Review

From Signals to Shields: Dual-Impact Germline Variants Implicated in FGF/FGFR Signaling in Cholangiocarcinoma

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From Signals to Shields: Dual-Impact Germline Variants Implicated in FGF/FGFR Signaling in Cholangiocarcinoma

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Abstract

Cholangiocarcinoma (CCA) is a cancer of the bile duct epithelium, which is increasingly associated with chronic inflammation and/or cholestasis. In recent years, fibroblast growth factor (FGF) signaling has gained substantial interest in CCA with three U.S. Food and Drug Administration (FDA)-approved drugs currently available for targeted inhibition of FGF receptors (FGFRs). FGF signaling is implicated in various aspects of CCA tumorigenesis, influencing immune responses and correlating with less favorable therapeutic outcomes. Despite some progress in our understanding of the genetic underpinnings of CCA over the past decade, the contribution of patient-intrinsic germline variations implicated in FGF/FGFR signaling had not been systematically explored. Here, we explore the hypothesis that the dual impact of germline variants pleiotropically associated with the FGFR signaling pathway acts both as signals promoting tumorigenesis (tumor-intrinsic) and as shields protecting tumors from targeted immune response (tumor-extrinsic). This dualistic behavior sheds insights into the intricate interplay between genetic predisposition and pleiotropic factors in CCA pathogenesis, potentially impacting the prognosis of targeted therapies and immune checkpoint blockade therapies.

Keywords: CCA; FGF; FGFR; dual-impact germline variants; risk factors; cancer signaling; immune signaling; genetics; environment; pleiotropy; pathway analysis; genetics; genomics

1. Introduction

CCA is a cancer of the bile duct, which constitutes approximately 15% of global liver cancer cases [1] . In recent years, the overall incidence and mortality rates of CCA have seen an upward trend [2] . CCA has its origin within the complex hepatobiliary system comprising the liver and bile ducts and is accordingly classified as intrahepatic CCA (iCCA), perihilar CCA and extrahepatic CCA. This intricate network serves as a critical component of digestion, as it facilitates bile acid (BA) production, storage, and transportation for the breakdown of dietary fats and waste elimination. The journey of bile begins with its synthesis in the liver, traversing a network of intrahepatic and extrahepatic ducts that culminate in the common hepatic duct at the hilum. From here, bile continues its path, merging with the gallbladder through the cystic duct before progressing through the sphincter of Oddi and, eventually, reaching the small intestine. The convergence of this orchestrated system forms the backdrop for the pathological emergence of CCA.

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BA are secreted from the canalicular surface of hepatocytes into canaliculi—narrow channels situated between neighboring liver cells that merge to create bile ducts [3] . From there, they move through a network of small ducts within the liver, which link to interlobular bile ducts. These ducts are located alongside branches of the portal vein and hepatic artery, collectively forming the portal triads. Eventually bile exits through the common hepatic duct and enters the gallbladder, where it is stored until needed for digestion. As bile flows through these ducts, its composition is modified by ductal epithelial cells known as cholangiocytes (CCs). These cells contain specific surface receptors and transport proteins that detect digestive hormones and ions, thus allowing them to regulate bile flow and alter it's composition. Ultimately, CCs respond by altering the composition of bile and regulating bile flow. In addition, CCs express a variety of growth factor receptors (GFRs) that play a variety of roles in CC biology, such as proliferation, apoptosis, and inflammation including de-differentiation. Furthermore, CC turnover is a dynamic process involving the renewal and replacement of damaged or aged cells. This process is crucial for maintaining the structural and functional integrity of the bile ducts. Various factors regulate the turnover of CCs, including signaling pathways mediated by growth factors, such as FGF. The important role of FGF and FGFR signaling in CCs has recently gained significant attention, as fibroblast growth factor receptor 2 (*FGFR2*) gene fusions occur almost exclusively in 10-15% of CCA cases. Additionally, various *FGFR2* insertions or deletions in tumor genomes create in-frame deletions in the FGFR2 extracellular domain, which are predicted to lead to oncogenic activation of the FGFR2 signaling pathway [4] . In addition, several point mutations have also been detected in the genomes of CCA tumors with unclear biological and clinical relevance. Therefore, next-generation sequencing (NGS), is now becoming part of the routine tumor diagnostics pipeline in order to detect tumor-specific *FGFR2* genetic alterations.

To date, three FDA-approved FGFR2-targeted therapies are available for clinical use, underscoring the therapeutic importance of FGF signaling in CCA [5–7] . However, there is increasing interest in developing treatment strategies for the majority of CCA patients—approximately 85%—whose tumors lack *FGFR2* gene fusions or rearrangements, with the overarching goal of extending the benefits of personalized, targeted therapies to all molecularly defined patient subgroups. Understanding the genetic basis of CCA has, therefore, become key for early detection and the development of targeted and personalized therapies. Tumor DNA sequencing has been instrumental in identifying targetable somatic mutations unique to CCA. However, determining the clinical relevance of polymorphic germline variants (PGVs), which are unique to individual patients, still remains a significant challenge. Moreover, the concept of polygenic risk scoring is not yet sufficiently validated for routine clinical implementation, as its potential role in clinical risk stratification and therapeutic decision-making continues to be explored in prospective studies. The somatic mutation profiles of CCA tumors have been thoroughly reviewed multiple times by various publications; [8–11] therefore, we will not revisit them in this paper.

Hence, in this review, we focus on the comprehensive body of scientific literature examining germline variations associated with CCA, aiming to reveal previously unrecognized connections among PGVs and their direct, pleiotropic effects on FGF/FGFR signaling pathways. We examine germline variants (GVs) potentially implicated in the FGF/FGFR signaling pathway and synthesize insights into their pleiotropic roles in CCA progression, with emphasis on the dual impact of tumor-intrinsic and tumor-extrinsic signaling mechanisms. Through these lines of inquiry, we suggest potentially targetable pathways for therapeutic interventions in patient cohorts that lack actionable tumor-specific genetic alterations in *FGFR2*.

With the growing accessibility of NGS in academic and clinical settings, it is now feasible to interrogate whole exome sequencing or whole genome sequencing (WGS) data from individual CCA patients to identify pathogenic or likely pathogenic GVs. A widely adopted approach to investigating GVs in cancer cohorts involves using pre-selected gene panels with established genotype–phenotype associations [12]. Germline testing panels typically include fewer than 4% of the approximately 20,000 protein-coding genes annotated in the GRCh38 reference assembly, with gene selection guided by established clinical relevance and expert consensus. For example, the American College of Medical

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Genetics and Genomics recommends reporting secondary findings in only 73 genes (0.36% of 20,000), underscoring the selective nature of clinically actionable targets.

2. Germline variants of FGFR Signaling in CCA: A Double-Edged Sword

About 1% of all human genes can be implicated as cancer drivers when mutated somatically, of which the largest subgroup of genes altered are protein kinases [13] . Among these, the FGF/FGFR signaling pathway is the most enriched in non-synonymous mutations [14] . Although only approximately 5–10% of human cancer genomes harbor genetic aberrations in *FGFRs [15]* , this frequency rises to 30–50% in iCCA cohorts, thus suggesting their role in CCA tumorigenesis [16,17] . GVs that converge on the FGF signaling pathway may, therefore, potentially contribute to CCA initiation and progression. Here, we explore the hypothesis that dual-impact GVs—affecting FGF signaling either directly or indirectly through biological pleiotropy by modulating DNA damage repair (DDR) and anti-tumor immune responses—may potentially contribute to CCA pathogenesis and could represent *bona fide* genetic risk factors.

2.1. Germline Variants Signaling Tumorigenesis (Tumor-intrinsic mechanisms)

Unlike other cancers such as breast, ovarian, and colorectal, routine germline genetic testing is not yet established for CCA. This is primarily due to limited data on the prevalence of germline cancer-predisposing variants and the currently restricted clinical utility of such testing. Existing germline analyses, conducted in both basic and translational research settings, have largely focused on a few hundred genes—primarily those involved in DDR pathways or associated with hereditary cancer syndromes, such as *ATM*, *CHEK2*, *BAP1*, *BRCA1*, *MLH1*, *BRCA2*, *PALB2*, *TP53*, *APC*, *CDH1*, *MSH6*, *PMS2*, and *MUTYH [18–20]* . Although progress has been made in other pathway-related genes, these insights have yet to translate into actionable gene panel testing for routine clinical use. This gap is largely attributable to the complex interplay between genetic and environmental factors that confound CCA pathogenesis, as well as persistent challenges in interpreting variants of uncertain significance in genetic testing [8,21,22] . Panel composition is often guided by established links between cancer syndromes and CCA, supported by clinical evidence, hereditary cancer registries, and curated genetic databases. Emerging associations may also inform iterative updates, based on evolving research and clinical observations. However, most panels remain focused on identifying alterations consistent with Knudson's 'two-hit' hypothesis in CCA [23] .

In the following sections, we examine studies that suggest pleiotropic effects of PGVs within the context of FGF/FGFR signaling in CCA [24] .

2.1.1. Cancer Syndrome Genes

Cancer syndromes, also referred to as hereditary or familial cancer syndromes, represent a collection of conditions marked by an elevated susceptibility to specific types of cancer. These syndromes usually arise from inherited mutations in particular genes, and individuals carrying these mutations face an increased probability of developing cancer compared to the general population [25] . The existence of a cancer syndrome within a family can result in the clustering of specific cancer types among relatives. By studying incidences of CCA in families with a history of cancer syndromes, novel associations can be uncovered, thus potentially revealing genes not previously linked to CCA. For instance, family members afflicted with Hereditary Nonpolyposis Colorectal Cancer syndrome have been found to have an elevated risk of extracolonic cancers, including CCA [26] . Furthermore, a case of Muir-Torre syndrome, which is considered a variant of Hereditary Nonpolyposis Colorectal Cancer syndrome, was also found to be linked with primary mucinous CCA and in this case a loss-of-function (LoF) germline mutation in *MSH2* chr2:g.47476387T>C; NM_000251.3:c.2026T>C; p.Ser676Pro; rs63751089 [27] . Similarly, *BRCA* syndrome patients show a statistically significant risk for CCA [28,29] . Results from additional case reports suggest a potential association between the BAP1 tumor predisposition syndrome and CCA, resulting in the identification of *BAP1*

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chr3:g.52404521G>A; NM_004656.4:c.1182C>T; p.Tyr394=; rs1364087255 [30] and a splicing site mutation in exon 4 of the *BAP1* chr3:g.52408467delAGCAGACC; NM_004656.4:c.255_255+6del [31] . Collectively, these case studies suggest a potential genetic association between CCA and GVs involved in DDR, highlighting the relevance of hereditary cancer syndromes in uncovering novel risk factors for CCA.

2.1.2. DNA Damage Repair Genes

DDR pathways comprise base excision repair (BER), homologous recombination of double-strand breaks, mismatch repair, non-homologous end joining repair of double-strand breaks and nucleotide excision repair (NER). The BER pathway is primarily responsible for repairing mutations caused by oxidized or reduced nucleotide bases. The human oxoguanine glycosylase 1 (*OGG1*) and *MUTYH* participate in the BER pathway. *OGG1* is involved in the repair of 8-oxoguanine (8-oxoG) lesions, common oxidative DNA damage products. The variant *OGG1* chr3:g.9757089C>G; NM_002542.6:c.977C>G; p.Ser326Cys; rs1052133 has altered activity, impacting the efficiency of repairing oxidative DNA damage. At least one study reported a significant association of *OGG1* p.Ser326Cys variant with the risk for CCA in individuals with the *GSTM1*-null genotype [32] . This suggests that the combined effects of *OGG1* and *GSTM1* variants may inform risk stratification, particularly in populations exposed to oxidative stressors. MUTYH is a DNA glycosylase that specifically targets adenine mispaired with 8-oxoG, recognizing and removing the adenine bases inappropriately paired with 8-oxoG. This, in turn, prevents mutations caused by 8-oxoG-induced G:C to T:A transversions. The *MUTYH* variants *MUTYH* chr1:g.45336998T>G; NM_001128425.2:c.37-2487T>G; rs3219476 and *MUTYH* chr1:g.45338378G>A; NM_001128425.2:c.36+1841G>A; rs3219472 in the promoter region are believed to impact the expression levels of *MUTYH*. Individuals homozygous for the c.36+1841G>A variant have been reported to have an increased risk of developing CCA, conversely, individuals heterozygous for the c.37-2487T>G variant have been associated with a reduced risk [33] .

