Review

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The spatiotemporal ecosystem of gallbladder cancer: From inflamed areas to invasive margins — Integrating biomechanical injury, the bile microenvironment, precursor lineages, and spatial niches

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SUMMARY: Gallbladder cancer (GBC) is usually described as the end point of chronic cholelithiasis-associated inflammation, and yet this linear formulation does not explain why only a small fraction of people with gallstones develop cancer, why histologically similar precursor lesions follow different evolutionary routes, or why invasion preferentially advances through anatomically and immunologically distinct interfaces. We propose a spatiotemporal ecosystem model that links five domains: gallstones and reflux as precipitating conditions; bile as a chemically active habitat; resident or transient microorganisms as context-dependent modifiers; metaplastic, biliary intraepithelial and intracholecystic precursor lineages as alternative epithelial trajectories; and stromal-immune niches that select for invasive phenotypes. Evidence from epidemiology, pathology, genomics, organoid and animal models, single-cell sequencing, and spatial profiling is integrated while preserving distinctions between association, mechanism, and clinical prediction. The strongest causal chain currently links prolonged mechanical or reflux injury with cycles of epithelial loss and repair, metaplastic or dysplastic changes, and acquisition of driver alterations. Microbial findings are biologically plausible and increasingly supported by functional Salmonella studies, but most human biliary microbiome datasets remain small and vulnerable to low-biomass contamination and treatment-related confounding. Precursor studies substantiate the existence of both BilIN progression in affected areas and alternative polypoid or BilIN-independent routes, arguing against a single obligatory sequence. Recent single-cell and spatial studies have identified organized, region-specific interactions among malignant epithelial states, macrophages, exhausted CD8 T cells, neutrophils, endothelial cells, fibroblasts, hepatocytes, and nerves at invasive margins. Liver-facing, serosal, perineural, lymphovascular, and nodal interfaces should not be assumed to share one ecology. These data shift the central question from which mutation is present to where a clone resides, which neighboring cells sustain it, and when the niche becomes permissive. The model suggests testable priorities: longitudinal sampling of high-risk gallbladders, paired bile-mucosa profiling with rigorous controls, lineage-resolved multi-section spatial analysis, and boundary-aware biomarkers validated in prospective cohorts.

Keywords: gallbladder cancer, gallstones and bile, spatiotemporal ecosystem model, stromal-immune niches, spatial transcriptomics, invasive margin

1. Introduction: Why gallbladder cancer requires a spatiotemporal model

Gallbladder cancer (GBC) is uncommon globally but highly lethal, with a pronounced geographic and ethnic concentration and a persistent tendency to present after invasion of the liver or regional structures. Contemporary syntheses have established its epidemiology, pathology, molecular alterations, and clinical management, but most frameworks still arrange these elements as a list of risk factors followed by a linear histological sequence (*1-4*). That arrangement is useful for describing the cancer but not for indicating the mechanism of its onset. Gallstones are common, whereas GBC is rare; chronic cholecystitis is widespread, whereas dysplasia is focal; and invasive behavior varies distinctly in regions of the same tumor. A

model that generalizes the entire organ or tumor therefore loses information that is central to causation.

The term "ecosystem" is used here in a restricted, testable sense. It refers to interactions among epithelial clones, bile constituents, microorganisms, immune cells, stromal cells, and the local anatomy of the gallbladder over time. An affected "area" denotes a mucosal region altered by repeated injury and repair, not proof that every cell in that area is premalignant. A invasive "margin" denotes the interface through which tumor cells enter adjacent liver or other tissues. This vocabulary helps separate three questions that are often conflated: what creates a susceptible mucosal region; which epithelial lineage acquires and retains malignant competence; and which local niche permits invasion.

This narrative review focuses specifically on GBC. PubMed-indexed and major peer-reviewed studies available through August 2026 were prioritized, with an emphasis on original human cohorts, systematic reviews, mechanistic infection models, precursor lesion studies, multi-region genomics, single-cell sequencing, and spatial profiling. Studies of cholangiocarcinoma or pan-biliary cancers are cited only when they provide an indispensable methodological or therapeutic context. Because the evidence base is heterogeneous, statements are explicitly calibrated as an epidemiological association, histological inference, experimental mechanism, prognostic association, or clinical-trial evidence (Figure 1).

2. Creation of an inflamed area

2.1. Gallstones are the predominant precipitator of an inflamed area's creation but not a sufficient cause

The most reproducible environmental factor associated with GBC is cholelithiasis. A recent field-wide review identified 215 studies and performed 350 meta-analyses, and its results confirmed that a biliary infection is closely associated with gallbladder cancer while adiposity and related metabolic factors were somewhat associated; however, heterogeneity, reverse causation, and residual confounding remained important limitations (*5*). A separate global analysis of 115 studies encompassing more than 32 million participants estimated a gallstone prevalence of 6.1%, with substantial geographic and sex variation, underscoring the mismatch between a common factor and a rare cancer outcome (*6*). Classic clinical reviews and modern pathology agree that gallstones should be understood as a prolonged source of mechanical abrasion, obstruction, bile stasis, and recurrent epithelial injury rather than as a direct cause of cancer in every carrier (*7,8*).

Evidence from cholecystectomy specimens substantiates the concept of an affected area. In a study of 350 gallstone-bearing gallbladders, hyperplasia, metaplasia, dysplasia, and carcinoma in situ were identified at different frequencies, and molecular loss-of-heterozygosity accumulated across the pathological continuum (*9*). A meta-analysis has also linked gallstones and their characteristics to the risk of biliary tract cancer, but estimates vary with stone ascertainment, latency, anatomical site, and the choice of stone-free or stone-bearing controls (*10*). These observations establish population-level associations and mucosal plausibility, but they do not show that an individual gallstone will initiate a particular tumor clone.

Obesity, diabetes, and smoking may make an affected area more susceptible through bile supersaturation, systemic inflammation, altered motility, oxidative stress, or shared lifestyle determinants. Meta-analyses reported an increased risk of GBC with obesity, with higher figures in women, and less of an association

