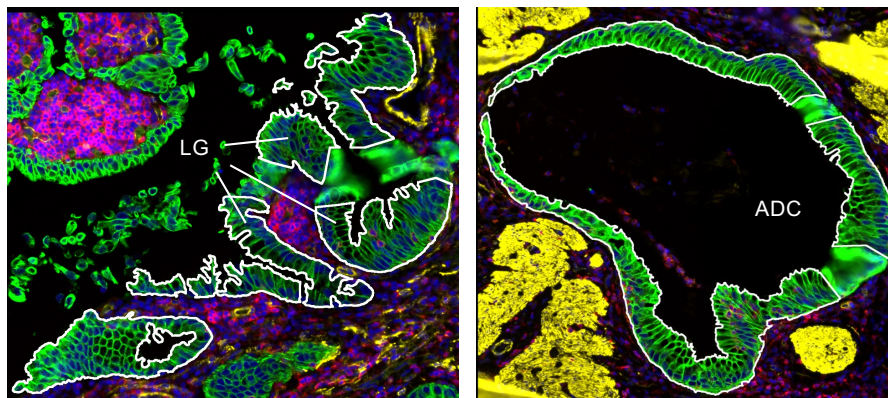
References

1. Merritt CR, Ong GT, Church SE, *et al.* Multiplex digital spatial profiling of proteins and RNA in fixed tissue. *Nat Biotechnol* 2020; 38: 586-99.
2. Rimland CA, Tilson SG, Morell CM, *et al.* Regional differences in human biliary tissues and corresponding in vitro derived organoids. *Hepatology* 2021; 73: 247-67.
3. Howe B, Umrigar A, Tsien F. Chromosome preparation from cultured cells. *J Vis Exp* 2014;e50203.
4. Kuipers H, de Bitter TJJ, de Boer MT, *et al.* Gallbladder Cancer: Current Insights in Genetic Alterations and Their Possible Therapeutic Implications. *Cancers (Basel)* 2021; 13: 5257.

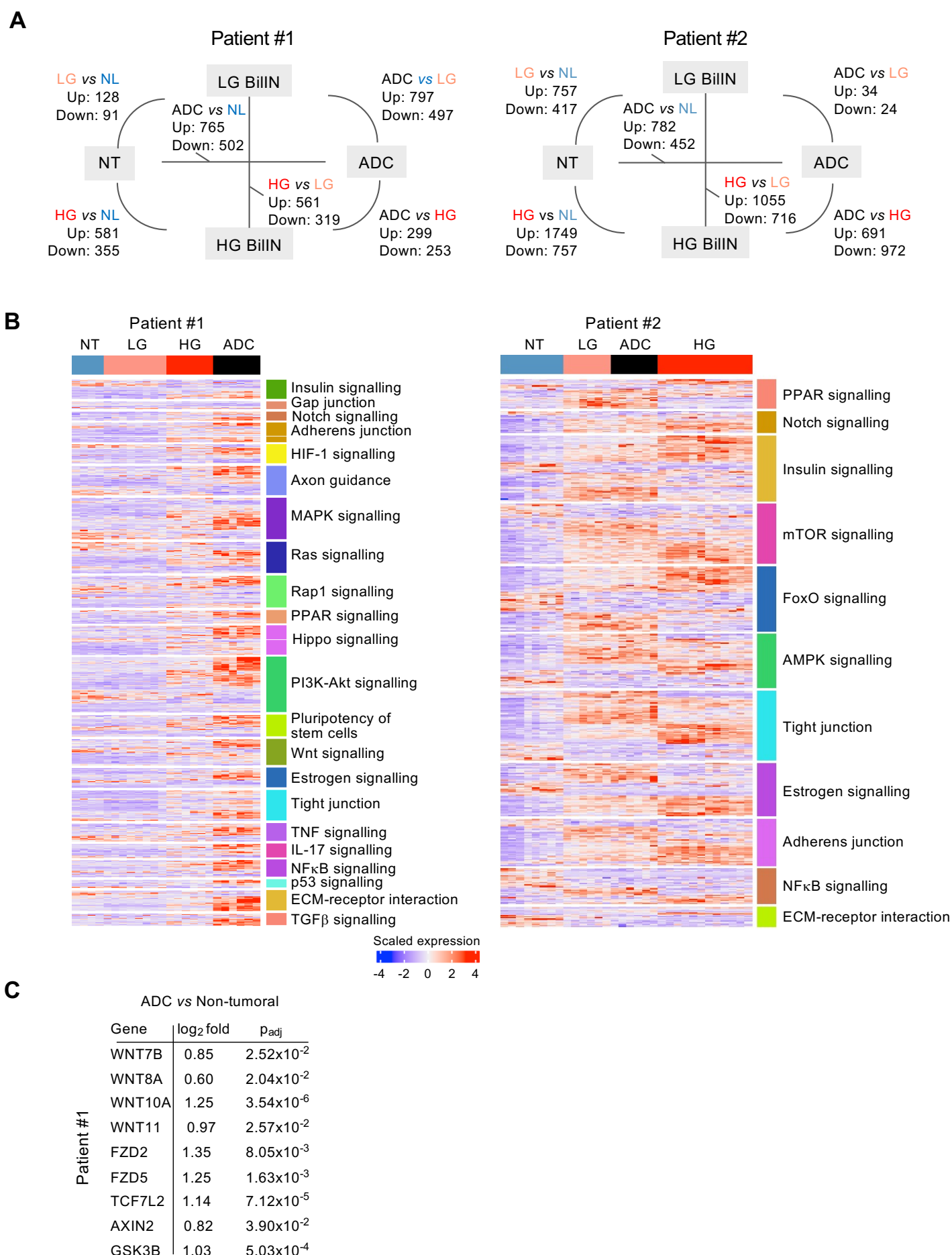
Target		Forward Primer	Reverse Primer
RPL19	H	5'-CGAATGCCAGAGAAGGTCAC-3'	5'-CCATGAGAATCCGCTTGTTT-3'
SEMA4A	H	5'-AATGGCTCCCTCTTGCTGAT-3'	5'-GACTGGAGGTGTTGCTCTCT-3'