Currently, the frequent use of NGS technologies in the clinic has led to the detection of an ever increasing number of pathogenic or likely pathogenic GVs in DDR genes in CCA patient cohorts. WGS or whole exome sequencing analyses in diverse CCA cohorts indicate that about 10%-20% of CCA patients carry pathogenic or likely pathogenic GVs within genes implicated in DDR pathways [34] . This proportion is consistent with findings from sequencing studies conducted in Asian cohorts. In a Chinese biliary tract cancer (BTC) patient cohort of 382 individuals, approximately 10.5% (40/382) harbored pathogenic or likely pathogenic GVs in genes related to DDR [35] . In a larger Chinese BTC cohort of 803 patients, 12% (96/803) carried pathogenic or likely pathogenic GV [36] . Meanwhile, in a smaller Chinese CCA cohort of 59 patients, targeted sequencing of 620 genes revealed germline mutations in 22% of patients [37] . Examining a BTC cohort of 412 patients (Japanese and Italian), 11% (14/146 Japanese) had germline variations in genes including *ATM* chr11:g.108203578_108203582delTTATA; NM_000051.4:c.7878_7882delTTATA; p.Ala2626fs; rs1450394308, *BRCA1* chr17:g.41243813_41243819insATAG; NM_007294.4:c.3593_3594insATAG; p.Ser1198fs, *BRCA1* chr17:g.41258147C>T; NM_007294.4:c.47T>A; p.Leu16Ter; rs80357086, *BRCA2* chr13:g.32930687C>T; NM_000059.4:c.7558C>T; p.Arg2520Ter; rs80358981, *BRCA2* chr13:g.32914066_32914069delAATT; NM_000059.4:c.5574_5577delAATT; p.Thr1858fs, *BRCA2* chr13:g.32954022insA; NM_000059.4:c.9090insA; p.Thr3030fs, *RAD51D* chr17:g.33428057A>T; NM_002878.4:c.964-2A>T; rs1403784434, *MLH1* chr3:g.37090506C>A; c.1378C>A; p.Gln460Lys, *MLH1* chr3:g.37038115A>G; NM_000249.4:c.122A>G; p.Asp41Gly; rs63751094, *MLH1* chr3:g.37067242C>T; NM_000249.4:c.430C>T; p.Arg144Cys, *MSH2* chr2:g.47643569G>A; NM_000251.3:c.1076+1G>A; rs267607940, *POLD1* chr19:g.50910674T>G; NM_002691.4:c.1775+1T>G, *POLE* chr12:g.133253971delG; c.779delG; p.Arg260fs, *TP53* chr17:g.7578406G>A; NM_001126116.1:c.128G>A; p.Arg43His; rs28934578 [38] . In a Japanese cohort of 269 patients with suspected genetic predisposition, only 1% (3/269) harbored pathogenic germline mutations in *BRCA1* chr17:g.43091891C>A; NM_007294.4:c.3640G>T; p.Glu1214Ter; rs80356923 and *BRCA2*

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chr13:g.32339928_32339931delCAAT; NM_000059.4:c.5576_5579del; p.Ile1859LysfsTer3; rs80359520, *BRCA2* chr13:g.32333365_32333366insA; NM_000059.4:c.1887_1888insA; p.Thr630AsnfsTer6; rs80359314 using a panel of 21 genes [39] . In a BTC cohort of 149 patients using an 88-gene panel sequencing approach, 16% (21/131) had pathogenic or likely pathogenic GVs. Among these, 14.5% (12/83) variations in 10 genes: *BRCA2*(5), *APC*(3), *MUTYH*(3), *BRCA1*(2), *FH*(2), *ATM* (1), *BAP1*(1), *MITF*(1), *NBN*(1), *PALB2*(1), *PMS2*(1) [40] . Additionally, a hepatobiliary cancer cohort of 205 patients, including BTC, exhibited 15.6% (32/205) of patients with pathogenic or likely pathogenic GVs in DDR genes (34 genes) analyzed using ~80 gene panel sequencing [18] . In another HCC cohort of 267 patients using ~135 gene panel sequencing, 15% (41/267) harbored pathogenic or likely pathogenic GVs [41] . In a BTC cohort of 124 patients 6% (6/92) had pathogenic or likely pathogenic GVs in genes including *MUTYH*(2), *ATM*(1), *BRCA1*(1), *BRCA2*(1), *MSH2*(1), *FH*(1) [42] . Furthermore, 11% (2/18) of iCCA patients harbored missense mutations in the *NBS1* gene. Functional analysis of *NBS1* genetic variants within the MRE11-interaction region revealed impaired nuclear localization of the MRE11 partner, consistent with the cellular phenotype of *NBS1* deficiency seen in Nijmegen breakage syndrome [43] . In a WGS investigation a BTC cohort by Holzapfel *et al.,* the pathogenic or likely pathogenic GV *MLH1* chr3:g.36996701G>C; NM_000249.4:c.199G>C; p.Gly67Arg; rs63750206 was found in 5% of patients [44] (1/20). Across all germline sequencing studies on BTC patients, including CCA, pathogenic or likely pathogenic GVs were consistently identified in DDR genes [45,46] . Remarkably, both *BRCA1* and *BRCA2* variants were found in over 10% of CCA patients with pathogenic or likely pathogenic GVs [45] . Furthermore, a recent study detected *BRCA* mutations in 3.6% of examined BTCs [47] . Additionally, large genomic rearrangements in *BRCA1/2* were identified in approximately 0.47% (2/425) of CCA patient cohorts [48] . These findings have some important therapeutic implications as there is emerging evidence suggesting potential benefits of Poly ADP-Ribose Polymerase (PARP) inhibitors in BTC patients with *BRCA* mutations [49] . However, it is crucial to emphasize that this association holds true not only for CCA, but is a prevalent theme across various cancer types [45] . Among DDR genes that act as cancer-causing drivers, most of them are tumor-suppressor genes rather than oncogenes. Despite numerous reported associations of DDR genes with CCA in the literature, only a limited number of studies have demonstrated the presence of second somatic hits that satisfy Knudson's "two-hit" hypothesis—a fundamental requirement to establish the pathogenicity of tumor suppressor genes as cancer drivers [50] .

2.1.3. Other Genes

While a large majority of investigations have focused on genes implicated in DDR pathways, a few studies have tested alternative hypotheses contributing to the development of CCA. For instance, in a study by Greer *et al*., a novel loss of function germline mutation in *ATG7* chr3:g.11426822C>T; NM_001349232.2:c.1975C>T; p.Arg659Ter; rs201706487, was found to interfere with autophagy in human bile duct cells and thereby to the development of CCA [51] . In another study, the role of mitochondrial pathways in the development of CCA was investigated using a small CCA cohort of 25 patients. In this study, the authors identified 161 mutations of germline origin in the mitochondrial DNA, including point mutations of substitutions, transversions and transitions, thus proposing a potential link to CCA development [52] . In a study involving 75 individuals with advanced bile-duct cancer who received Epirubicin, Cisplatin, and Capecitabine chemotherapy, those genotyped homozygous for the major allele *EZH2* chr7:g.148808210; NM_001203247.3:c.211A>G; p.Thr71Ala; rs887569 variant lived significantly longer [53]. In another study involving 158 individuals without disease and 198 patients diagnosed with CCA, individuals genotyped homozygous for the *NFE2L2* chr2:g.177238501; NM_006164.5:c.46-4230T>C; rs6726395 variant correlated with improved survival outcomes [54].

Collectively, these findings indicate that approximately 10–15% of CCA cases are attributable to pathogenic or likely pathogenic GVs, primarily in genes involved in DDR pathways. However, this likely reflects only a partial view of CCA heritability, as current germline testing panels predominantly target DDR genes, potentially overlooking relevant genetic modifiers and pleiotropic

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variants. Importantly, genetic alterations beyond canonical DDR genes—particularly those not typically captured in standard germline panels—may exert indirect yet biologically meaningful effects on CCA risk. In particular, variants in genes that influence cellular signaling dynamics may converge on the FGF signaling axis, thereby modulating oncogenic processes in ways that are not readily detectable through conventional somatic gene panel sequencing. In the following sections, we examine published variants implicated in FGF/FGFR signaling and discuss their potential contribution to CCA susceptibility through mechanisms that extend beyond traditional DDR-focused germline frameworks.

2.1.4. Genes participating in the FGF Signaling Nexus

2.1.4.1. DDR genes leading to FGF signaling and vice versa→ FGF2/FGFR1

The DDR pathway is crucial for preserving the accuracy of DNA replication, preventing mutations and ensuring genomic stability [55] . When DDR fails, replication errors persist, producing abnormal microsatellite repeat numbers and consequent microsatellite instability [56] . At least, one study suggests that ionizing radiation, which induces DNA damage, stimulates the production of FGF2 in human epithelial cells [57] . Moreover, several studies have identified the role of FGF2 in a survival pathway, which shields cells from radiation-induced death [58–62] , and activating DDR in response to irradiation across various cell types [63,64] , including human cancer cells [65,66] .

Furthermore, FGF signaling also impacts the expression of DDR genes in specific cellular contexts. For instance, studies investigating the FGFR2 inhibitor Ki23057 have shown that platinum drug-resistant gastric cancer and HCC cell lines become sensitive to various drugs when combined with FGFR2 inhibitors [67] . Inhibition of FGFR2 suppresses excision repair cross-complementary gene 1 (*ERCC1*) gene expression, an effect that is attributed to reduced ERK1/2 activation [68] . During the NER process, ERCC1, the rate-limiting enzyme, is overexpressed, thus reflecting increased NER-related resistance to platinum drugs [69,70] . Although many studies have investigated the association of *ERCC1* polymorphisms with various cancers, no conclusive evidence has been yet established with CCA. However, one study identified two GVs: *ERCC1* chr19:g.45409478; NM_001983.4:c.*197G>T; rs3212986 and *ERCC1* chr19:g.45423658; NM_001983.4:c.-8+123G>T; rs2298881 associated with an increased risk of CCA in a cohort of Chinese patients [24].

Ionizing radiations → FGF2 → DNA Repair Pathways → Resistance to Radiation-induced apoptosis.

2.1.4.2. Inflammatory pathway genes leading to FGF signaling

Many cytokine mediators associated with chronic inflammation have demonstrated their involvement as fibrogenic, mitogenic, and survival factors in CCA pathogenesis, operating both through autocrine and paracrine mechanisms [71–73] . The exploration of genetic variants influencing these signaling pathways is relevant for our understanding of CCA pathogenesis particularly those that are driven by chronic inflammation.

PTGS2 (COX2)→ FGF2/FGFR-1:

Prostaglandin-endoperoxide synthase 2 (PTGS2), commonly referred to as cyclooxygenase-2 (COX2), is an enzyme centrally involved in mediating inflammatory processes. It is considered a potential target for antitumor chemoprevention in CCA [74] . It is responsible for converting arachidonic acid into prostaglandin E2 (PGE2), which is a precursor to various prostaglandins and thromboxanes. These molecules regulate pain, inflammation, fever, and gastric acid secretion. PGE2 also plays a role in vascular remodeling and is considered protumorigenic mediated via its multifaceted role in inflammation and angiogenesis [75–78] . Several germline variations in the coding and non-coding regions of the *PTGS2* gene have been reported to be associated with high levels of COX2. The variant *PTGS2* chr1:g.186681619T>G; NM_000963.4:c.-1195A>G; rs689466 disrupts a MYB-binding motif in the promoter region and is associated with reduced *PTGS2* mRNA levels in esophageal tissue [79]. The C-allele of the variant *PTGS2* chr1:g.186681189; NM_000963.4:c.-

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765G>C; rs20417, located in the Stimulatory Protein 1 binding site, is linked to markedly reduced promoter activity relative to the G-allele in lung tissue [80] and in the normal duodenal mucosa of patients [81] . Additionally, the C-allele of the variant *PTGS2* chr1:g.186673926A>G; NM_000963.4:c.8473T>C; rs5275, which disrupts miRNA-mRNA interaction in the 3′ untranslated region of the *PTGS2* mRNA, results in increased half-life of the *PTGS2* mRNA [82] . As a result, carriers of these GVs show altered *PTGS2* transcript levels in tissues that express the relevant transcription factors or miRNAs. While various studies investigating the relationship between *PTGS2* genetic polymorphisms and CCA risk have produced inconsistent results [83,84] , a recent systematic review by Wang *et al.* suggested a potential association after adjusting for multiple comparisons with CCA susceptibility [24] . FGF signaling has been implicated in the upregulation of *PTGS2* mRNA and proteins in specific types of gastric epithelial cells [85,86] . Interestingly, COX2 inhibition has been shown to suppress *FGFR2* expression [87] . Additionally, PGE2, a product of COX2 enzyme activity, regulates angiogenesis via activation of FGFR1. PGE2 indirectly regulates FGFR signaling by activating prostaglandin E2 receptor 3 receptors. This activation initiates a signaling cascade comprising Proto-oncogene tyrosine-protein kinase Src (SRC) leading to the shedding of active MMP2, which acts on membrane-anchored FGF2 to release it for binding to FGFR1, either on the same cell or on neighboring endothelial cells, thereby activating them [88] . These findings collectively support an autocrine-paracrine mechanism linking the *PTGS2* GVs with the FGF2/FGFR1 signaling pathway.