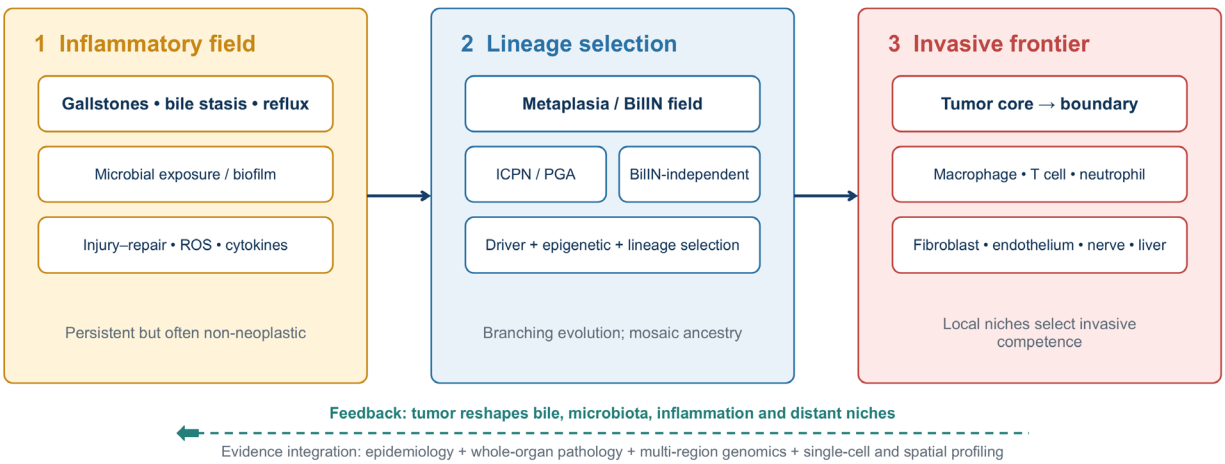


Figure 1. A spatiotemporal ecosystem model of gallbladder carcinogenesis. Prolonged injury creates an inflamed area; epithelial lineages then branch through BilIN-associated, polypoid precursor-associated, or apparently independent routes; and local stromal-immune niches select for invasive competence. The dotted arrow indicates feedback from established cancer to bile, microbiota, inflammation, and distant niches. *Abbreviations:* BilIN, biliary intraepithelial neoplasia; ICPN, intracholecystic papillary neoplasm; PGA, pyloric gland adenoma; ROS, reactive oxygen species.

with overweight (*11,12*). Type 2 diabetes was associated with an approximately 1.5-fold pooled risk in observational studies, although confounding by adiposity and gallstones could not be fully eliminated (*13*). Smoking was associated with a pooled relative risk of approximately 1.45 in an analysis dominated by case-control studies (*14*). These effect sizes are materially smaller than those reported for chronic biliary infection or selected anatomical abnormalities and should not be presented as equivalent causal drivers.

Host susceptibility is also non-random. A genome-wide association study in India implicated variants near ABCB1 and ABCB4, genes related to hepatobiliary transport, while population studies in Shanghai showed that a family history of gallstones clustered with gallstone and biliary cancer risk (*15,16*). The most defensible interpretation is interaction: inherited or acquired differences in bile handling and inflammation may determine which stone-bearing mucosa sustains prolonged injury, but no current germline marker alone provides support for population screening for GBC.

2.2. Pancreaticobiliary maljunction (PBM) as a natural experiment in reflux-driven carcinogenesis

PBM provides unusually coherent evidence that anatomy can create an area where cancer is more likely to develop. In a Japanese nationwide survey of 2,529 patients, adults with congenital biliary dilatation had a bile duct cancer frequency of 6.9% and a GBC frequency of 13.4%; among adults with PBM without dilatation, the corresponding frequency of bile duct cancer was 3.1% and that of GBC was 37.4% (*17*). Because the pancreatic and bile ducts join outside sphincter control, pancreatic juice can reflux into the gallbladder, where activated enzymes, secondary bile acids, and prolonged stasis repeatedly injure the mucosa (*18*).

Histopathological studies of "reflux-associated cholecystopathy" describe diffuse mucosal hyperplasia and proliferative changes that precede carcinoma, providing a morphological bridge between abnormal anatomy and neoplasia (*19*). Importantly, PBM-associated GBC is not restricted to East Asian practice: a Western pathological series identified comparable lesions and distinctive clinicopathological features, corroborating PBM as a general model of anatomy-induced physicochemical carcinogenesis rather than a regional curiosity (*20*). PBM nevertheless represents a specific high-risk pathway and should not be used to infer that pancreatic reflux drives most gallstone-associated cancers.

2.3. Chronic infection: Strongest for Salmonella, uncertain for a universal biliary microbiome

Chronic Salmonella carriage has the most substantiated evidence chain for infection leading to GBC. Using anti-Vi serology and culture-based definitions, a Chilean case-control study combined with a meta-analysis found an increased GBC risk, although confidence intervals in the primary study were wide (*21*). An earlier systematic review of 17 studies estimated an overall odds ratio of 4.28 for chronic Salmonella typhi carriage, with stronger associations in South and Southeast Asian datasets (*22*). Detection of non-typhoidal Salmonella DNA in tumor specimens widened the question beyond S. typhi (*23*).

Mechanistic work has shifted the association beyond serology. In epithelial and organoid models, Salmonella manipulation of MAPK and AKT signaling promoted transformation-related phenotypes, and chronic infection accelerated tumor formation in susceptible experimental settings (*24*). More recent triangulation across human microbiomes, cultured S. typhimurium isolates, gallstone-bearing mice, GBC cells, and xenografts implicated KDM6B-dependent host epigenetic remodeling; KDM6B inhibition reduced tumor growth in the experimental model (*25*). This does not prove that Salmonella is required for human GBC, but it establishes a biologically coherent route by which persistent bacteria can cooperate with gallstones and inflammation.

Evidence for Helicobacter species is less consistent. A multicountry analysis detected Helicobacter DNA in some gallbladder and extrahepatic biliary cancers, but differences in sampling, assays, geography, and contamination control preclude a universal conclusion (*26*). The literature on infection therefore supports a hierarchy: Salmonella has convergent epidemiological and functional evidence; other taxa are candidates whose effects may be restricted to particular ecological and geographic contexts (Table 1).

3. Bile and microbiota: A coupled but technically fragile habitat

3.1. Human sequencing studies show dysbiosis but have yet to demonstrate a reproducible cancer-specific consortium

Bile is not merely a carrier fluid. It is a concentrated mixture of bile acids, phospholipids, cholesterol, proteins, microbial products, and host-derived inflammatory molecules. Gallstone obstruction, infection, fasting, antibiotics, endoscopic instrumentation, and tumor-related cholestasis can each alter that mixture. Consequently, a cross-sectional difference between cancer bile and control bile may be a cause, a consequence, or both.

Small human studies nevertheless provide useful signals. Metagenomic analysis of gallbladder bile from seven GBC and 30 cholelithiasis patients identified distinct microbial communities and candidate organisms, but the small cancer group limited inference (*27*). Shotgun metagenomics of mucosal biopsies subsequently described microbial shifts along the chronic cholecystitis-to-GBC progression, shifting the sampled compartment from free bile towards the tissue-associated habitat

Table 1. Evidence linking major factors or conditions field-forming exposures to the development of gallbladder cancer

Factor or condition	Human evidence	Quantitative signal	Mechanistic interpretation	Principal limitation
Gallstones/chronic cholecystitis	Meta-analyses, case-control studies, cholecystectomy pathology	Strong association, but estimates vary by stone phenotype and comparator (*5,9,10*)	Repeated abrasion, stasis, necrosis-repair cycles, oxidative injury	Common exposure; low absolute progression rate; reverse causation in short-latency studies
Obesity/metabolic disease	Cohort and case-control meta-analyses	Obesity RR about 1.6; diabetes RR about 1.5 (*11-13*)	Cholesterol supersaturation, motility, systemic inflammation	Residual confounding by gallstones and lifestyle
PBM/pancreatic reflux	Nationwide survey and pathology series	Gallbladder cancer reported in 37.4% of adults with non-dilated PBM in the Japanese survey (*17*)	Enzyme-rich reflux, secondary bile acids, diffuse hyperplasia	Rare anatomy; referral and registry effects
Chronic Salmonella carriage	Case-control studies, meta-analysis, cellular and animal models	Pooled odds ratios around 4 in historical meta-analyses (*21,22*)	Persistent colonization, inflammatory signaling, epigenetic remodeling	Geographic concentration; exposure misclassification
Other biliary bacteria	Small sequencing cohorts	Taxa differ across studies (*26-30*)	Biofilm, metabolite production, innate immune activation	Low biomass, antibiotics, obstruction, instrumentation, contamination

(*28*). Bile microbiome profiling across normal, chronic cholecystitis, and GBC groups also found disease-associated dysbiosis (*29*), while a study spanning pancreaticobiliary and gallbladder cancers associated gallbladder microbial composition with prognosis (*30*). A 2024 mechanistic review summarized the emerging bacteria-metabolite-inflammatory pathways but also noted that study designs and taxonomic signals remain heterogeneous (*31*).