PLXNB1	H	5'-CCTCACCTTTGATGGGACCT-3'	5'-GCACATCACACCAGAACCAG-3'

Additional File 2: Supplementary figures

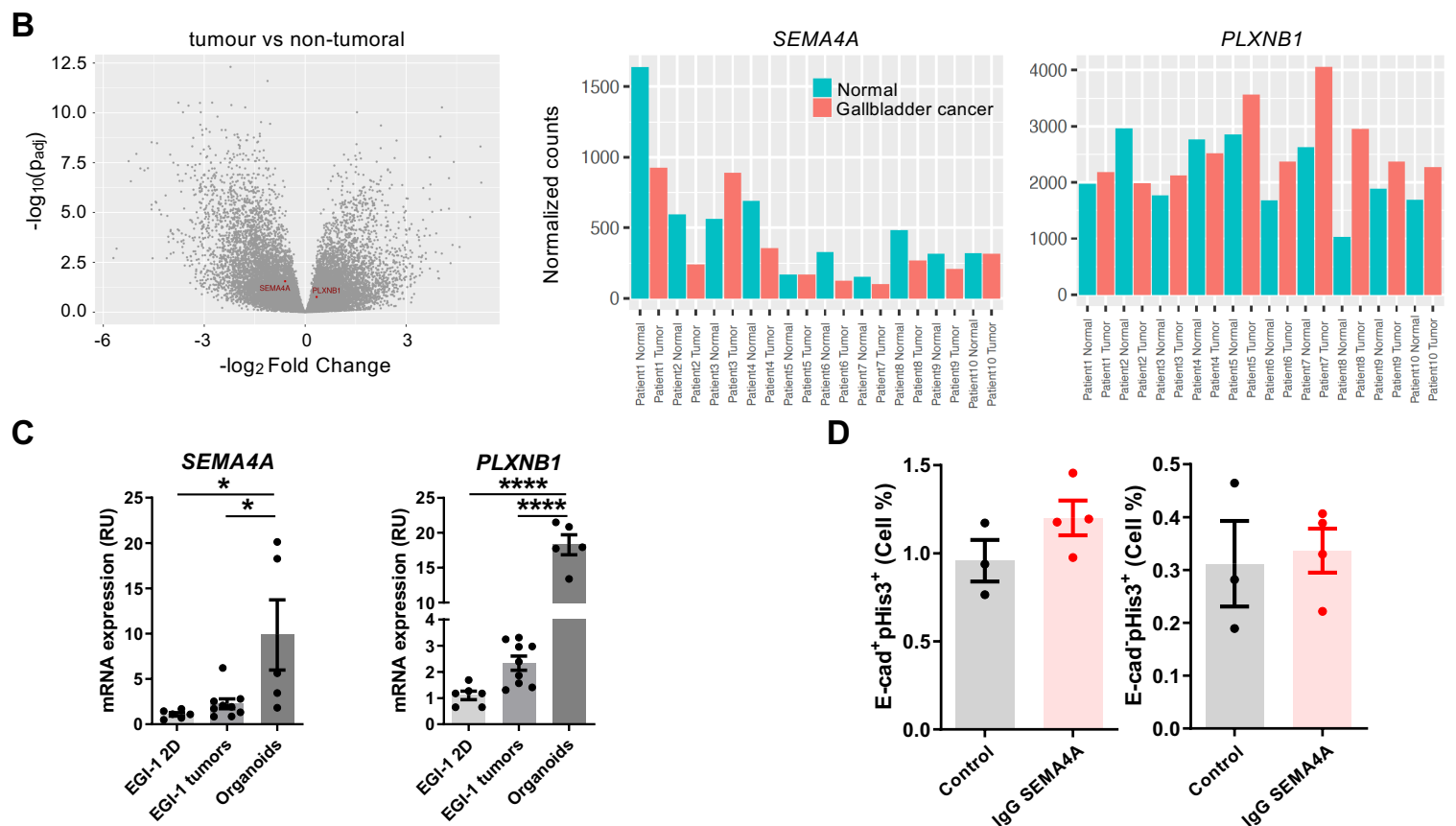
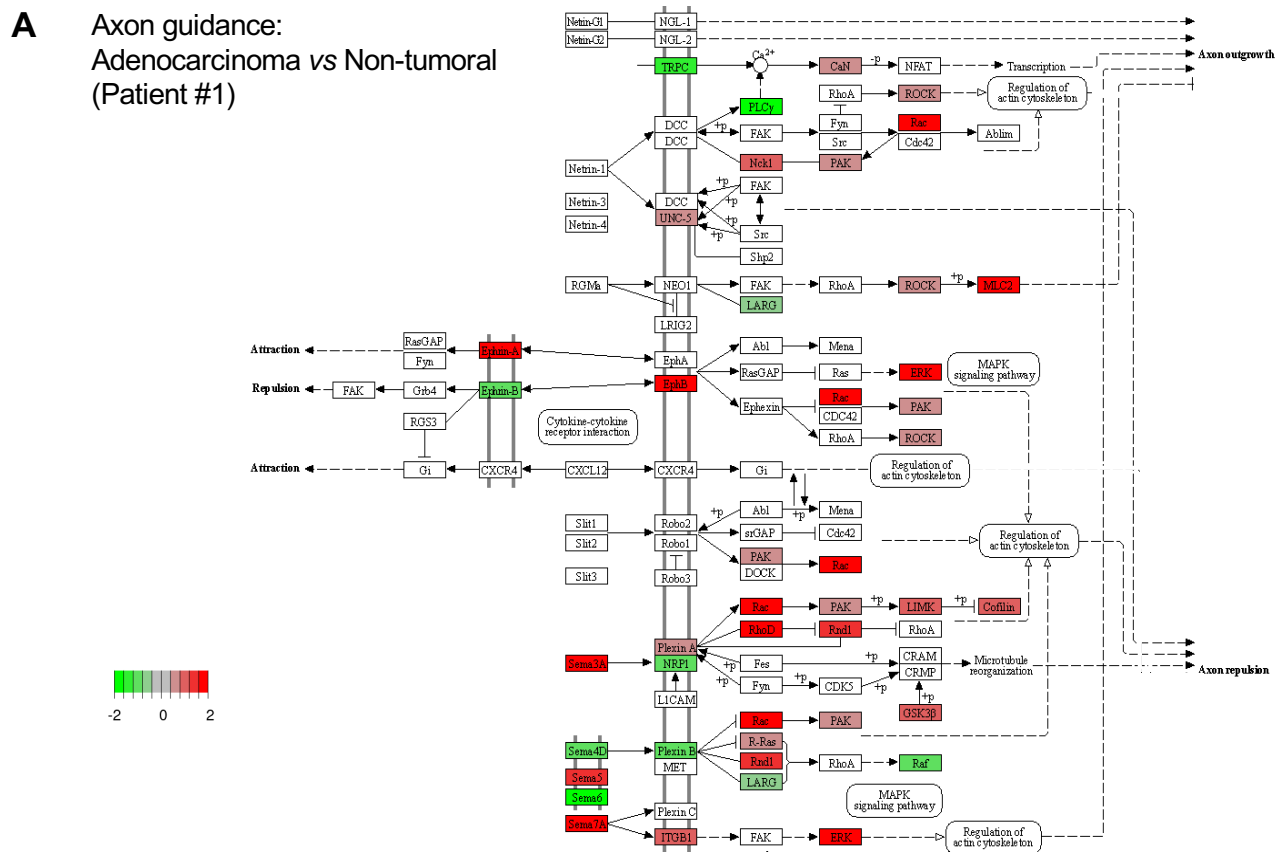
pan-cytokeratin / CD45 / aSMA / HuR



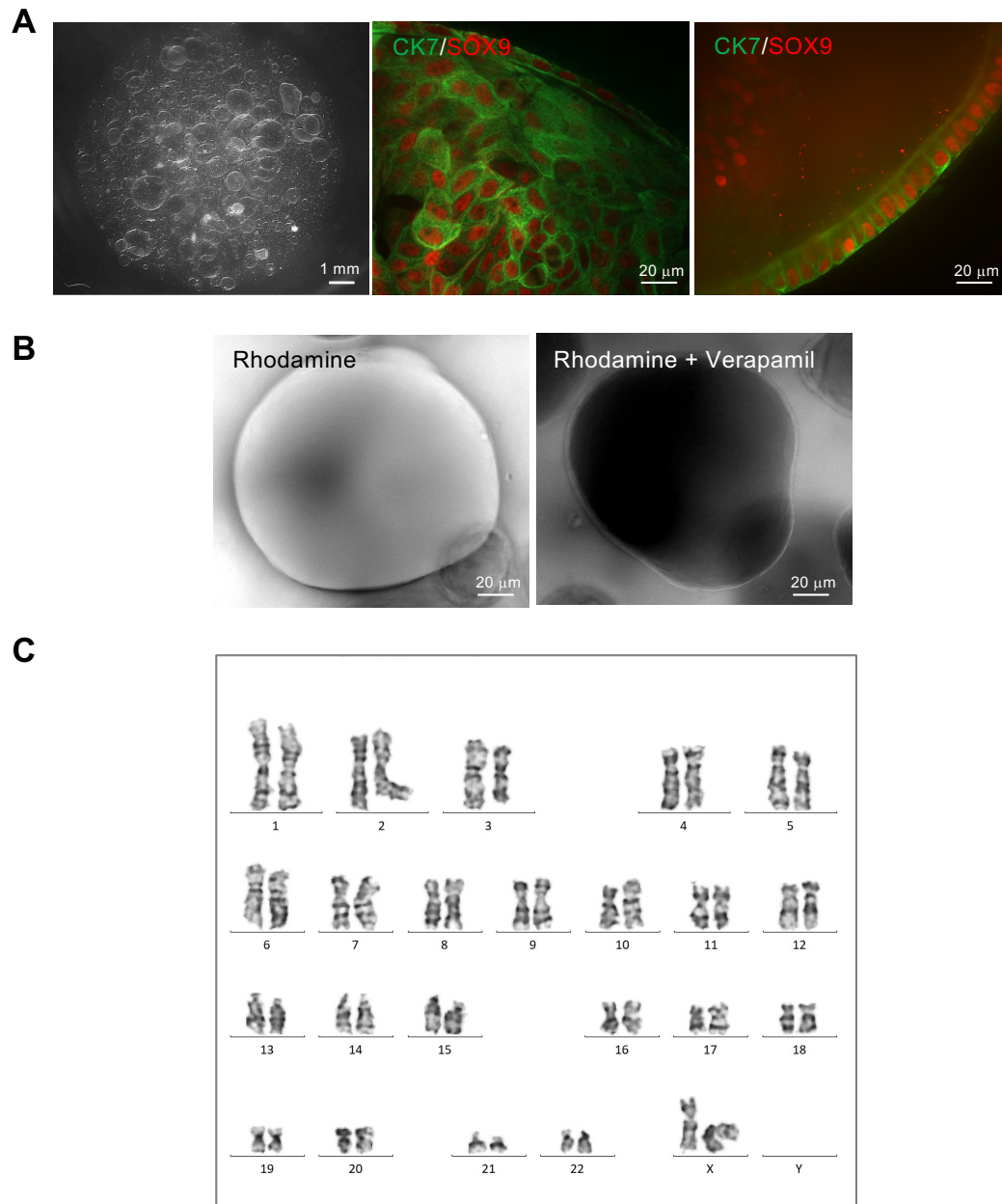
Supplementary Fig. S1. Delineation of ROIs for spatial transcriptomic analysis. Illustrative examples of gallbladder sections of Patient #2 with delineation (white line) of epithelial ROIs subjected to spatial transcriptomic analysis. ADC, area with adenocarcinoma; LG, area with low-grade biliary intraepithelial neoplasia (BillN); NI, area with histologically normal epithelium.



Supplementary Fig. S2: Differential gene expression in spatial transcriptomics. A Number of genes up- or downregulated in cross-comparison of tissue types ($\log_2$ fold change ≥ 1.0 ; $p_{adj} \leq 