COX2 activity → PGE2 (lipid) → EP3 receptor (lipid receptor) → SRC → MMP2 (release membrane anchored FGF2) → FGF2/FGFR-1 (CCs)

IL6→ FGF2/FGFR3:

Interleukin-6 (IL6) is a pleiotropic cytokine and has been well described to play important roles in the pathogenesis of various inflammation-associated cancers [71,72,89–92] . It is detected at high levels in the tumor microenvironment (TME) of various cancers, including CCA [93,94] , and promotes tumorigenesis via multiple signaling pathways. These pathways regulate almost all hallmarks of cancer, such as cellular apoptosis, survival, proliferation, angiogenesis, invasiveness, metastasis [95] and, most importantly, the innate and adaptive immune responses [96,97] . Germline variations in the *IL6* are associated with altered levels of IL6 in the blood of healthy individuals [98] . In a North-Indian cohort meta-analysis, the *IL6* chr7:g.22727026C>G; NM_000600.5:c.-174G>C; rs1800795 variant emerged as a possible cancer risk modifier with ethnicity- and site-specific patterns [99] . And in a study of liver fluke-infected patients in Thailand, both heterozygous and homozygous genotypes for the *IL6* chr7:g.22727026C>G; NM_000600.5:c.-174G>C; rs1800795 variants showed elevated levels of IL6 and were also found to be significantly associated with CCA[100–102].

IL6 signaling begins by binding to the IL6 receptor (IL6R), which comes in two forms: membrane bound form (mIL6R), which mediates classical signaling, and the soluble form (sIL6R) of the IL6R, which mediates trans-signaling [103]. IL6 trans-signaling occurs when sIL6R binds with IL6 and IL6ST, enabling IL6 to activate cells lacking mIL6R. This, in turn, allows IL6 to signal cells that do not express mIL6R, playing a crucial role in regulating systemic inflammation. Several germline variations in the *IL6R* gene have been found to impact IL6 signaling activity [104–107] . Among these a missense variant *IL6R* chr1:g.154454494A>C; NM_000565.4:c.1073A>C; p.Asp358Ala; rs2228145 results in an amino acid substitution from Asparagine to Alanine at the proteolytic site [108,109] . This variant is associated with increased levels of sIL6R and the IL6 ligand [104] . This GV is associated with various inflammatory diseases, such as rheumatoid arthritis [110,111] and periodontitis ; metabolic syndrome such as obesity [112,113] and diabetes [114,115] , as well as cancers such as melanoma and CCA [116] .

Several studies have indicated a potential cross-talk between FGF signaling and IL6 signaling pathways. For instance, In LNCaP and PC3-PSMA prostate cancer cell lines, levels of *IL6* expression are increased after treatment with FGF2 *[117]* . In myeloma cells, stimulation of IL6Rα/IL6ST by IL6 induces tyrosine phosphorylation of Signal transducer and activator of transcription 3 (STAT3), whereas stimulation of FGFR3 by FGF2 induced not only activation of ERK1/2 and PI3K signaling

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pathways, but also the serine phosphorylation of STAT3. Upon blocking the FGF/FGFR3 pathway, the effect of IL6 was abolished, suggesting a cross-talk between IL6/IL6R and FGF2/FGFR3 signaling pathways proliferation [118] . IL6 signaling is predominantly mediated by the STAT3 and p65 subunit of NF-κB encoded by the *RELA* gene. Activation of both RELA [119] and STAT3 [120] have been shown to upregulate FGF2, therefore suggesting a synergistic link between IL6 and FGF2 signaling pathways in CCs, which are known to express both genes. Therefore, germline variations that alter levels of IL6 may potentially impact the FGF signaling pathway.

IL6/IL6R→ STAT3 ← FGF2 ←RELA (NF-κB p65)← IL6/IL6R (CCs)

IFNγ → FGF2/FGFR-1:

Interferon-gamma (IFNγ), a type II interferon, is a potent activator of the innate and adaptive immune system, and is also a pro-inflammatory cytokine. In addition, it has anti-viral effects and can inhibit the replication of certain viruses, including Hepatitis B Virus (HBV) [121] . Its most notable effects include the induction of major histocompatibility complex (MHC) molecules [122–124] and the promotion of apoptosis [125,126] . CCs are not typically known for producing IFNγ. IFNγ is primarily produced by immune cells, such as T cells [127] , natural killer (NK) cells [128] , macrophages [129] , dendritic cells (DCs) [130] , neutrophils [131] , eosinophils [132] and NK1.1+ T cells [133] , in response to various stimuli, including infections or inflammatory signals. Specific germline variations in the *IFNG* gene, which are associated with high levels of IFNγ in blood or tumor tissues, have not been well established, although a link between the intron variant *IFNG* chr12:g.68158742T>A; NM_000619.3:c.+874A>T; rs2430561 and high serum levels of IL6 have been suspected at risk of metabolic syndrome, primarily in women[134] . However, there appears to be a signaling link between IL6 and IFNγ. Elevated levels of IL6 have been associated with increased frequencies of IFNγ+ pro-inflammatory CD4+ T cells in Peripheral Blood Mononuclear Cells of patients with PSC [135] . Compared with peripheral blood, bile and peribiliary liver tissue exhibited higher frequencies of IFNγ-positive proinflammatory CD4+ T cells and elevated IL6 concentrations, consistent with a localized inflammatory immune response around the bile ducts [136,137] . Moreover, IFNγ potentiates TLR-driven IL6 production by interrupting a negative feedback loop orchestrated by the canonical Notch targets *HES1* and *HEY1*. IFNγ signaling downregulates the levels of the Notch2 Intracellular Domain (NICD2), a component of the Notch2 receptor, thereby inhibiting the expression of *HES1* and *HEY1*. Both HES1 and HEY1 function as transcriptional repressors that negatively regulate IL6 gene expression [138] . Therefore, it is anticipated that germline variations in *IL6* and/or *IL6R* may modulate the local production of IFNγ by immune cells in a context-dependent manner. Increased serum levels of IFNγ and increased numbers of intrahepatic IFNγ-producing NK cells and CD8+ T cells have been found to contribute to increased cytotoxicity and liver injury in patients with PSC [139] . IFNγ signaling begins by binding to the ubiquitously expressed IFNγ receptor, which is composed of two subunits: IFNGR1 and IFNGR2. Phosphorylated tyrosine residues on the receptor subunits serve as docking sites for STAT protein family, including STAT1 *[140]* and STAT3 *[141]* .

Germline variations in the *IFNGR* gene have been associated with susceptibility to chronic HBV infection in the Chinese population [142] and variable susceptibility to various cancers. For example, the germline variation in the *IFNGR1* promoter increases susceptibility to HBV infection. The GV *IFNGR1* chr6:g.137219383; NM_000416.3:c.-56C>T; rs2234711 is considered a host susceptibility factor in the early development of gastric cancer [143,144] . Furthermore, the GV *IFNGR2* chr21:g.33,403,281; NM_005534.4:c.-128C>T was shown to increase the risk of developing gastric cancer in a population-based study in Poland [145]. Genetic variations in *IFNGR1* subunits, namely chr6:g.137198643; NM_000416.3:c.862-4A>G; rs3799488 and *IFNGR1* 6: 137219383; NM_000416.3:c.-56T>C; rs2234711 were also associated with an increased risk of developing CRC [146–148] . Additionally, the variant *IFNGR1* chr6:g.137219938C>T; NM_000416.3:c.-611A>G; rs1327474 is a valuable biomarker for predicting the risk of developing HCC [149] .

Several studies have reported a crosstalk between IFNγ and FGF-signaling pathways. IFNγ signaling is capable of inducing the synthesis of FGF23 during pro-inflammatory stimulation through

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activation of NF-KB and STAT1 pathways in M1 RAW264.7 macrophages. FGF23 is upregulated in LPS/IFNγ-induced pro-inflammatory M1 macrophages, but not in IL4-induced M2 anti-inflammatory macrophages [150] . Interestingly, human CCs can also upregulate components of the TLR4 signaling pathway in response to IFNγ [133] . In studies using renal carcinoma cell lines, activation of the FGF2/FGFR pathway was found to suppress the JAK/STAT signaling cascade triggered by IFNγ, leading to reduced expression of downstream genes such as *B2M*, *CXCL10*, and *CD274* (also known as PD-L1). Conversely, blocking FGFR signaling with lenvatinib reestablished the tumor's responsiveness to IFNγ within the TME of a syngeneic RCC mouse model lacking mature lymphocytes [151] .

IFNG → IFNGR → STAT1/STAT3 (CCs)

IFNG → GSK3 (↑) →suppress CREB/AP1 →IL10→IL10(↓) → STAT1/STAT3 (immune cells)

TLR→IL6(↑)→IL6(↑) ⊣ HES & HEY ←NICD1 ←NOTCH1 (immune cells)

IFNG / TLR →STAT1/NFkB →FGF23(↑) →FGF23(↑) (immune cells)

TNF/TNF-α (Tumor Necrosis Factor-alpha)→ FGF2/FGFR-1:

Tumor Necrosis Factor-alpha (TNFα) is a pro-inflammatory cytokine that belongs to the family of tumor necrosis factor (TNF) superfamily. A wide variety of immune cells produce TNFα during infection and inflammatory conditions, including CCs. In particular, these are sensitive to extracellular secretory products of *Clonorchis sinensis*, leading to the secretion of TNFα in a TLR4-dependent manner [152] . TNFα is produced as a precursor pro-TNFα, a type II transmembrane protein which is subsequently proteolytically processed by the multidomain metalloprotease TNFα-converting enzyme, yielding a 17-kDa soluble, mature TNFα species.

Two well known functions of TNFα are 1) to trigger apoptosis during CC injury [153,154] and 2) to induce expression of adhesion molecules, such as ICAM1 and MHC molecules [155,156] . TNFα-mediated upregulation of adhesion molecules in endothelial cells allows pro-inflammatory cells, such as neutrophils, macrophages and T cells, to infiltrate into the sites of bile duct injuries [157,158] .

Several germline variations in the promoter regions of the *TNF* gene, such as *TNF* chr6:g.31575254; NM_000594.4:c.−308G>A; rs1800629, *TNF* chr6:g.31574699; NM_000594.4:c.−863A; rs1800630, *TNF* chr6:g.31574531; NM_000594.4:c.−1031C; rs1799964, *TNF* chr6:g.31574705; NM_000594.4:c.−857T; rs1799724 have also been associated with different levels of TNFα production among healthy individuals [159,160].