The central limitation is low microbial biomass. Contaminating DNA from extraction kits, reagents, endoscopes, the room environment, or batch effects may constitute a substantial fraction of the apparent signal. Salter and colleagues experimentally demonstrated that reagent contamination can critically distort sequence-based microbiome studies, and later guidance for low-biomass research emphasized negative controls, randomized extraction batches, absolute microbial quantification, and transparent contaminant removal (*32,33*). These are not optional refinements for GBC: bile is often collected after antibiotics or instrumentation, and cancer cases frequently differ from stone controls in obstruction, jaundice, hospitalization, and drainage.

3.2. Bile acids, lipids, and microbial products may link injury to selection

Bile acids regulate epithelial physiology through membrane effects and receptor-mediated signaling, while microbial transformation modifies the primary-secondary bile acid pool. General hepatobiliary physiology supports links among bile acids, barrier function, inflammation, FXR/TGR5 signaling, and epithelial stress (*34*). In the gallbladder, however, direct longitudinal evidence that a defined bile acid species initiates a human precursor clone is sparse.

Multi-omics has increased resolution. A 2022 study used a training cohort of 87 bile samples, a metaproteomic subset of 42, and a validation cohort of 38 to derive lipid and microbial-peptide signatures that distinguished GBC from benign gallbladder disease; the reported eight-lipid panel achieved very high diagnostic performance in the study setting (*35*). The result is encouraging but should be treated as a biomarker-development signal and not as a population-ready test because spectrum effects, sample handling, biliary obstruction, and independent external validation determine transportability.

Older biochemical studies also reported increased lipid peroxidation products in GBC bile, consistent with oxidative injury but not specific for carcinogenesis (*36*). Together, these findings justify paired chemical and microbial profiling, but they do not establish that any single bile constituent leads to cancer in humans.

The most plausible synthesis is a feedback loop. Stones or reflux alter flow and damage epithelium; altered bile chemistry and inflammation change microbial fitness; selected organisms or biofilms modify metabolites and innate immune signaling; and the resulting habitat imposes selection on clones repairing the epithelium. The direction and magnitude of each arrow are patient-specific. This formulation avoids the unsupported claim that there is one "GBC microbiome," while preserving the experimentally substantiated possibility that particular microbes, under appropriate chemical and anatomical conditions, can become causal cofactors (Figure 2).

4. Precursor lineages: One area, several evolutionary routes

4.1. The flat metaplasia-BilIN-carcinoma route

The fifth edition of the WHO Classification of Digestive System Tumours recognizes pyloric gland adenoma, biliary intraepithelial neoplasia (BilIN), and intracholecystic papillary neoplasm (ICPN) as distinct gallbladder epithelial precursor or preinvasive entities (*37*). BilIN is a microscopic flat or micropapillary lesion graded in a two-tier system: low-grade BilIN incorporates the former BilIN-1 and BilIN-2 categories, whereas high-grade BilIN corresponds broadly to the former BilIN-3. High-grade BilIN requires extensive sampling to exclude invasion and is difficult to detect by imaging or gross examination (*38*). ICPN, in contrast, is a grossly visible intraluminal mass-forming neoplasm; any associated invasive carcinoma should be documented separately. Contemporary reviews emphasize that BilIN, ICPN, and pyloric gland adenoma are related but not interchangeable, with different morphology, lineage markers, and associated invasive cancers (*39,40*).

The classic route associated with an inflamed area begins with repeated epithelial loss and repair in a stone-bearing gallbladder. Pseudopyloric, foveolar, or intestinal metaplasia may occur, followed by low-grade and high-grade BilIN and then invasive carcinoma. A mapping study showed that intestinal metaplasia and dysplasia cluster in gallbladders with carcinoma and that routine limited sampling under-detected focal lesions (*41*). Cross-sectional age differences led to an estimate of roughly 15 years from dysplasia to advanced carcinoma, but this is an ecological inference from different patients rather than a direct longitudinal observation (*42*). A histological review similarly corroborated a sequence while cautioning that not every metaplasia progresses and that pseudopyloric change is common in benign inflammation (*43*).

Whole-organ mapping reinforces the spatial nature of an affected area. Dysplasia may be patchy, multifocal, and more extensive than the gross lesion, which is why complete or additional sampling is required when high-grade dysplasia is found (*44*). The area therefore contains multiple epithelial states at the same time. Histology alone cannot determine which patch is ancestral to the invasive tumor.

4.2. Polypoid precursors and alternative lineages

ICPN is a macroscopically visible, mass-forming intraepithelial neoplasm. In a clinicopathological study of 123 cases, ICPNs displayed biliary, gastric, intestinal, or oncocytic differentiation and were frequently associated with invasive carcinoma, but the invasive component and outcome differed from conventional flat-pathway GBC (*45*). Gallbladder adenomas also display molecular heterogeneity. The observation that CTNNB1 exon 3 mutations were more frequent in adenomas than carcinomas supports the view that many conventional adenomas are not obligatory intermediates of ordinary GBC (*46*).

Rare invasive phenotypes further caution against collapsing all endpoints into one lineage. A 2024 population-based analysis found that gallbladder signet-ring cell carcinoma presented with more aggressive clinicopathological features and poorer survival than conventional adenocarcinoma (*47*). That study established prognostic heterogeneity but not a distinct precursor sequence: registry morphology cannot