0.05$). **B** Heatmap of genes from the KEGG pathway gene sets that are differentially expressed between adenocarcinoma and normal epithelium ROIs in the two patients ($p_{adj} \leq 0.05$). **C** List of upregulated Wnt signalling genes in Patient #1. ADC, adenocarcinoma; HG, high-grade biliary intraepithelial neoplasia (BiIN); LG, low-grade BiIN; NT, non-tumoral epithelium.



Supplementary Fig. S3: Expression of axon guidance genes and function of SEMA4A. **A** Axon guidance pathway (KEGG) enriched in Patient #1 during tumour progression. **B** Volcano plot illustrating differentially expressed genes between gallbladder cancer and adjacent non-tumoral tissue. *SEMA4A* expression is significantly reduced in tumour samples ($\log_2$ fold change = -0.597; p_{adj} = 0.0282), but no significant difference between cancer and non-tumoral tissue was noticed for *PLXNB1* ($\log_2$ fold change = 0.339; p_{adj} = 0.173) (left panel). Pairwise comparison of tumour and matched non-tumoral tissues shows the expression of *SEMA4A* and *PLXNB1* in individual patients (middle and right panels). Raw counts from gallbladder cancer and adjacent normal tissue were downloaded from GEO (GSE139682). Differential expression analysis was performed with DESeq2 (v 1.44) using default parameters and a paired design (tissue + patient). **C** Expression of *SEMA4A* and its receptor *PLXNB1* in cultured EGI-1 cells, EGI-1-derived subcutaneous tumours, and cultured gallbladder organoids, as measured by RT-qPCR. *SEMA4A* and *PLXNB1* primers are human-specific and do not detect potential expression in murine cells colonising EGI-1-cell derived tumours. One-way ANOVA was used to compare means (*, $p < 0.05$; ****, $p < 0.0001$). **D** Percentage of E-CADHERIN (E-CAD)-positive cells and E-CADHERIN-negative cells expressing phospho-Histone H3 (pHis3) in EGI-1-cell-derived tumours at day 8 of treatment with PBS (= control) or blocking anti-SEMA4A IgG antibodies. No significant difference was detected using unpaired t-test.



Supplementary Fig. S4. Characterisation of gallbladder organoids. **A** Gallbladder organoids were grown in Matrigel and visualised using light microscopy (left panel). Immunostaining revealed expression of the biliary-specific proteins CYTOKERATIN 7 (CK7) and SRY-related HMG box transcription factor 9 (SOX9). **B** Intraluminal accumulation of Rhodamine (100 μ M) that was added to the organoid medium was inhibited by Verapamil (10 μ M), demonstrating the functionality of MDR1-mediated transport. **C** Normal karyotype of the selected organoid line.