TNF signals by interacting with two membrane-bound receptors TNFR1 (p55) and TNFR2 (p75), of which TNFR1 is ubiquitously expressed and is believed to be the major mediator of cytotoxicity of TNFα, whereas TNFR2 is found typically on cells of the immune system and mainly contributes to inflammation [161] . TNF/TNFR signaling is primarily mediated by the NFkB signaling pathway. However, a plausible molecular link between TNF/TNFR signaling and FGF2 production via NFkB signaling has not yet been established. Experiments with human endothelial cells have demonstrated a direct link between FGF2 signaling and the subsequent transcription and translation of *FGF2*, which occurs in a dose-dependent manner when the cells are incubated with TNFα [162] . Perhaps, this could be a feedback inhibition loop in order to dampen TNFα-mediated upregulation of adhesion molecules as, evidently, pre-treatment of endothelial cells by FGF2 inhibits TNFα-induced *ICAM1* expression [163] . Interestingly, FGF2 has been observed to also inhibit TNFα-mediated apoptosis through the upregulation of both *BCL2A1* and *BCL2L1* genes in mouse chondrocyte cell lines [164] .

TNF/TNFR →FGF2 →FGF2 → FGF2/FGFR → Feedback inhibition of TNFα-driven cell adhesion or apoptosis

2.1.4.3. Metabolism genes leading to FGF signaling → FGF2/FGFR1:

The Cytochrome P450 (CYP) enzyme family, which includes cytochrome P450 1A1 (CYP1A1) and cholesterol 7α-hydroxylase (CYP7A1), is involved in the metabolism of a wide range of substrates, including cholesterol, pharmaceutical agents, and polycyclic aromatic hydrocarbons. The primary function of CYP7A1 is to catalyze the initial and rate-limiting step in the classical or neutral pathway of BA synthesis, converting cholesterol into 7-alpha-hydroxycholesterol [165] . This

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conversion is the first step in the formation of primary BAs, such as cholic acid and chenodeoxycholic acid, which activate Farnesoid X Receptor (FXR)-mediated signaling pathways in enterocytes throughout the small intestine and colon [166,167] . Activated FXR and SHP both induce the expression and secretion of FGF19 in enterocytes, which then circulates via the blood to the liver, where it binds to FGFR4 and signals the transcriptional repression of *CYP7A1 [168–170]* . This endocrine signaling pathway of FGF19/FGFR4 is essential for the feedback regulation of CYP7A1 by BAs. Interestingly, besides primary BAs, FGF19 can also activate FXR by phosphorylating tyrosine-67 via the FGF19/SRC signaling pathway [171] , suggesting a constitutive FXR activation loop mediated by FGF19. Polycyclic aromatic hydrocarbons from environmental toxins or food additives induce the expression of *CYP1A1* via the activation of AHR. AHR is a nuclear factor that has multiple roles in regulating pro-inflammatory and adaptive immune responses [172–174] and also plays a key role in HCV infection [175] . Interestingly, the FGF19/SHP pathway is also involved in the activation of AHR [176] . Although some studies suggest FGF19 levels are associated with incidences of liver tumorigenesis [177–179] , there are others that indicate protective roles of FGF19 in cancer [180,181] . AHR activation, in turn, is associated with the increased production of FGF23 in hepatocytes and tumor cells [182] . Although some studies suggest an association of germline variations in *CYP7A1* or *CYP1A1* with cancer risk [183–185] , no studies to date suggest that genetic polymorphisms in *CYP7A1* or *CYP1A1* might influence the levels of FGF19 or FGF23.

CYP7A1 →Primary BAs→FXR activation →FGF19 synthesis →FGFR4 signaling →AHR activation →FGF23

2.1.4.4. Genes encoding receptor tyrosine kinases leading to FGF signaling→ FGF2/FGFR1:

The regulation of RTK activity is influenced by both the expression levels and the interaction with their specific ligands [186,187] . Because FGF/FGFR signaling extensively interacts with other RTK pathways, blocking FGF/FGFR can be compensated for by activation or upregulation of other RTK-driven pathways, and the reverse is also true *[188,189]* . Remarkably, resistance to EGFR inhibition by Gefitinib emerges through the upregulation of FGF/FGFR signaling components in various human cancer cell lines. Gefitinib-resistant cancer cells exhibit higher expression levels of both FGF ligands and FGFRs. Moreover, upon inhibition of either FGF or FGFR, using siRNA or the FGFR inhibitor PD173074, gefitinib sensitivity is fully restored [190–192] . These results suggest a common molecular signaling node among various RTK signaling pathways [193] . Similarly, FGFRs and EphA family receptor tyrosine kinases can trans-phosphorylate each other, eventually leading to the activation of shared downstream signaling pathways and simultaneous stimulation of both receptors, which, in turn results in the potentiation of MAPK activity [194] . Similarly, associations of ephrin receptors EphB2 and EphB3 with receptor-like tyrosine kinase (RYK), an atypical RTK which lacks detectable kinase activity of its own, have also been found [195,196] . RYK and some members of RTKs are atypical due to their role in WNT signal transduction [197] . The extracellular domain of RYK contains a WNT inhibitory factor 1 domain, which enables RYK to directly bind WNT1 and WNT3A [198] . In addition, RYK can also directly interact with FZD8 and act as a WNT co-receptor [199] . Other RTKs that bind to WNT ligands are muscle associated receptor tyrosine kinase (MUSK) and the receptor tyrosine kinase like orphan receptor (ROR) subgroup of nuclear receptors, which includes an extracellular cysteine-rich domain analogous to the WNT-binding region of Frizzled receptors [200] . As a result, WNT5A can also bind to MUSK [201,202] , ROR1 *[203]* and ROR2 *[204]* . WNT binding to RORs can result in one of several signaling cascades, such as down-regulation of β-catenin via the E3 ubiquitin ligase SIAH2 [205] , or downstream inhibition of β-catenin [206] , or activation of JNK signaling pathways [207] . When WNT signaling is triggered, the destruction complex consisting of Axin, APC, Casein Kinase 1 and Glycogen Synthase Kinase 3β is disassembled, targeting its components for ubiquitination and proteasomal degradation. In the absence of this complex, β-catenin phosphorylation is prevented and thereby its subsequent degradation, allowing it to bind to Lymphoid Enhancer Factor/T-Cell Factor transcription factors. In human cells, activated LEF/TCR results in elevated expression of *FGF18* and *FGF20* by directly

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binding to their promoters [208–210] . Both FGF18 and FGF20 have been implicated to signal via FGFR2 in the context of human gastric cancers [211–213] .

Several germline variations in the *EGFR* gene have been identified that can regulate the expression and activity of EGFR *[214]* . CA-SSR1, a polymorphic 14–21 CA repeat at the 5′ end of *EGFR* intron 1 near a secondary enhancer, shows that a reduced repeat number correlates with higher *EGFR* mRNA and protein levels *[215,216]* . Two other well-studied GV in the promoter region linked to elevated promoter activity and *EGFR* mRNA expression are *EGFR* chr7:g.55019062G>T; NM_005228.5:c.-216G>T; rs712829 and *EGFR* chr7:g.55019087A>C; NM_005228.5:c.-191A>C; rs712830 [217,218] . Among germline variations in the coding regions, two SNVs are known to alter the activities of EGFR, the *EGFR* chr7:g.55146678A>G; NM_005228.5:c.497A>G; p.Asp166Gly; rs11543848 located in exon 13, which leads to the replacement of the amino acid arginine with lysine [219] and reduced activity of EGFR *[220]* . The *EGFR* chr7:g.55154050A>G; NM_005228.5:c.787A>G; p.Thr263Ala; rs1057519829 SNV, located in exon 20 within the tyrosine kinase (TK) domain, has been associated with better survival in treatments using TK inhibitors [221] .

While GVs in ephrin receptors are not well studied, a few studies suggest that germline variations in the promoter region or the kinase domain may influence the expression of these transcripts. The GV *EPHA2* chr1:g.16156481T>C; NM_004431.5:c.-549T>C; rs6603883, which is located in the promoter region, has been shown to influence the transcriptional activity of *EPHA2* by reducing PAX2 binding affinity [222] . Moreover, the missense variant *EPHA2* chr1:g.16132227; NM_004431.5:c.2162G>A; p.Arg721Gln; rs116506614 located in the TK domain alters the functions of EPHA2 in cellular and biochemical assays [223] . For *EPHB4*, at least one study found the association of *EPHB4* chr7:g.100824547; NM_004444.5:c.53-274A>G; rs314310 with the protein expression of EPHB4 in an NSCLC cohort [224] . Interestingly, the intron variant *RYK* chr3:g.134163307C>T; NM_002958.4:c.1583-3934C>T; rs4470517 was found to be correlated with high expression levels in a glioma cohort [225] . At least two GVs in *MUSK* are described in autoimmune patients, *MUSK* chr9:g.110687034; NM_005592.4:c.220insC; p.Trp74Serfs in exon 3 and a missense mutation *MUSK* chr9:g.110800746G>A; NM_005592.4:c.2368G>A; p.Val790Met; rs199476083 in exon 14, which can either lead to an absence of the protein, or the expression of a functionally abnormal MUSK *[226]* .

Several germline variations such as *ROR2* chr9:g.91737448G>A; NM_004560.4:c.565C>T; p.Arg189Trp; rs199975149 in the cysteine-rich domain, *ROR2* chr9:g.91730997; NM_004560.4:c.1096C>T; p.Arg366Trp in the kringle domain [227] , *ROR2* chr9:g.91730070; NM_004560.4:c.1378+5del3ins19; p.Gln460fs [228] and *ROR2* chr9:g.91724819C>T; NM_004560.4:c.1675G>A; p.Gly559Ser; rs117134265 [229] have been described in the *ROR2* gene that affect its expression and functional aspects of TK activities.

The interrelationships of these germline variations in RTKs that regulate expression of FGFs and FGFRs are potential candidates for further exploration in the context of CCA. At least one study has found a significant association between the variant *EGFR* chr4:g.104224087; NM_005228.5:c.-186_187+87G>C; rs2107000 and an increased susceptibility to BTC. However, the specific role of rs2107000 in the context of the expression of *EGFR* has not yet been investigated [230,231] .

Decreased RTK activities →upregulation of FGFs & FGFRs

RTKs →*WNT* signal transduction pathway →Elevated expression of *FGF18* and *FGF20* → *FGFR1* and *FGFR2*

2.1.5. Genes linked to chronic onslaughts on the biliary epithelium.

In the previous section, we reviewed how various GVs in multiple genes can contribute to elevated levels of FGFs or FGFRs, especially in the context of tumorigenesis. Here, we further explore GVs within the genetic loci that influence susceptibility to diverse challenges faced by the biliary epithelium, arising from environmental factors, metabolic influences, and/or chronic immunological responses. The key contributors to the development of CCA are factors that continuously assault bile duct cells [232] . These factors include chronic infections with parasites such as liver flukes and viruses [233,234] ; autoimmune-mediated bile duct scarring such as PSC [235,236] , obstructions to

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bile flow caused by choledochal cysts or congenital biliary dilatation [237] , and gallbladder stones [238,239] , which ultimately lead to BA-induced damage.

The pathogenesis of PSC has been attributed to an interaction between genetic predisposition and environmental factors, mediated by immune mechanisms that cause chronic injury to CCs [240–242] . Genome Wide Association Studies have identified multiple GVs, including variants in or near the *MST1* and *BCL2L11* genetic regions, which increase susceptibility to PSC. Both genes are highly relevant to immune regulation: *MST1* is abundantly expressed in multiple immune cell lineages, controlling T cell adhesion, trafficking, homeostasis, DC cytokine signaling, and B cell maturation [243,244] . On the other hand, *BCL2L11* is a pro-apoptotic regulator widely expressed in lymphoid and myeloid cells, thus maintaining immune tolerance and limiting aberrant cellular proliferation through apoptosis [245] .

Environmental factors, such as chronic infections and persistent immune-mediated injury, further exacerbate local inflammation [240–242] . This chronic inflammatory state in PSC patients not only damages CCs but also promotes dysplastic transformation, where cells undergo abnormal proliferation and genetic alterations that increase malignant potential, altogether leading to CCA [246,247] . Given these links, alterations in these immune pathways may disturb immune homeostasis and bile duct epithelial integrity, predisposing CCs to injury and malignancy. Consequently, the genetic predispositions underlying PSC may likewise heighten susceptibility to CCA development.