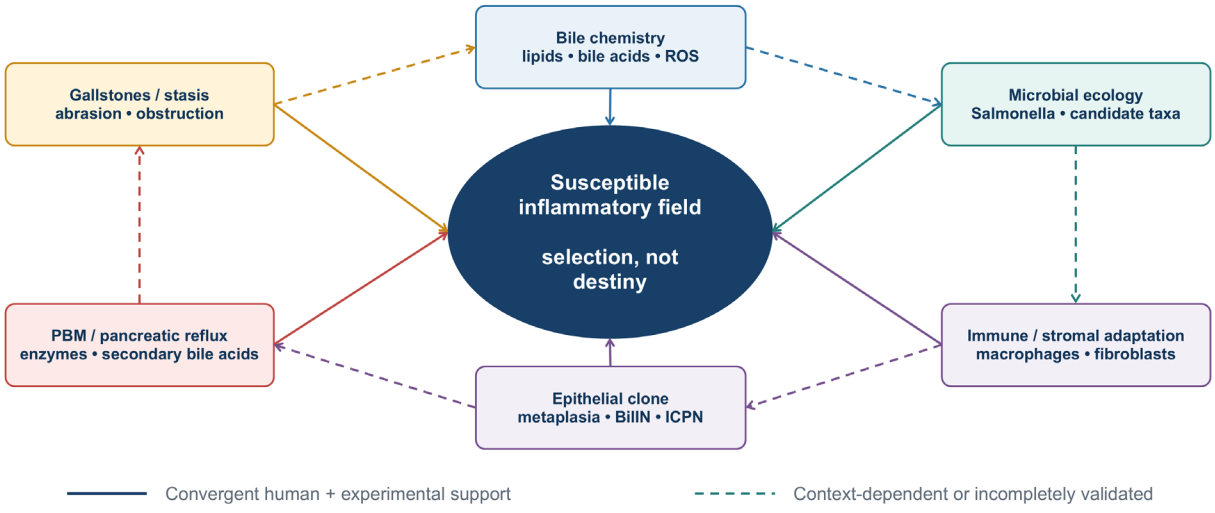


Figure 2. The gallstone-bile-microbiota-mucosa feedback system. Gallstones and pancreaticobiliary reflux have convergent human and mechanistic factors that create affected areas. Microbial, metabolic, immune, stromal, and epithelial components interact bidirectionally, but many individual taxa and metabolites remain context-dependent candidates rather than validated universal causes. *Abbreviations:* PBM, pancreaticobiliary maljunction; BilIN, biliary intraepithelial neoplasia; ICPN, intracholecystic papillary neoplasm; ROS, reactive oxygen species.

determine whether a signet-ring phenotype has arisen through BilIN, ICPN, or an unsampled route.

Early molecular pathology further indicated pathway divergence. KRAS mutations were detectable in subsets of dysplasia and carcinoma (*48*), microsatellite alterations were found in chronic cholecystitis and preneoplastic lesions (*49*), and loss of E-cadherin expression or gene alterations increased across precursor-to-carcinoma progression (*50*). Those studies were limited by small cohorts and older assays, but they anticipated the contemporary conclusion that histologically similar lesions may have different clonal histories.

4.3. Multi-region genomics and spatial transcriptomics reveal branching evolution

Multi-region sequencing provides more direct evidence of alternative routes. Whole-exome analysis of 44 microdissected regions from 11 T1 GBCs with coexisting lesions found both BilIN-dependent and apparently BilIN-independent cancers; six tumors were classified as BilIN-independent and five as BilIN-dependent, although the rarity and early stage of the cohort limited generalization (*51*). A two-patient spatial transcriptomic study sampled 24 and 32 regions of interest spanning normal epithelium, low-grade BilIN, high-grade BilIN, and adenocarcinoma. The sequence of pathway activation differed between patients, and SEMA4A repression emerged as a shared potential event, with functional support in organoids and cell-line-derived tumors (*52*). That study is mechanistically informative precisely because it does not claim population prevalence based on two cases.

A 2025 study of papillary neoplasms of the bile duct and gallbladder added genomic and spatial-transcriptomic evidence for sequential evolution from intraductal or intracholecystic papillary neoplasm to invasive cancer, while also showing that molecular trajectories differed by anatomical site (*53*). Because the cohort combined biliary locations, its gallbladder findings support the plausibility of an ICPN-associated route but should not be generalized to flat BilIN-associated GBC.

Mouse models supply controlled tests of potential drivers. Concurrent Kras and canonical Wnt activation induced premalignant gallbladder or extrahepatic biliary lesions resembling human papillary-tubular precursors and allowed progression under defined genetic conditions (*54*). More recent human pathology studies are attempting to refine high-risk metaplastic states: CLDN18 expression was absent in normal epithelium and TFF2-positive pseudopyloric metaplasia but was detected in intestinal metaplasia, BilIN, ICPN, and a subset of GBC; however, most lesions were cancer-adjacent and the 37-cancer cohort was unable to establish prospective risk (*55*). Single-cell analysis of adenomyomatosis and GBC likewise identified epithelial and macrophage states of interest, and yet adenomyomatosis should not be reclassified as a premalignant condition without longitudinal data on its incidence (*56*) (Table 2).

5. Genomic evolution: Drivers are necessary information but not the whole explanation

Exome sequencing established recurrent disruption of the ErbB signaling pathway in GBC, including ERBB2 and ERBB3 alterations, alongside TP53, KRAS, PIK3CA, and other context-dependent events (*57*). Functional work later linked ERBB2/ERBB3 mutations to PD-

Table 2. Precursor routes and the limits of a single linear sequence

Precursor route	Typical spatial form	Representative molecular features	Evidence substantiating progression	Boundary of inference
Metaplasia → BilIN → invasive adenocarcinoma	Flat, patchy, often within thickened stone-bearing mucosa	TP53/cell-cycle disruption, variable KRAS, adhesion and chromosomal changes (*38,41,48-50*)	Whole-organ mapping, age-stratified pathology, coexisting lesions, multi-region genomics	Cross-sectional sequence is not a patient-level clock; many metaplasias never progress
ICPN-associated carcinoma	Grossly visible polypoid/ papillary-tubular lesion	Lineage-dependent alterations; heterogeneous gastric, intestinal, biliary, oncocytic phenotypes (*39,40,45*)	Large clinicopathological series and associated invasive components	ICPN biology should not be merged with flat BilIN
Conventional adenoma/pyloric gland adenoma	Localized polypoid lesion	CTNNB1 enriched in some adenomas; lineage-specific programs (*39,46*)	Morphology and molecular divergence	Many adenomas may be evolutionary side branches
PBM-associated hyperplasia-dysplasia	Diffuse reflux-associated mucosal hyperplasia	Proliferative and injury-response programs, followed by driver acquisition (*17-20*)	Strong anatomical exposure and characteristic pathology	Specific to reflux anatomy; not a model for all gallbladder cancer
BilIN-independent/de novo route	Invasive focus without clonally related sampled BilIN	Distinct regional mutation profiles (*51*)	Multi-region sequencing of early gallbladder cancer	"Independent" may reflect unsampled or extinct precursors

L1-mediated immune escape, directly connecting an epithelial driver to microenvironmental selection (*58*). An integrative study of 190 patients, including 92 whole exomes and a multi-omic subset, reported a low median mutation burden, TP53 as the most frequently mutated gene, and three transcriptomic survival groups distinguished more strongly by hypoxia, desmoplasia, myeloid suppression, and T-cell dysfunction than by a single driver (*59*).

Large targeted sequencing has reinforced both diversity and actionability. In 244 samples from 233 patients, TP53, CDKN2A, ERBB2, and KRAS were among the common alterations; potentially actionable changes were identified in 35% of those patients, and 18% of patients with metastatic disease received genomically guided off-label or trial therapy (*60*). Smaller targeted studies in high-incidence populations identified overlapping somatic alterations but also highlighted limited cohort size and geographical variation (*61*). Older allelotyping and morphological synthesis had already suggested stepwise chromosomal loss and divergent histogenetic pathways (*62*).