2.1.5.1. Genes associated with chronic infection

Liver fluke infestation occurs through the consumption of contaminated food or water, commonly freshwater fish or vegetables. While there are currently no definitive, well-established GV associations directly linked to an increased susceptibility to fluke infestations, studies suggest that genes like *IL10, IL4*, and MHC class II genes could play a role in susceptibility to specific helminths [248] . The role of variations in the HLA loci in liver fluke infections is unclear, however, consistent associations have been observed between HLA alleles and viral resistance and persistence [249,250] . Among several liver flukes known to infest the liver, *Opisthorchis viverrini (O. viverrini)*, which is commonly found infesting the bile ducts, is associated with an increased risk of CCA development [251] . *O. viverrini* infestation induces chronic inflammation, periductal fibrosis, and CCA disease [252] . Several studies have investigated the link between genetic variations in inflammatory cytokines and their contribution to altering the risk of CCA development. At least one study reported a genetic association of *O. viverrini* infection with CCA risk. Patients with a heterozygous or homozygous genotype for *LTA* chr6:g.31572536A>G; NM_000595.4:c.+252A>G; rs909253, *IL6* chr7:g.22727026G>C; NM_000600.3:c.-174G>C; rs1800795, and *TNF* chr6:g.31575254G>A; NM_000594.3:c.-308G>A; rs1800629 variants, who showed significantly elevated levels of these pro-inflammatory cytokines, and for *IFNG* chr12:g.68158742T>A; NM_000619.2:c.+874T>A; rs2430561 variants , who exhibited fewer cytokines than the wild-type (WT), were all associated with an increased CCA risk [100] . Furthermore, the *IFNG* chr12:g.68160508G>C; NM_000619.2:c.-764G>C; rs2069707 variant, which has a higher binding affinity for NFkB, causing enhanced promoter activity and increased IFNG production, correlates with enhanced viral clearance, observed in cases of natural spontaneous remission and following antiviral treatment [253] .

Moreover, several viruses have also been associated with an increased risk of CCA, including Epstein-Barr Virus, HBV, HCV, Human Papillomavirus, and Hepatitis E Virus. Among these, HBV and HCV are directly associated with CCA, while other viruses may indirectly contribute to biliary damage or chronic inflammation, thereby exacerbating the susceptibility to CCA pathogenesis [253–255] . Remarkably, the association of CCA with HBV infection is much stronger among Asian populations, in contrast to Caucasian populations that are more strongly associated with HCV infections [256] . Several factors are considered when studying susceptibility to HBV and HCV, such as varying prevalence of viruses in different geographical regions, lifestyle choices, dietary habits, environmental exposures, and differences in surveillance or early detection practices. In addition,

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certain genetic variations in host populations could also play a major role in varying susceptibility to viral infections.

The sodium taurocholate cotransporting polypeptide (NTCP), encoded by *SLC10A1*, is the main receptor mediating HBV entry and is expressed not only in hepatocytes but also in CCs. In addition, other co-receptors that contribute to HBV entry are glycosaminoglycans and heparan sulfate proteoglycans. Two genetic variants of NTCP, including the loss of function variant *SLC10A1* chr14:g.69778476C>T; NM_003049.4:c.800C>T; p.Ser267Phe; rs2296651 [257–259] and the *SLC10A1* chr14:g.69796931G>A; NM_003049.4:c.225G>A; p.Thr75=; rs4646285 variant, which affects the expression of NTCP [260], have been reported to be associated with HBV immune recovery. Whereas, the intron variant *SLC10A1* chr14:g.69796098G>A; NM_003049.3:c.356+702G>A; rs4646287 *[261]* and *SLC10A1* chr14:g.69794608C>T; NM_003049.3:c.356+2192C>T; rs943277 [262] were linked to a higher risk of HBV infection.

The immune checkpoint molecule CTLA4 is primarily expressed in effector T cells and its function is crucial for immune regulation to prevent autoimmune inflammatory conditions. The *CTLA4* variant chr2:g.203867991; NM_005214.5:c.49A>G; p.Thr17Ala; rs231775 is linked to lower risk of persistent HBV infection [263] .

Carbonic anhydrases (CAs) are a family of enzymes that play a crucial role in regulating acid-base balance and pH homeostasis. Carbonic Anhydrase IX also known as CA9, is a membrane-bound CA detected in the epithelium of the biliary tree including CCs [264] . The *CA9* variant chr9:g.35674056; NM_001216.3:c.97G>A; p.Val33Met; rs2071676 was found to be associated with increased risk for chronic HBV infection [265] .

DOCK8 is a member of the DOCK family of guanine nucleotide exchange factors, with highest expression in immune-lineage cells, particularly T cells, NK cells, and B cells [266,267] . The *DOCK8* chr9:g.271638C>T; NM_203447.4:c.65C>T; p.Ala22Val; rs506121 results in hypomorphic function, which retains partial function but is insufficient for normal immune response. Interestingly, this variant was associated with chronic HBV infection [265] .

Toll-like receptors (TLRs) are transmembrane or intracellular proteins that recognize specific molecular patterns or damage-associated molecular patterns found in pathogens including viruses. They are primarily expressed in macrophages and DCs [268] . However, some TLRs are also expressed in CCs, which upon activation can lead to the production of pro-inflammatory cytokines and chemokines [269,270] . Loss of TLR-driven pathways limits generation of HBV-specific adaptive immunity (T and B cells), which facilitates ongoing HBV infection [271] . Conversely, activation of TLR signaling diminishes HBV replication via both IFNγ-dependent and IFNγ-independent mechanisms [272] . Several genetic variants of the *TLRs* were also found to be associated with either clearance or persistence of HBV infection. The *TLR2* variants chr4:g.153703504; NM_003264.3:c.597T>C; p.Asn199=; rs3804099 and chr4:g.153704257; NM_003264.3:c.1350T>C, p.Ser450=; rs3804100 and the *TLR3* variants chr4:g.186068179; NM_003256.4:c.-926T>A; rs5743305 and chr4:g.186076613; NM_003265.3:c.-7C>A; rs3775296 were shown to promote the progression of HBV clearance [273] . The genetic variant *TLR3* chr4:g.186079167; NM_003265.2:c.633+136G>T; rs1879026 of the receptor TLR3, which recognizes the double-stranded RNA, showed a significant association with HBV carriers [274] , while the *TLR3* chr4:g.186083063; NM_003265.3:c.1377C>T, p.Phe459=; rs3775290 variant showed an association with decreased risk for chronic HBV [275] . Furthermore, the *TLR3* chr4:g.186082920; NM_003265.3:c.1234C>T; p.Leu412Phe; rs3775291 variant was observed to be linked with increased risk of chronic HBV infection [276] . Several variants of the TLR7 receptor, which detect GU-rich short single-stranded RNA, including the *TLR7* chrX:g.12884766; NM_016562.3:c.4-746T>C; rs179010, *TLR7* chrX:g.12885330; NM_016562.3:c.4-182T>C; rs2074109 and the *TLR7* chrX:g.12885361; NM_016562.3:c.4-151A>G; rs179009 variant, were all associated with chronic HBV [277] . TLR9 recognizes unmethylated CpG oligodeoxynucleotides, which are prevalent in microbial DNA. Remarkably, individuals heterozygous for the variant *TLR9* chr3:g.52222681; NM_017442.4:c.1635G>A; rs352140 were associated with increased risk of chronic HBV infection [278] .

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Several proinflammatory cytokines such as TNFA, IL6 and IL1B downregulate *SLC10A1* gene expression, the entry receptor for HBV, and may, therefore, may plausibly contribute to the inhibition of HBV entry the CCs [279] . Genetic variations in these and a number of other cytokine genes, including *IL1B [278]* , *IL4 [280]* , *IL6 [281]* , *IL10/IL10RB [282–289]* , *IL12A [290]* , *IL12B [278]* , *IL16 [291]* , *IL18 [292]* , *IL21/ IL21R [293]* , *IL28B [294]* , *EBI3 [290]* , *TNF [282,283,295,296]* , *IFNG/IFNGR [278,297]* , *IFNAR2 [284,287]* , *IFNLR1 [298]* , and *TGFB1 [299]* were associated with susceptibility to HBV infection.

HCV initially attaches to host cells via LDLR and glycosaminoglycans, then sequentially engages CD81, scavenger receptor class B member 1 , claudin 1, occludin , and the cholesterol uptake receptor Niemann–Pick C1-like 1 to complete entry [300] .

The blood mononuclear cell surface expression of LDLR in patients with HCV chronic infection positively correlates with high HCV viral loads [301] . Moreover, the genetic variant *LDLR* chr19:g.11116926C>T; NM_000527.5:c.1773C>T; p.Asn591=; rs688, which decreases *LDLR* expression and impairs LDLR activity [302] , was associated with decreased susceptibility to HCV infection [303] . Several other variations in the *LDLR* gene are associated with modifying the susceptibility to HCV. The *LDLR* chr19:g.11111624G>A; NM_000527.5:c.1171G>A; p.Ala391Thr; rs11669576 was associated with IFNλ treatment response and progression to fibrosis [304] and the *LDLR* chr19:g.11113589A>G; NM_000527.5:c.1413A>G; p.Arg471=; rs5930 variant in exon 10 correlates with viral clearance [305] . In addition, the *LDLR* chr19:g.11120205T>C; NM_000527.5:c.1959T>C; p.Val653=; rs5925 variant was found to show a Caucasian ethnicity-specific association with lower HCV susceptibility [306] .

Among other cell surface receptors required for HCV entry, the *CD81* chr11:g.2388869C>T; NM_004356.3:c.67-1543C>T; rs708564 variant, either alone or combined with the *CLDN1* chr3:g.190321812C>A; NM_021101.4:c.223+172C>A; rs893051 variant, has been associated with lower susceptibility to HCV [307] . Furthermore, functional assays using hepatic cell lines led to the identification of three additional mutations in the fourth transmembrane segment of *CD81* chr11:g.2390506C>T; NM_004356.4:c.161C>T; p.Ala54Val; rs368611423, *CD81* chr11:g.2396697G>A; NM_004356.4:c.631G>A; p.Val211Met; rs139884987 and *CD81* chr11:g.2396815G>A; NM_004356.4:c.660G>A; p.Met220Ile; rs371516232 all of which rendered cells less susceptible to HCV infection. Regarding genetic variants that confer increased susceptibility to HCV infections, individuals homozygous for *IL6R* chr1:g.154416720A>T; NM_000565.3:c.85+11006A>T; rs4075015 and *HNF4A* chr20:g.44399750A>G; NM_001287184.2:c.41-6308A>G; rs3212172 variants were associated with an increased risk of HCV infection [308] .

IL28B, also called IFN-λ3, is a cytokine upregulated by HCV infection, contributing to protection against viral infection by signaling via the IL28RA/IL10RB heterodimer receptor. Several genetic variations in the *IL28B* gene have been found associated with various aspects of HCV infection [309,310] , including apparent resistance to HCV infection [311] . Among these two are notable the *IL28B* variant, which is linked to spontaneous and treatment-induced viral clearance [312,313] , and the *IFNL3* (*IL28B)* chr19:g.39252525T>G; NM_172139.4:c.-7587T>G rs8099917 variant, which is linked to the progression of chronic HCV infections [314] . In addition, the promoter variants *IL18* chr11:g.112164735; NM_001562.4:c.-607C>A rs1946518 and *IL18* chr11:g.112164265; NM_001562.4:c.-137G>C rs187238, which results in elevated levels of IL18, were significantly overrepresented in individuals with resolved HCV infection as compared to those with chronic HCV infections [315] . And the 5-UTR variant *IL12B* chr5:g.159315942; NM_002187.3:c.1188A>C; rs3212227 that enhanced IL12 production was associated with resistance to HCV infection [311] .

In addition, TLRs play a key role in HCV infection as evidenced by the antiviral effects demonstrated in early clinical trials using TLR agonists [316,317] . GVs within the promoter regions of *TLR7* and *TLR8*, such as *TLR7* chrX:g.12885361A>G; NM_016562.3:c.4-151A>G; rs179009, *TLR8* chrX:g.12906707A>T; NM_138636.5: c.1A>T; p.Met1Leu; rs3764880 and *TLR8* chrX:g.12906578C>T; NM_138636.5:c.1420+129C>T; rs3764879, all of which modify gene expression levels, have been associated with chronic HCV infection [318,319] .