Epigenetic and RNA-regulatory layers provide another possible link between an affected area and phenotype. Epigenome-wide profiling across cholelithiasis, dysplasia, and GBC identified recurring methylation changes along the pathological sequence, but bulk cross-sectional specimens cannot establish when or in which epithelial clone those changes arose (*63*). Functional studies suggested lncRNA-HGBC, through a HuR/miR-502-3p/SET/AKT axis, and circAATF, through AKT/miR-142-5p/PD-L1 signaling, as regulators of proliferation, invasion, or immune signaling (*64,65*). The circAATF study combined paired tissue analyses with humanized mouse models rather than a prospective immunotherapy cohort. Those data justify integrating methylome and non-poly(A) RNA assays with lineage and spatial methods; they do not yet show that noncoding RNA creates an inflamed area or predicts clinical response.

Metabolic selection can also alter both tumor fitness and treatment sensitivity. In paired human specimens, cell lines, and xenografts, fatty-acid synthase promoted GBC progression and reduced gemcitabine sensitivity through PI3K/AKT signaling (*66*). A contemporaneous GBC-focused review catalogued additional resistance mechanisms but emphasized that most remain preclinical (*67*). These findings justify incorporating lipid metabolism into ecosystem models, while avoiding the stronger claim that FASN defines a clinically validated immune-metabolic subtype.

These data argue against using the mutation list as the complete model of carcinogenesis. The same driver can occur in different epithelial lineages, and the same histological lesion can be embedded in different inflamed areas and stroma. Genomics is most informative when paired with location and state: whether an alteration is clonal or subclonal, whether it is present in adjacent BilIN, whether the clone resides near reflux-injured mucosa or an invasive margin, and which immune or stromal cells are present.

6. Single-cell reconstruction of ecosystem remodeling

6.1. From an inflamed gallbladder to an immunosuppressive tumor

Single-cell studies have changed the level of analysis from average gene expression to interacting cell states. A 2022 atlas of 24 fresh gallbladder samples profiled 140,870 cells across chronic cholecystitis, primary GBC, and metastasis. Chronic cholecystitis contained MDSC-like macrophages, tissue-resident CD8 T cells, and CCL2-positive regulatory fibroblasts, whereas GBC contained an abundance of tumor-associated macrophages, regulatory and exhausted T-cells, and STMN1-positive tumor-promoting fibroblasts (*68*). This indicates continuity between an adapted inflamed area and a more suppressive tumor ecosystem but does not prove that each inflammatory cell state is a direct ancestor of its cancer counterpart.

Earlier immunological synthesis had already implicated macrophages, neutrophils, cytotoxic T cells, regulatory T cells, and checkpoint pathways in GBC (*69*). The newer atlases refine that picture by separating cell states and neighborhoods: immune cells are not simply recruited in increasing numbers but are progressively reorganized from injury-associated repair toward myeloid dominance, T-cell dysfunction, and stromal exclusion.

In ErbB-pathway-mutated GBC, single-cell analysis identified an MDK-dependent communication program linking malignant cells to immunosuppressive myeloid populations; the study provided a concrete example of genotype-to-niche coupling (*70*). An early five-patient atlas profiled 24,887 cells from primary tumors, lymph-node metastases, and adjacent normal tissue, identifying altered T-cell, macrophage, and ACKR1-positive endothelial interactions across primary and nodal compartments (*71*). Its mixed histologies and small patient number preclude a general metastatic trajectory, but the study showed why a draining lymph node should not be treated as a duplicate of the primary tumor. Separate work indicated reactivation of human endogenous retroviral elements in selected malignant and immune states, suggesting an additional layer of transcriptional plasticity (*72*).

A larger 2024 study profiled 230,737 cells from 43 specimens spanning 15 GBCs, cholecystitis, polyps, adenomas, and adjacent tissues. It identified distinct stromal ecosystems, including an iCAF-tip endothelial interaction associated with poor outcome, and implicated epithelial OLFM4 in immune evasion through MAPK-AP1-dependent PD-L1 regulation (*73*). Focused T-cell profiling further resolved 12 T-cell states and described

tumors dominated by exhausted CD8 cells, Tregs, or macrophage-rich suppressive patterns (*74*). A 2025 study of 98,113 cells spanning normal tissue, high-grade intraepithelial neoplasia, and GBC extended the temporal axis and emphasized changing intercellular communication during malignant transformation (*75*).

Pan-biliary comparisons highlight the need for anatomical specificity. Single-cell RNA/T-cell receptor sequencing of 36 samples from 16 patients, integrated with 355 pathological slides, showed that T-cell states, tertiary lymphoid structures, and desert phenotypes differed among intrahepatic, extrahepatic, and gallbladder cancers (*76*). These data are a valuable context for GBC but cannot substitute for GBC-only validation. Likewise, integrated immunogenomic analysis of 31 resected biliary tract cancers associated a KRAS mutation with fewer CD8 T cells and antigen-presenting cells and greater tumor-APC-T-cell separation (*77*); whether the same spatial relationship predicts adjuvant outcome specifically in GBC still needs to be verified.

The largest current ecosystem analysis combined single-cell, spatial, and other multi-omic data from 102 patients, encompassing 1,117,245 cells, and described five tumor microenvironmental ecosystems associated with progression. An AREG-EGFR-pERK-EGR1-CXCL5 circuit linked malignant epithelial signaling to myeloid recruitment and adverse ecology (*78*). Its scale strengthens the taxonomy, but the ecosystems still require prospective testing as predictors of treatment response.

6.2. Immune suppression is spatially heterogeneous

Before single-cell profiling, immunohistochemistry had already shown that immune context matters. A spatiotemporal analysis of 139 biliary precursor lesions documented changing immune infiltration across carcinogenesis, although the cohort was not limited to GBC (*79*). In 211 GBCs, lower CD8-cell density and higher BTLA/CD8, Cbl-b/CD8, and FOXP3/CD4 ratios were associated with worse survival (*80*). Classification by CD8 infiltration and PD-L1 expression provided an additional prognostic immune framework (*81*), and a separate series found that high stromal CD3 or CD8 infiltration favored survival mainly in smaller tumors, suggesting stage- and geometry-dependent effects (*82*).

Multiplex immunohistochemistry later demonstrated co-expression of PD-1 and TIM-3 and differences among the tumor core, hepatic invasive margin, and liver metastases (*83*). These observations caution against treating a single core biopsy as a complete immune phenotype. Mechanistic studies also identified stromal-metabolic regulators: nicotinamide N-methyltransferase promoted M2 macrophage polarization through IL-6 and myeloid-derived suppressor-cell conversion through GM-CSF (*84*). The recurrent pattern is not simply "more" or "less" immunity; it is the presence of specific suppressive cell states in specific locations.

Methodological limits must be treated as part of the biological interpretation. Current GBC includes small precursor-mapping and multimodal liver-front cohorts (*51,52,85-87*). Standard 10x Visium capture spots are approximately 55 μm in diameter and usually contain transcripts from multiple cells, so assignment of a 'niche' or ligand-receptor interaction depends on deconvolution, the reference single-cell atlas, histological annotation, and section orientation (*88*). Dissociation can under-represent fragile cell states in scRNA-seq, while a tissue section remains a thin two-dimensional sample of a three-dimensional boundary. RNA quality, archival fixation, treatment exposure, and batch effects further constrain sensitivity and comparability. For retrospective GBC cohorts, probe-based FFPE-compatible single-nucleus RNA sequencing can recover cell-state information from archived blocks, while imaging-based platforms such as Xenium and CosMx can localize targeted transcripts at a single-cell or subcellular resolution (*89,90*). These methods expand access to historical specimens but remain sensitive to block age, RNA fragmentation, panel design, segmentation error, and platform-specific detection bias; they complement rather than replace fresh-tissue whole-transcriptome discovery. Spatial candidates therefore require orthogonal immunohistochemistry or in situ validation, multi-section sampling, multicenter replication, and prospective treatment cohorts (Table 3).

6.3. Immune editing is dynamic across progression and treatment

The transition from a premalignant inflamed area to an early tumor microenvironment is bidirectional, gradual, and temporally overlapping: chronic inflammation can select for epithelial clones, while emerging dysplastic or malignant clones progressively remodel the same area. The available evidence supports a staged, but not strictly linear, model of immune editing. During chronic cholecystitis, innate recruitment and tissue repair may initially contain epithelial injury while simultaneously exposing regenerating cells to cytokines, reactive species, and growth signals. With dysplasia and clonal expansion, epithelial programs such as ERBB-MDK, OLFM4-PD-L1, AREG-CXCL5, and NNMT-dependent myeloid polarization can convert this inflammatory setting into selective immune pressure (*58,70,73,78,84*). During invasion, suppressive macrophages, metabolically adapted neutrophils, dysfunctional CD8 T cells, Tregs, fibroblasts, and abnormal endothelium assemble in different ways at each anatomical boundary. This is better described as immune-state replacement and spatial redistribution than as a uniform transition from "hot" to "cold."

Clinical observations are consistent with this model but do not yet validate its mechanisms. In a single-center retrospective cohort of 80 ICI-treated GBC patients, the objective response rate was 13.75%; high

Table 3. Representative single-cell and spatial studies defining gallbladder cancer ecosystems

Study	Material and scale	Spatial/temporal coverage	Principal ecosystem finding	Key limitation
Wang *et al.*, 2022 (*68*)	24 fresh tissues; 140,870 cells	Cholecystitis, primary gallbladder cancer, metastasis	Transition from adapted inflammatory macrophage/fibroblast states to TAM-, Treg-, exhausted-T-cell-rich tumors	Cross-sectional patients; state continuity is inferred
Zhang *et al.*, 2021 (*70*)	Gallbladder cancer single-cell atlas with ErbB stratification	Tumor and adjacent compartments	MDK-dependent immunosuppressive communication in ErbB-mutated tumors	Genotype-selected context; external validation needed
Chen *et al.*, 2021 (*71*)	Primary tumors, lymph-node metastases, and adjacent normal tissue from 5 patients; 24,887 cells	Primary-to-nodal metastatic compartments	Immunosuppressive T-cell/ macrophage remodeling and ACKR1-positive endothelial interactions	Five patients, mixed histologies, no spatial boundary map
He *et al.*, 2024 (*73*)	43 samples; 230,737 cells plus spatial/histological validation	Normal, cholecystitis, polyps, adenomas, gallbladder cancer	OLFM4-MAPK-AP1-PD-L1 axis; heterogeneous stromal ecosystems	Observational ecosystem-outcome links
He *et al.*, 2025 (*75*)	98,113 cells	Normal, high-grade intraepithelial neoplasia, gallbladder cancer	Transformation-associated cell-state and communication trajectories	Limited number of patients per pathological stage
Zhou *et al.*, 2025 (*78*)	102 patients; 1,117,245 cells with multi-omics	Multiple disease stages and spatial contexts	Five ecosystems; AREG-EGFR-pERK-EGR1-CXCL5 myeloid-recruiting circuit	Predictive utility not prospectively established
Rao *et al.*, 2024 (*85*)	scRNA-seq, spatial transcriptomics, proteomics, mIHC	Tumor core and liver-invasive front	KRT17-positive tumor-fibroblast niche supports OLR1-positive oxLDL-absorbing neutrophils	Functional model requires clinical intervention testing
Hou *et al.*, 2025 (*86*)	Single-cell and experimental validation	Primary tumors and metastatic phenotypes	CD34-positive/CD90-positive endothelial cells acquire mesenchymal features through TGF-β signaling	Preclinical target evidence; clinical prevalence is uncertain
Li *et al.*, 2026 (*87*)	27 scRNA-seq samples from 8 patients (140,213 cells); 40 spatial slides from 4 patients; validation cohort	Tumor-to-liver interface	CXCL9-positive macrophage niches recruit or exhaust CD8 T cells according to position and LGALS4	Small discovery cohort; retrospective response analysis
He *et al.*, 2026 (*91*)	86 treatment-naive Gallbladder cancers profiled with 16-marker multiplex IHC; transcriptomics in 9 cases	Tumor core, margin, peritumor, and nerve-centered regions	Inflamed, excluded, and desert phenotypes; PNI-associated PD-L1-positive myeloid/exhausted-CD8 niche with IL-6/IGF1 signaling	Retrospective treatment associations; transcriptomic subset was small

CD8 infiltration and an inflamed phenotype were associated with disease control and longer survival, but only 31 archival tumors underwent molecular and immunohistochemical analysis, and treatment regimens were not randomized (*91*). Conversely, a 2026 single-cell study of successfully downstaged advanced GBC reported treatment-associated expansion of T follicular helper cells, tertiary lymphoid structures in half of treated tumors, cytotoxic/exhausted CD8 states, and NK cells, with fewer tumor-promoting macrophages and angiogenic fibroblasts (*92*). That analysis selected successful responders and lacked a randomized pretreatment-to-posttreatment design, so it revealed that therapy can remodel the ecosystem but did not indicate which baseline niche caused that response.

7. The invasive margin: Local anatomy becomes an ecological selector

The gallbladder has a thin wall, lacks a conventional submucosa, and lies directly against the liver. Once epithelial clones cross the muscular layer, they encounter vascular, lymphatic, neural, fibrous, and hepatic compartments over very short distances. The invasive margin is therefore not just the outermost part of the tumor; it is a new habitat that selects for motility, matrix remodeling, immune evasion, and metabolic adaptation.

7.1. The surface facing the liver

Multi-modal profiling of liver-invasive GBC identified

enrichment of neutrophils at the front. KRT17-positive tumor cells and surrounding fibroblasts formed a niche that promoted OLR1-dependent uptake of oxidized LDL by neutrophils, reducing cytotoxicity and enhancing invasion-related behavior (*85*). Single-cell analysis also identified a CD34-positive/CD90-positive endothelial population with endothelial-to-mesenchymal features, TGF-β activation, and association with metastasis; inhibition of TGF-β-related signaling attenuated the pro-migratory effect in experimental systems (*86*). The two studies linked local lipid availability and vascular plasticity to invasion, but both are based on preclinical hypotheses.

Pan-biliary experimental work provides an adjacent mechanism linking the vascular state to immune resistance: adrenomedullin disrupted endothelial function and reduced the efficacy of combined immunotherapy and targeted therapy in biliary tract cancer models (*93*). This finding is conceptually compatible with the endothelial states observed in GBC, but it should not be offered as GBC-specific clinical evidence.