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2.1.5.2. Genes associated with metabolite toxicity

CYPs are haem-containing enzymes, which belong to a protein superfamily and form the main components of phase I metabolism in the liver. Some of the CYP enzymes, specifically CYP1A1, CYP2E1, and CYP3A4 are also expressed in CCs. The CYP1A1 enzyme metabolizes exogenous compounds (e.g. drugs, tobacco, polycyclic aromatic hydrocarbons, nitrosamines and aromatic amines) to carcinogenic intermediates [320] . Individuals with the homozygous genotype for the WT prototypical allelic variant *CYP1A2*1A* are considered normal metabolizers, and were associated with a decreased CCA risk when compared to individuals homozygous for the *CYP1A2*1F* variant *CYP1A2*, chr15:g.74749576; NM_000761.5:c.−163C>A; rs762551 [321] . In turn, this alteration in the promoter region of the *CYP1A2* gene results in increased transcriptional activity and higher CYP1A2 enzyme activity.

Another class of enzymes involved in xenobiotic metabolism in CCs are N-Acetyltransferases (NATs). NATs catalyze the transfer of acetyl groups from acetyl-CoA to various substrates, including arylamines, arylhydroxylamines, and arylhydrazines. Certain genetic polymorphisms in NATs have been associated with a high CCA risk, such as NAT2 variants, which are considered to result in slower acetylator phenotypes by negatively affecting the acetylation rates of NAT2 through reduced enzyme stability and/or diminished protein expression. Remarkably, subjects harboring the alleles *NAT2*5* chr8:g.18400344T>C; NM_000015.3:c.341T>C; p.Ile114Thr; rs1801280, *NAT2*6B* chr8:g.18400593G>A; NM_000015.3:c.590G>A; p.Arg197Gln; rs1799930, and *NAT2*7A* chr8:g.18400860G>A; NM_000015.3:c.857G>A; p.Gly286Glu; rs1799931 were associated with a decreased CCA risk [321] .

Glutathione S-transferases (GSTs) are enzymes belonging to a protein superfamily and serve as vital components of phase II metabolism in the liver. They play a crucial role in detoxifying significant environmental carcinogens, such as benzo[a]pyrene and other polyaromatic hydrocarbons, through the GST system [322,323] . As dimeric proteins, they catalyze addition of glutathione to electrophilic mutagens, facilitating detoxification and their excretion. GSTs protect CCs by detoxifying harmful compounds, including environmental toxins and reactive oxygen species.

There are four major subfamilies of GSTs—GSTα, GSTμ, GSTθ, and GSTπ [324] —and each of these subfamilies is composed of several members. The GSTμ subfamily member GSTM1 carries a deletion polymorphism that, when homozygous (GSTM1-/-), abolishes detectable enzyme activity [325] . An analogous deletion affecting GSTT1 occurs in the GSTθ subfamily [326] . By contrast, GSTP1 (GSTπ) coding region variants chr11:g.67585218A>G; NM_000852.4:c.313A>G; p.Ile105Val; rs1695 and chr11:g.67586108C>T; NM_000852.4:c.341C>T; p.Ala114Val; rs11382723 alter the enzyme's catalytic properties [327–330] .

Reduced GST activity due to genetic GVs in the gene encoding GST may impair glutathione conjugation, thus leading to impaired detoxification and allowing carcinogens to accumulate in the liver. Several studies have shown that LoF variations in *GSTM1* could increase the risk of various cancers [331–335] . At least one study reported an association with the development of CCA in *O. viverrini*-positive individuals harboring predicted LoF (pLoF) variations in *GSTT* and who were also alcohol drinkers [336] . Another study found that the *GSTO1* chr10:g.104263031C>A; NM_004832.3:c.419C>A; p.Ala140Asp; rs4925, which has significantly lower deglutathionylation activity [337] , was associated with an increased risk of CCA [338] .

The 5,10-methylenetetrahydrofolate reductase (MTHFR) is a folate metabolism enzyme that directs metabolites toward either methylation reactions (crucial for DNA methylation) or nucleotide synthesis (critical for DNA replication and repair). Two extensively studied *MTHFR* GVs are *MTHFR* chr1:g.11796321C>T; NM_005957.5:c.665C>T rs1801133, resulting in reduced MTHFR enzyme activity, and *MTHFR* chr1:g.11794419A>C; NM_005957.5:c.1286A>C; rs1801131, associated with altered levels of DNA methylation and nucleotide synthesis. Genetic polymorphisms in the *MTHFR* gene can modify the availability of methyl groups for DNA methylation or influence nucleotide pools, potentially negatively impacting cancer susceptibility and response to therapy.

MTHFR variants, specifically *MTHFR* chr1:g.11796321C>T; NM_005957.5:c.665C>T; p.Ala222Val; rs1801133 and *MTHFR* chr1:g.11794419A>C; NM_005957.5:c.1286A>C; p.Glu429Ala;

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rs1801131, result in hyperhomocysteinemia and the development of liver steatosis. These variants have been associated with an increased risk for non-alcoholic steatohepatitis, which raises the risk for progression to CCA physiopathology [339] .

The homozygous genotype of the genetic variation in *MTHFR* chr1:g.11794407T>G; NM_005957.5:c.1298A>C; p.Glu433Ala; rs397507444 has been proposed as a factor that increases the risk of CCA in individuals positive for *O. viverrini* infection when compared to those with the WT genotype [340] . Another variant, *MTHFR* chr1:g.11796309; NM_005957.5:c.677T>C; p.Ile226Thr; rs1801133 has been documented to elevate CCA risk, particularly when combined with GVs in the thymidylate synthase enhancer region. This region competes with MTHFR for 5-methyltetrahydrofolate as a substrate for thymidylate synthesis [341] .

The multidrug resistance-associated protein 2, encoded by ABCC2, is expressed on the apical membrane of CCs. It plays a key role in the excretion of conjugates of carcinogens into the bile. The *ABCC2* chr10:g.99844450C>T; NM_000392.5:c.3972C>T; p.Ile1324=; rs3740066 variant, although a synonymous alteration, may alter protein folding and shift substrate specificity toward the ABC transporter ABCB1, by altering the translational kinetics and cotranslational folding [342] . In the Caucasian population, the *ABCC2* chr10:g.99844450C>T; NM_000392.5:c.3972C>T; p.Ile1324=; rs3740066 has been found to occur at a higher frequency in patients with CCA (32%) compared to healthy subjects [343] .

Chronic exposure to heavy metals through drinking water is a serious public health issue and has been associated with various cancers [344,345] . Epidemiological studies implicate arsenic-contaminated drinking water as a possible carcinogen for CCA, with stronger associations observed in Asian populations [346] . More than 70 countries around the world have an elevated arsenic content of groundwater exceeding the 10μg/L standard set by the World Health Organization [346] . Two pathways have been proposed for the detoxification of inorganic arsenic: (1) the classic oxidative methylation pathway [347] and (2) the reductive methylation pathway [348] . According to the classical pathway, arsenic enters the cells via the phosphate transporter, undergoes sequential methylation to generate only one end product, dimethyl arsenite (DMA). The alternative pathway suggests that arsenic either binds to certain proteins or conjugates with glutathione, followed by methylation to generate two end products, methyl arsonate (MMA) and DMA. In both pathways, purine nucleoside phosphorylase (PNP) reduces arsenic, and GSTO1 and GSTO2 reduce all the pentavalent arsenic species (i.e., As(v), MMA(v), and DMA(v)). Conversely, arsenic (+3) methyltransferase (AS3MT) methylates the trivalent arsenic species (As(iii), MMA(iii), and DMA(iii). Several genetic variations in *PNP*, *AS3MT*, *GSTO1* and *GSTO2* have been shown to contribute to inter-individual variations in arsenic metabolism and, thus, susceptibility to arsenic toxicity [349–351] . It has been found that individuals homozygous for the *AS3MT* chr10:g.102900499A>G; NM_020682.4:*c.1460A>G; rs10748835 variant had about half the MMA compared with WT homozygotes [352] . Remarkably, individuals heterozygous for the genetic variants *GSTO1* chr10:g.104263072-1042630786; NM_004832.3:p.Glu155del; rs72323784 and *GSTO1* chr10:g.104267301G>A; NM_004832.3:c.622G>A; p.Glu208Lys; rs11509438 displayed significantly higher inorganic arsenic levels in urine [353] . Higher inorganic arsenic and MMA levels were also associated with the LoF variations of *GSTM1 [354,355]* .

2.1.5.3. Genes associated with chronic autoinflammatory conditions

Although rare, autoimmune attacks on CCs are known to increase the risk of CCA development [356] . While both PSC and PBC are chronic autoinflammatory diseases that can damage CCs, PSC predisposes to CCA, whereas PBC is associated with reduced incidence of CCA [357] . PSC involves ongoing inflammation and damage to the bile ducts, primarily intrahepatic and extrahepatic large bile ducts [358] . PBC, on the other hand, primarily affects the small bile ducts within the liver, characterized by immune-mediated destruction of these ducts [359] . Genome Wide Association Studies have identified a number of genetic susceptibility loci to PSC, mostly in the HLA complex genes [360–365] and genes expressed in various immune cells, such as *BLTP1 (KIAA1109)*

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chr4:g.122194347A>C; NM_001384125.1:c.148-8422A>C; rs13151961, *IL2/IL21* intergenic region chr4:g.122588266G>C/T; NM_000586.5:n.5746G>C/T; rs6822844, *IL21-AS1* chr4:g.122633552C>T; NM_001300994.2:n.2423-1463C>T; rs6840978, *CARD9* chr9:g.136372044C>T; NM_052813.5:c.35G>A; p.Ser12Asn; rs4077515, *PUS10* chr2:g.60943910A>G; NM_001243084.1:c.103-212A>G; rs6706689, *IL2RA* chr10:g.6072893A>G/T; NM_000417.4:c.264+2389A>G/T; rs10905718, *MST1* chr3:g.49684099G>A; NM_020998.4:c.2107C>A; p.Arg703Cys; rs3197999 [366–369] . The NK receptor G2D (CD314), encoded by KLRK1, is an activating immune receptor expressed on NK cells, γδ T cells, and subsets of CD8+ T cells; its ligands are upregulated on stressed or virus-infected cells and trigger lymphocyte-mediated cytotoxicity upon engagement. At least two non-coding GVs *KLRK1* chr12:g.10384670C>T; NM_007360.5:c.148+2233G>A; rs11053781 and *KLRK1* chr12:g.10406632G>A; NM_007360.5:c.*17+1004C>T; rs2617167 have been associated with increased CCA risk in PSC patients [370,371] .

Glypicans, such as GPC5 and GPC6, belong to the glypican family, which consist of cell surface heparan sulfate proteoglycans that also function as cofactors for FGFRs. There is some evidence indicating a role of glypican in CCs, as silencing of *GPC6* in a CC cell line resulted in the upregulation of the FXR involved in xenobiotic metabolism. The *GPC6* chr13:g.93861537; NM_005708.5:c.711+30992A>T; rs9524260 variant was found to show significant association with the susceptibility to PSC [366,372] .

2.1.5.4. Genes associated with bile toxicity

Over 95% of secreted BAs are recycled through the enterohepatic circulation. Reabsorbed bile that returns to the liver contains primary s and secondary BAs, the latter ones being modified by gut microbiota. Thus, bile composition is influenced by both exogenous (secondary BAs) and endogenous (xenobiotics) molecules, which have the potential to act as mediators of inflammation. Under normal homeostasis, CCs are maintained in a mitotically inactive state via the expression of a number of proteins regulating the cell cycle [373] . However, under pathological circumstances, CCs react to mediators of inflammation and become activated. Consequently, reactive CCs begin to proliferate [374,375] and exhibit changes in the expression of several protein molecules involved in inflammation and fibrosis, collectively termed as the 'ductular reaction' [376] . Reactive CCs also undergo apoptosis alongside proliferation [374,377] , resulting in loss of integrity to the biliary ductular epithelium.

Genetic factors influencing bile stasis also contribute to CCA risk. Variants in genes such as *SLC10A2*, which encodes the apical sodium-dependent BAtransporter, have been linked to gallstone formation and impaired BAtransport, predisposing to chronic biliary injury [378] . Similarly, the germline variant *VEGFA* chr6:g.43784799; NM_001025366.2:c.*237C>T; rs3025039 may influence susceptibility to biliary atresia [379] and in turn genetic predisposition to gallstones and thereby an increased risk of CCA [378] .