The most direct boundary map comes from paired single-cell and spatial transcriptomics of the tumor-liver interface. Twenty-seven single-cell samples from eight patients and 40 spatial slides from four patients were used to identify CXCL9-positive macrophage-rich niches. A liver-adjacent CXCL9-positive/TRAC-positive niche recruited CD8 T cells through CXCL9-CXCR3, whereas a more tumor-proximal CXCL9-positive/C1QB-positive niche was associated with LGALS4-mediated T-cell exhaustion. In a validation cohort, high CXCL9 and low LGALS4 at the boundary correlated with a better outcome and anti-PD-1 response (*87*). The initial and validation cohorts were small, so the marker pair cannot be used for routine selection yet, but that study established a crucial principle: the same chemokine-rich interface can contain spatially adjacent immune recruitment and immune failure.

7.2. Parallel surfaces are non-equivalent anatomically

The serosal/peritoneal surface, lymphovascular channels, cystic-duct mucosa, draining lymph nodes, and perineural spaces impose different physical and cellular constraints. A liver-facing front includes hepatocytes, sinusoidal endothelium, liver-resident immune cells, and a lipid-rich parenchyma; a serosal breach entails entry into a mesothelial and peritoneal compartment. Direct GBC spatial comparisons of these two interfaces in GBC are limited, so mechanisms defined at the edge of the liver should not be generalized to the serosal edge without matched sampling.

Lymphovascular and cystic-duct routes add continuity with mucosa, endothelium, and regional immune tissue. The aforementioned five-patient single-cell atlas that included lymph-node metastases demonstrated compartmental immune and endothelial remodeling but did not indicate the anatomical route from a primary tumor to a node (*71*). A matched primary tumor, invasive lymphatic channel, cystic-duct margin, sentinel or regional node, and distant metastasis therefore need to distinguish between dissemination-associated selection and organ-specific adaptation.

Perineural invasion (PNI) represents another specialized aspect. Spatial profiling of 86 treatment-naive GBCs identified inflamed, immune-excluded, and immune-desert phenotypes and a nerve-centered niche rich in PD-L1-positive myeloid cells, exhausted CD8 T cells, and IL-6/IGF1-related signaling with lipid-metabolic rewiring (*94*). Retrospective identification of an association with survival and treatment suggested clinical relevance, but causal therapeutic benefit cannot be inferred from non-randomized comparisons. Together, the liver, serosal, lymphovascular/nodal, and neural interfaces show that invasion should be sampled by boundary type, not collapsed into a generic "tumor margin."

Mechanistic evidence published in 2026 further showed that GBC-derived extracellular vesicles can transfer O-GlcNAcase to neurons, induce neuronal necroptosis, and facilitate PNI in experimental systems (*95*). This tumor-neuron mechanism complements, rather than proves, the spatially observed immune-evasion niche: neuronal injury and immune exclusion were measured in different studies and require joint validation in the same specimens (Figure 3).

8. An integrated three-stage model

The evidence supports a three-stage model with branching rather than obligatory progression.

First, a susceptible inflamed area is created by prolonged injury. Gallstones, PBM-related reflux, chronic Salmonella carriage, metabolic bile changes, and host susceptibility contribute to this, with different effect sizes and in different populations. The area is characterized by repeated epithelial death and repair, altered bile chemistry, stromal activation, and immune adaptation. This stage can persist for decades without cancer developing.

Second, epithelial lineages diversify. Metaplastic cells, BilIN, ICPN, pyloric gland adenoma, and apparently de novo clones acquire different combinations of driver, chromosomal, epigenetic, and lineage alterations. Selection occurs within a mosaic mucosa, so the most atypical lesion sampled histologically is not necessarily the ancestor of the invasive tumor. Multi-region sequencing and spatial transcriptomics are required to distinguish shared ancestry from coexistence.

Third, invasive competence is selected for at boundaries. Malignant epithelial states interact with fibroblasts, macrophages, neutrophils, endothelial cells, T cells, hepatocytes, and nerves. The local ecology may simultaneously recruit immune cells and disable them. This stage explains why molecular profiling of the tumor

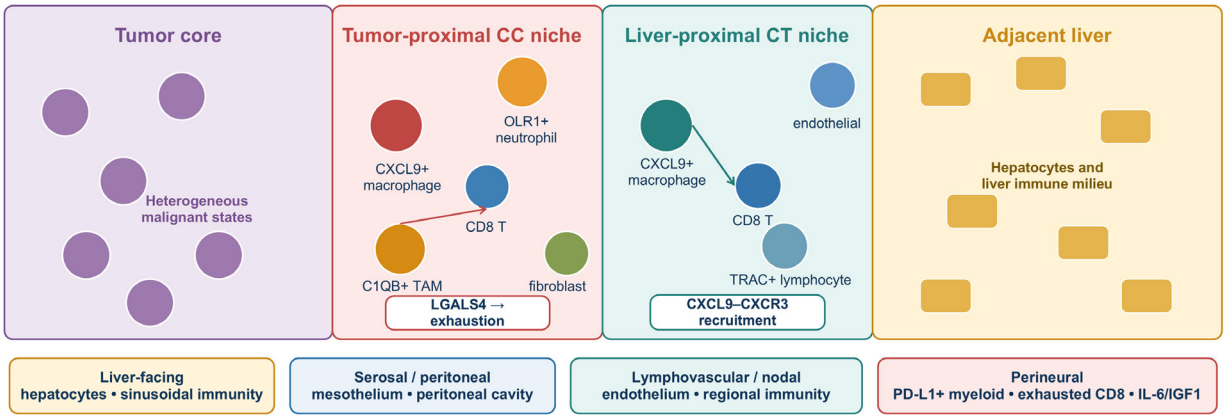


Figure 3. Anatomically distinct invasive margins in gallbladder cancer. Cancer cell (CC) niche denotes the CXCL9-positive/C1QB-positive macrophage-rich compartment, and connective tissue (CT) niche denotes the CXCL9-positive/TRAC-positive T-cell-recruitment compartment. At the best-mapped liver interface, a tumor-proximal CXCL9-positive/C1QB-positive macrophage niche is associated with LGALS4-mediated T-cell exhaustion, whereas a more liver-proximal CXCL9-positive/TRAC-positive niche encourages CXCL9-CXCR3-dependent CD8 T-cell recruitment. OLR1-positive neutrophils, fibroblasts, and specialized endothelial cells provide additional invasion-associated states. Serosal/peritoneal, lymphovascular/nodal, and perineural routes impose different anatomical constraints and require matched sampling; they should not be inferred from the edge of the liver. This diagram is a synthesis, not a diagnostic assay. *Abbreviations:* CC, cancer cell; CT, connective tissue; TAM, tumor-associated macrophage; CD8, cluster of differentiation 8; C1QB, complement C1q B chain; CXCL9, C-X-C motif chemokine ligand 9; CXCR3, C-X-C motif chemokine receptor 3; OLR1, oxidized LDL receptor 1; TRAC, T-cell receptor alpha constant; LGALS4, galectin-4; PD-L1, programmed death ligand-1; IL-6, interleukin-6; IGF1, insulin-like growth factor 1.

core may fail to predict behavior at the liver-facing edge and why spatially resolved biomarkers may outperform whole-tumor averages.

The stages overlap. A tumor can continue to reshape bile and microbiota; an invasive niche can feed back on core epithelial states; and treatment can remodel every compartment. The model is therefore a set of testable relationships, not a fixed chronology for every patient.