The ATP-binding cassette subfamily B member 4 (ABCB4) is a membrane transporter protein that is primarily expressed in the canalicular membrane of CCs belonging to the ABC superfamily. In specific, it is involved in the bile formation by transporting phosphatidylcholine from the cytoplasm to the canalicular membrane, where BAs and other biliary components are secreted into the bile canaliculi. Genetic variations within the *ABCB4* locus, such as *ABCB4* chr7:g.87451711A>C; NM_000443.4:c.620T>G; p.Ile207Arg; rs2116802789 and *ABCB4* chr7:g.87422135T>TA; NM_000443.4:c.2301dupT; p.Thr768Tyrfs26Ter: rs2546785164 were shown to impair transporter activity and increase susceptibility to cholestatic liver disorders in carriers [380] . The *ABCB4* chr7:g.87450090; NM_000443.4:c.711A>T; p.Ile237Ieu; rs2109505 variant has been associated with a cholestatic phenotype and an increased risk for the progression of PBC [381] .

Similarly, polymorphisms in the *VEGFA* gene, particularly the *VEGFA* chr6:g.43784799C>T; NM_003376.4:c.936C>T; rs3025039, may influence susceptibility to biliary atresia, a neonatal cholestatic disorder that increases long-term bile duct injury risk as well [379] .

During homeostasis, the apical primary cilium of the CCs detects bile flow, osmolarity changes, and chemical signals via several membrane proteins. Genetic variations in the G protein-coupled bile

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acid receptor 1 (*GPBAR1*), which is expressed in CC cilia, have been associated with PSC [372] . GPBAR1 plays a versatile role during bile duct injury. It is also expressed in macrophages and is, thus, involved in BA-induced suppression of macrophages [382] . Interestingly, there is a functional crosstalk between GPBAR1 and CFTR. GPBAR1 is not only colocalized with the CFTR in CCs, but also prompts the activation of CFTR via GFR stimulation of GPBAR1. Furthermore, it was also found that LoF genetic variations in *CFTR* results in reduced chloride concentrations and bicarbonate levels within the bile ducts [383–385] , leave the biliary epithelium vulnerable to the toxic effects of bile [386,387] , possibly via compromised GPBAR1 activity.

2.1.6. Genes associated with dedifferentiation pathways

In previous sections, we examined the intricate relationship between genetic factors and the activation of the FGF/FGFR signaling pathway in CCs. We also explored how genetic variations may influence the chronicity of biliary epithelial damage caused by environmental exposures, metabolic disturbances, bile toxicity, autoimmune responses, and viral infections.

Tumorigenesis often co-opts core embryonic developmental and patterning programs, with epithelial-mesenchymal transition (EMT) serving as a hallmark example [388] . This observation underpins the concept of onco-ontogeny recapitulating phylogeny, which posits that oncogenic transformation mirrors the embryonic developmental trajectory of the affected organ. The broader idea of ontogeny recapitulating phylogeny suggests that embryonic development stages reflect the evolutionary history of a species [389,390] . In cancer, this principle is echoed by the reactivation of embryonic programs during tumor progression [388,391,392] . The pervasive cellular plasticity observed across cancer types further reinforces this developmental parallel [393–395] .

During normal embryogenesis, EMT is essential for gastrulation, occurring around three weeks post-fertilization, when cells migrate through the primitive streak to form the mesoderm and endoderm. In malignancy, EMT contributes to dedifferentiation, therapy resistance, and metabolic reprogramming, including a shift toward glycolytic metabolism [396]. In human CCs, EMT can be induced via FGF signaling [397]. FGF signaling—alongside NOTCH, YAP, WNT/CTNNB1, and TGFB1 pathways—is critical for early liver and bile duct development. Specifically, FGF signals from the cardiac mesoderm initiate hepatic diverticulum invagination into the septum transversum mesenchyme, where cells undergo hepatic specification to form hepatoblasts. These progenitors later differentiate into hepatocytes and CCs. FGF also regulates bile duct branching and morphogenesis through interactions with BMP and TGFβ pathways. FGFR1 and FGFR2 are selectively expressed in ductal plates and intrahepatic bile ducts, underscoring their role in bile duct formation [398,399] . Given these developmental roles, it is plausible that CCA tumors arising from bile epithelium recapitulate aspects of ductal plate development. Indeed, many CCA tumors exhibit molecular and cellular features of both hepatocytic and cholangiocytic lineages [400–404] , suggesting an intermediate progenitor origin. While mature hepatocytes predominantly express FGFR4, CC and gallbladder epithelial progenitors rely more on FGFR1 and FGFR2 signaling.

The biliary epithelium, positioned between hepatocytes and bile ducts, adapts dynamically to injury from bile toxicity, autoimmune insults, or viral infections. The nature and duration of these insults shape the epithelium's plasticity and repair response. Three primary modes of biliary repair have been identified:

Proliferation of Mature CCs: Acute injury prompts mature CCs to proliferate and restore epithelial integrity.

Activation of Progenitor or Stem-Like Cells: Chronic injury activates progenitor reservoirs capable of differentiating into both CCs and hepatocytes.

Dedifferentiation and Transdifferentiation: Prolonged damage induces CCs or hepatocytes to adopt a reactive ductular cell (RDC) phenotype, reverting to bipotent hepatic progenitor cell (HPC) states **[405]**·[406,407] .

At injury sites, RDCs engage in autocrine and paracrine signaling with immune cells. Damaged CCs release chemokines and cytokines that recruit and activate innate and adaptive immune cells

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[408–415] . These immune cells orchestrate repair by inducing apoptosis in damaged CCs, modulating adjacent cell proliferation, expanding HPCs [416–420] , and influencing hepatocyte plasticity [421] . Interactions within the TME may also drive oncofetal reprogramming, a process distinct from fetal development due to its lack of regulatory control.

Chronic biliary injury thus triggers a repair response characterized by dedifferentiation and reversion to progenitor-like states. Mouse models show that FGFs are upregulated during liver injury [422,423] , and also play important roles in liver regeneration [424–427] . FGF7, which signals via FGFR1 and FGFR2, has been shown to be a critical regulator of HPCs [428] . FGF7, signaling through FGFR1 and FGFR2, is a critical regulator of HPCs. FGFs also maintain liver homeostasis via paracrine, autocrine, and endocrine signaling modes [429,430] .

The dual role of FGF/FGFR signaling in both embryonic development and oncogenic transformation underscores its vulnerability to germline perturbations. Variants affecting ligand-receptor affinity, downstream signaling fidelity, or feedback regulation may predispose individuals to aberrant repair responses, excessive plasticity, or oncofetal reprogramming. These germline alterations could serve as molecular “shields” or “triggers,” modulating susceptibility to CCA through developmental reactivation and dedifferentiation pathways.

2.2. Germline Variants Shielding Tumorigenesis (Tumor-extrinsic mechanisms)

The development of CCA is not an isolated event stemming from a single oncogenically transformed neoplastic cell. Rather, it involves a prolonged interaction between the neoplastic cells and various immune and non-immune cell types within the TME, which is profoundly influenced by germline determinants. These germline variations can impact vasculature formation, stromal fibroblast recruitment, EM deposition, and, importantly, the responsiveness of innate and adaptive immune cells. Tumor-extrinsic influences, particularly those encoded by the germline, contribute substantially to how the TME evolves and responds to immune surveillance. GVs significantly influence cellular interactions and signaling pathways central to CC biology. These patient-specific GVs likely shape the TME by modulating processes such as neovascularization, stromal fibroblast recruitment, and EM deposition. Innate immune cells, as early responders at tumor initiation sites, act as gatekeepers for the subsequent infiltration of adaptive immune cells, including T-regulatory and cytotoxic CD8+ T cells. Importantly, the crosstalk between FGF/FGFR signaling and immune pathways represents a key mechanism by which GVs contribute to tumor immune evasion, shielding malignant cells from effective anti-tumor immune responses. Building on these insights, therapeutic targeting of the TME is now being explored as a promising strategy for treating various cancers, including CCA [431,432] .

The FGF/FGFR pathway, while central to regeneration, also participates in shaping an immunosuppressive stroma when aberrantly activated. GVs that augment or stabilize receptor signaling can thereby enable tumor immune evasion.

2.2.1. FGF/FGFR signaling regulates PD-L1 expression

FGF signaling enhances PD-L1 expression through direct transcriptional activation and modulation of tumor–stroma immune interactions. FGF/FGFR signaling activates several canonical downstream pathways, including JAK/STAT3, PI3K/AKT, and MAPK cascades, all of which influence PD-L1 transcription. In CCA, FGFR-driven activation of STAT3 can enhance PD-L1 transcription, facilitating immune escape by suppressing cytotoxic T lymphocyte activity. Concurrently, activation of MAPK and PI3K cascades not only support cell proliferation and survival but also promote immune checkpoint expression through convergent signaling mechanisms [433] . Intrahepatic CCA frequently harbors FGFR2 fusions or over-expression of *FGFR* mRNA. Approximately 20–25% of ICCs display FGFR2 fusions, whereas up to 60% of fusion-negative cases show elevated FGFR1–4 expressions. However, studies show a nuanced relationship between FGFR activity and PD-L1 expression. FGFR2 fusion–positive ICCs often exhibit low or absent PD-L1 expression, while fusion-negative tumors with high *FGFR* mRNA expression can display PD-L1

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positivity in a subset of cases. This suggests that PD-L1 regulation in CCA may depend more on the functional activation of FGFR signaling rather than specific genetic fusions [434] .

2.2.1. Combined actions of FGF/VEGF signaling in T cell exhaustion

VEGFA overexpression in CCA has been linked to increased FGF activity through shared downstream signaling pathways such as PI3K/AKT and MAPK/ERK. These pathways potentiate tumor survival, invasion, and angiogenesis, creating a positive feedback loop that sustains vascular remodeling and tumor cell proliferation. In particular, *VEGFA* can enhance FGFR signaling indirectly by upregulating MMPs, which release matrix-bound FGFs into the TME, thus amplifying FGFR activation [435] . The combined activation of FGF and VEGF signaling components within the TME is a potent driver of T cell suppression and exhaustion. TME-derived VEGFA increases PD-L1 and other inhibitory checkpoints, promoting CD8+ T cell exhaustion, which anti-angiogenic VEGFA–VEGFR inhibitors can counteract [436] . FGF7 additionally modulates immune cell infiltration, with a pronounced effect on CD4+ T-cell recruitment. VEGFA and FGF2 significantly upregulate the expression of PD-L1, CTLA4, and TIM2 on T cells, while inhibiting the secretion of IFNγ, GZMB and cytotoxic T cell functions. Surprisingly, this immunosuppressive effect is reverted by lenvatinib but not sorafenib [437] . Moreover, FGF2 signaling deficiency augments T cell responses, as evidenced by experimentally elevated CD4 and CD8 T cell counts in FGF2 signaling–deficient mice [438] . Studies in related cancer patients have shown that combined stimulation of VEGFA and FGF2 results in inhibition of IFNγ and GZMB secretion by T cells [437] . On the other hand, the use of anti-VEGFR and anti-FGFR monoclonal antibodies increases the percentage of IFNγ and GZMB secretion by activated CD8+ T cells [439] . VEGFA has been shown to drive T cell exhaustion by inducing inhibitory checkpoint pathways. Furthermore, VEGFA also enhances expression of PD-L1, CTLA4, TIM3 and LAG3 on $CD8^+$ T cells in a dose-dependent manner, resulting in co-expression patterns characteristic of exhausted T cells [436] . The effect occurs through VEGFR2-NFATC1 signaling, and inhibition of VEGFR2 can reverse this phenotype. Subsequent work confirmed that VEGFA signaling directly increases expression of PD-L1, CTLA4, and TIM3 on T cells, promoting exhaustion via the VEGFR2–PLCγ–calcineurin–NFAT pathway [440] . Taken together, combined signaling actions of VEGF/FGF elicits anti-tumor immunity.