9. Clinical and research implications

9.1. Prevention and early detection should be risk-structured

The model does not justify prophylactic cholecystectomy for all asymptomatic gallstones. Absolute cancer risk remains low for most carriers, and surgery has its own harms. It does support more precise risk stratification regarding the duration and phenotype of stones, PBM, suspicious wall thickening or polyps, geography, infection history, and host factors. When dysplasia is found, extensive histological sampling is essential because the area is patchy and occult invasion may be focal.

Bile multi-omics and microbial assays are attractive because they sample the organ habitat, but current signatures require prospective validation with standardized pre-analytic variables, antibiotic and drainage metadata, negative controls, and benign controls matched for obstruction and inflammation. A clinically useful assay must distinguish cancer-associated signals from the consequences of cholestasis and instrumentation.

Imaging can identify where the area has become morphologically abnormal, although it cannot depict an immune niche. In a 2025 retrospective study of 151 patients with gallbladder lesions, contrast-enhanced ultrasound and contrast-enhanced computed tomography had broadly comparable diagnostic performance, with important limitations due to unpaired testing and small subgroups (*96*). Such data support the multimodal assessment of suspicious wall or polypoid lesions but do not support population screening or assignment of a histological lineage.

9.2. Use of biomarkers must bear compartments in mind

Routine molecular profiling identifies actionable alterations in a subset of advanced GBC, but tissue location should be recorded. A core biopsy, primary resection, invasive margin of the liver, and metastasis may contain different immune and stromal states even when driver alterations overlap. Future trials should predefine where tissue is obtained and should not validate a spatial marker in whole-tumor homogenates alone.

Incidental GBC creates a distinct sampling problem. Approximately half of GBCs may be detected during or after cholecystectomy in some contemporary series, although the proportion varies by geography and referral pattern (*97*). Once the index operation has removed the intact organ, the untreated invasive margin is no longer prospectively accessible. Spatial analysis should therefore remap the archived cholecystectomy specimen—hepatic-side wall, cystic-duct margin, serosal breach, lymphovascular and perineural foci—and, when completion radical re-resection is performed, pair these findings with the gallbladder bed, regional nodes, and any residual disease. Such maps should complement,

not replace, pathological stage, margin status, cross-sectional imaging, and the assessment of resectability. In unresectable GBC, image-guided biopsy protocols should record whether tissue represents the tumor core, liver-facing edge, nodal disease, or distant metastasis and, when safe, obtain multiple cores for orthogonal histological and molecular assays.

Trials of systemic treatment in biliary tract cancer provide proof that immune modulation can improve outcomes, but they do not validate the proposed GBC niches. Gemcitabine-cisplatin established a systemic-therapy backbone in advanced biliary tract cancer (*98*). Adding durvalumab improved overall survival in TOPAZ-1, with a hazard ratio of 0.80 and 24-month survival of 24.9% versus 10.4% (*99*). KEYNOTE-966 similarly indicated a median overall survival of 12.7 versus 10.9 months with pembrolizumab plus gemcitabine-cisplatin and a hazard ratio of 0.83 (*100*). Those trials enrolled mixed biliary tract cancers; GBC-specific, spatially stratified predictive analyses need to be performed before CXCL9, LGALS4, OLFM4, MDK, OLR1, or neural-interface signatures guide therapy.

Updated TOPAZ-1 follow-up confirmed a durable overall-survival benefit without a new safety signal (*101*), and a 2024 network meta-analysis of seven phase III first-line trials (4,033 patients) indicated that durvalumab plus gemcitabine-cisplatin ranked high in progression-free and overall survival (*102*). These analyses strengthen the pan-biliary standard but do not deal with the smaller and statistically uncertain GBC subgroup effects. In a separate phase II pembrolizumab study of 32 metastatic biliary tract cancers, integrated tissue and blood profiling sought to identify response biomarkers in microsatellite-stable disease (*103*); sample size and mixed anatomy again limited any GBC-specific inferences. The orphan disease perspective remains relevant because a low incidence constrains dedicated trials and drug development (*104*), but rarity should encourage the assembly of coordinated GBC cohorts rather than justifying uncritical extrapolation from cholangiocarcinoma.

Spatial findings offer a plausible mechanistic explanation for heterogeneous checkpoint-inhibitor benefit but not a validated resistance test. At the edge of the liver, a CXCL9-rich liver-proximal niche can recruit CD8 T cells, whereas the adjacent tumor-proximal CXCL9-positive/C1QB-positive macrophage niche and LGALS4-positive malignant cells are associated with T-cell exhaustion; high CXCL9 with low LGALS4 correlated with anti-PD-1 response only in a small retrospective cohort (*87*). PNI-associated excluded or desert regions likewise place exhausted T cells beside PD-L1-positive myeloid cells (*94*). These observations suggest that the tumor core and an invasive edge may contain opposing immune programs that are averaged out in bulk assays. However, TOPAZ-1 and KEYNOTE-966 neither selected patients by these niches nor prospectively sampled boundary-specific tissue, so the spatial states cannot yet be called mechanisms of clinical resistance.

9.3. A practical research agenda

Four study designs would materially strengthen this field. First, longitudinal cohorts should enroll patients with PBM, selected high-risk gallstones, or suspicious mucosal lesions before cancer, banking serial plasma, bile when clinically indicated, imaging, and eventual whole-organ pathology. Second, bile and mucosa should be sampled as paired but non-equivalent compartments, with absolute microbial load and rigorous contamination controls. Third, precursor studies should combine laser microdissection, multi-region DNA sequencing, single-cell or single-nucleus profiling, and spatial transcriptomics to reconstruct lineage rather than infer it from morphology alone. Fourth, invasive margin studies should standardize orientation and collect a matched tumor core, gallbladder-facing margin, liver-facing margin, serosal/peritoneal breach, lymphovascular channel, cystic-duct margin, PNI, draining lymph node, distant liver, and metastasis sample. For retrospective cohorts, pathology-guided serial sections should pair FFPE-compatible single-nucleus profiling with targeted imaging-based spatial transcriptomics, while fresh tissue should be reserved for broader examination whenever feasible. Clinical endpoints and treatment exposure must be prospectively defined.

The decisive validation step is intervention. A niche marker becomes clinically meaningful only if it reproducibly predicts an outcome or treatment response in an independent cohort, and a proposed driver becomes actionable only if perturbing it changes disease behavior without unacceptable toxicity. Until then, spatial and microbial signals should be presented as mechanistic or prognostic candidates, not as established clinical tests.

10. Conclusions

Gallbladder carcinogenesis is not adequately explained by a single mutation, a single organism, or a single histological staircase. The strongest evidence supports a layered process in which stones or reflux create an area of chronic injury; bile chemistry, selected microorganisms, host susceptibility, and immune-stromal adaptation modify that area; epithelial precursors follow branching lineages; and invasive clones are selected for within anatomically distinct regions. Single-cell and spatial studies have made this framework measurable by depicting cell states and their neighborhoods, but the cohorts in which they were found remain small relative to the biological and geographical heterogeneity of GBC.

The immediate opportunity is methodological rather than speculative: sample the correct compartments, preserve orientation, distinguish association from

causation, and validate proposed biomarkers prospectively. If those standards are met, the transition from an inflamed area to an invasive margin may become not only a conceptual account of GBC but also a practical map for prevention, early detection, and context-specific therapy.

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