Germline Variations in both *VEGFA* and *VEGFR* genes have been shown to influence VEGF pathway activity. Notably, several GVs in the *VEGFA* gene have been extensively characterized for their impact on VEGFA expression and signaling. For instance, *VEGFA* chr6:g.43770613; NM_003376.4:c.-634G>C; rs2010963, located in the 5′-UTR, influences VEGFA expression and circulating protein levels [441] , thereby potentially affecting angiogenesis and potentially predicting prognosis of CCA [435] . Similarly, *VEGFA* chr6:g.43784799C>T; NM_003376.4:c.936C>T; rs3025039 in the 3′-UTR regulates *VEGFA* mRNA stability, hence altering gene expression [442] . Furthermore, promoter variants such as *VEGFA* chr6:g.43770093A>G; NM_003376.4:c.-1154G>A; rs1570360 and *VEGFA* chr6:g.43768652A>C; NM_003376.4:c.-2578C>A; rs699947 modify transcription factor binding, thus impacting total VEGFA synthesis [441] . Additionally, variants such as chr6:g.43957870; NM_001318876.2:c.945+428599G>A; rs6921438 and chr6:g.43982716; NM_001318876.2:c.945+453445T>C; rs4416670 serve as expression quantitative trait loci, correlating with lower circulating VEGFA concentrations [443] . *FGFR* gene polymorphisms, incNM_213647.3:c.1162G>Aluding the intronic variants *FGFR2* chr10:g.121591810; NM_022970.3:c.109+1899A>G; rs2420946, *FGFR2* chr10:g.121586676; NM_022970.3:c.109+7033T>A; rs1219648 [444,445] and the signaling variant *FGFR4* chr5:g.177093242; NM_213647.3:c.1162G>A; p.Gly388Arg; rs351855 [446] , contribute to variations in receptor function and expression. These GVs may influence ligand binding affinity and the downstream activity of both PI3K/AKT and MAPK pathways, thus reinforcing the tumor survival and immune evasion mechanisms mediated by FGF signaling. For *VEGFR2 (KDR)*, functional GVs such as *KDR* chr4:g.55113391A>T; NM_002253.4:c.889A>T; p.Val297Leu; rs2305948 and *KDR* chr4:g.55106807T>A; NM_002253.4:c.1416A>T; p.Gln472His; rs1870377 alter receptor activity, ligand binding, and

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dimerization, which altogether modulates signal transduction [447,448] . The promoter variant *KDR* chr4:g.55126199A>G; NM_002253.4:c.-604T>C; rs2071559 affects *VEGFR2* expression, while rs7667298, an intronic variant, is implicated in regulatory modulation of *VEGFR2* expression [449] . Collectively, these GVs modulate transcriptional regulation, receptor binding, and signaling efficiency, which in turn impact angiogenesis, tumor progression, and immune regulation (including aspects relevant to T cell exhaustion through VEGF/FGF pathways). Their interplay can influence susceptibility to cancer progression and responses to therapy, highlighting their potential as biomarkers for personalized treatment strategies in CCA pathophysiology.

3. Persistent FGF/FGFR signaling - a contributor of CCA pathogenesis

FGF/FGFR signaling→Bile accumulation & toxicity→Chronic Injury→Dedifferentiation

During bile duct formation, FGF signaling plays an important role in the branching and morphogenesis of the bile duct network [450] . Germline variations causing disruptions in FGF/FGFR signaling can, therefore, lead to a variety of bile duct disorders including biliary atresia and intrahepatic cholestasis, which may indirectly contribute to the development of CCA. In fact, a great degree of variations in the biliary tree structure is observed in the general population reflecting heterogeneity in biliary duct formation [451] . Certain genetic mutations in the FGF/FGFR signaling pathway have been attributed as the contributing factor to a range of congenital abnormalities in biliary tree formation [452] . Biliary atresia is a rare congenital condition in which the bile ducts are underdeveloped [453] . This can lead to a buildup of bile in the liver, which can ultimately lead to chronic injury to CCs & HCs. The human bile in addition to BAs also contains FGF19 *[454]* . Under normal physiological conditions, FGF19 signals primarily via FGFR4 and triggers a signaling cascade that leads to the production of BAs [455–457] . However, prolonged BA production is blocked via a feedback loop, which regulates bile production, flow and reabsorption from the intestine. BAs from the reabsorbed bile can activate a variety of receptors on hepatocytes and CCs, including the FXR and the pregnane X receptor. Altogether, these receptors regulate the expression of genes that are involved in BA transport and synthesis. For instance, in the intestine both FXR and pregnane X receptor inhibit the expression of FGF19 and the BA transporter NTCP, and in the liver, they suppress transcription of *CYP7A1*, the rate-limiting enzyme for de novo BA synthesis from cholesterol [458,459] . In addition, excessive FGFR-RAS signaling can also lead to the accumulation of tricarboxylic acid (TCA) cycle enzymes such as 2-hydroxyglutarate (2-HG) and isocitrate dehydrogenase 1 (IDH1), both of which are oncometabolites that can directly inhibit the activity of key enzymes involved in BA synthesis. In cholestatic patients, increased serum FGF19 levels and the abundant expression of hepatic FGF19 were observed, indicating that FGF19 signaling is involved in some of the adaptations that protect the liver against BA toxicity [460] .This suppression of BA synthesis as a negative feedback mechanism prevents excessive BA buildup in the liver, which can lead to BA-induced toxicity to CCs.

However, under chronic conditions, decreased BA production and compensatory mechanisms are unable to prevent long-term liver damage. Particularly, reduced BA synthesis over an extended period can inadvertently cause numerous problems including fat malabsorption and deficiencies in vitamins A, D, E, and K, which may ultimately lead to gallstone formation and increase the likelihood of BA–induced toxicity, thereby elevating the risk of CCA. Furthermore, accumulation of TCA cycle metabolites, particularly 2-HG, can induce DNA damage by inhibiting DDR enzymes such as ATM and ATR. This, in turn, promotes genomic instability through disruption of one-carbon metabolism, resulting in altered DNA methylation patterns that can affect chromatin structure and drive dedifferentiation in CCs. These processes altogether contribute to cancer development by reactivating primitive cellular programs reminiscent of early evolutionary stages, often summarized as onco-ontogeny recapitulating phylogeny.

In addition, increased bile accumulation also modifies the immune cells in the liver that interact with CCs [459] . Bile that is reabsorbed in the intestine is modified by intestinal microbiota, thereby altering the composition of this secondary bile. Secondary bile contains proinflammatory

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components, which activate various receptors in CCs in the liver, such as the plasma membrane- and nuclear-bound TGR5 and FXR, respectively. In addition, *FXR* and *TGR5* are also expressed in various immune cells, including Kupffer cells [382] , NK and NK-T cells [461] , monocytes, macrophages and DCs [462] . Remarkably, the activation of these immune cells creates a proinflammatory environment, which under chronic conditions could potentially favor a cytokine-mediated dedifferentiation pathway [463,464] . The TGFβ family of cytokines is a well-established inducer of EMT [465] . Liver-specific deletion of TGFβ results in truncation or complete absence of the biliary tree network, thus establishing its prominent role in bile duct repair [466] . During chronic inflammation of the bile duct, TGFβ is produced by various cell types including damaged CCs, Kupffer cells and infiltrated macrophages [467,468] .

4. Limitations in studies investigating genetic predisposition to CCA

It is imperative to recognize that investigations relying on associations derived from clinical case studies or NGS analyses of patient cohorts may introduce an ethnicity bias, potentially complicating the interpretation of genetic associations with dubious clinical significance. This bias is evident in multiple instances, such as the incomplete replication of the association between BRCA syndrome and CCA within an Asian cohort [35] . In the study by Maynard *et al.*, inherited mutations were distributed fairly evenly across intrahepatic, extrahepatic, and gallbladder cancers; the highest frequency was in *BRCA1* and *BRCA2* (33.3%), with *PALB2*, *BAP1*, and *PMS2* also observed. *[40]* . In a comprehensive analysis, Cao *et al.* investigated the genomic heterogeneity between Chinese and North American populations, profiling 164 Chinese and 283 North American patients with iCCA. This analysis found significant genetic differences, Chinese patients having higher rates of alterations in *TP53, KMT2C, BRCA1/2, DDR, TERT, TGFBR2, RBM10, NF1, SPTA1*, and *RB1*. In contrast, the Western cohort displayed dominance in *IDH1/2, BAP1*, and *CDKN2A/B,* attributing this genetic diversity to variations in underlying disease risk factors [469] . Furthermore, the underrepresentation of individuals of African ethnicity in the majority of published CCA cohorts raises concerns when linking pathogenic/likely pathogenic GVs to CCA susceptibility. Notably, common observations from germline sequencing of BTCs indicate that bi-allelic loss is not overly prevalent, and a family history is often absent in patients harboring pathogenic/likely pathogenic variants. Importantly, up to 85% of patients in hepatobiliary, BTC, or CCA cohorts fail to meet the criteria for suspected cancer predisposition. Thus, clarity regarding the clinical utility of the pathogenic GVs associated with an elevated risk for CCA has not yet been established for the implementation of predictive genetic testing in clinical settings.

5. Conclusion

CCA is a malignancy increasingly associated with chronic inflammation and elevated FGF signaling. While significant therapeutic advances have been made with the availability of FDA-approved targeted inhibitors for FGFRs, the contribution of patient-intrinsic germline variations to the pathogenesis of CCA has not been previously explored. This review addresses this gap by establishing a conceptual framework where GVs associated with FGF/FGFR signaling exhibit a dual impact on tumor and immune cells. These pleiotropic variations promote cancer progression through both tumor-intrinsic mechanisms and tumor-extrinsic mechanisms that shield the tumors from anti-tumor immune responses. The signaling role of these GVs is evident through their convergence on mechanisms that potentiate FGF/FGFR pathway activity. Germline variations in genes governing chronic inflammation (i.e., *PTGS2, IL6, IFNγ, TNFα*) or DDR pathways are implicated in up-regulating FGF ligands and/or receptors. Furthermore, genetic predisposition to chronic onslaughts on the biliary epithelium—including viral infections (i.e., HBV, HCV, liver flukes), BA toxicity, and autoimmune conditions like PSC—induces persistent injury. This chronic state drives CCs and HC into a plastic, dedifferentiated state, often referred to as RDCs or HPCs. FGF signaling, particularly via the FGFR1/FGFR2 nexus, is a critical regulator of this cellular plasticity and plays a vital role in

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bile duct development and regeneration. The reactivation of these primitive cellular programs, consistent with the concept of onco-ontogeny recapitulating phylogeny, serves as a key pathway for CCA progression.

Conversely, the "shield" function underscores how germline variations shape the unique TME of each patient, influencing immune surveillance and anti-tumor responses. Germline alterations in immune signaling genes, such as those related to T cell activity — *CTLA4, RBM20, FRAS1* — or immune recognition — *NKG2D, TLRs, HLA* —modulate both innate and adaptive immunity. The combined stimulation of FGF/VEGF signaling components in the TME has been shown to induce T cell exhaustion by upregulating inhibitory checkpoints — *PD-L1, CTLA-4, TIM3* — and suppressing cytotoxic effector molecules — *IFNγ, GZMB*. Altogether, these interactions suggest that patient-specific germline profiles may determine the vulnerability of CCA tumors to immune-mediated cell destruction.

Understanding this dualistic behavior is essential for improving personalized medicine in CCA, potentially impacting the prognosis of both targeted and immune checkpoint blockade therapies. While three FGFR2-targeted drugs are FDA-approved, this personalized framework is especially critical for approximately 85% of CCA patients who lack actionable tumor-specific genetic alterations in *FGFR2*. Identifying these pleiotropic GVs allows for the discovery of new therapeutic vulnerabilities in these specific patient cohorts. For example, germline factors that induce a DNA repair deficiency phenotype, or "BRCAness," (such as in *IDH1/2* mutant tumors) may sensitize patients to PARP inhibitors, thus offering a complementary therapeutic strategy.

In summary, our comprehensive literature analysis presented here highlights the critical, previously unrecognized role of germline variations in governing FGF/FGFR signaling and the immune TME in CCA pathology. Future research must focus on validating these dual-impact variants in diverse patient cohorts, moving beyond single-gene studies and addressing ethnicity biases. Ultimately, establishing the clinical utility of these germline cancer biomarkers is warranted for stratifying patient risk and guiding truly personalized pharmacological interventions in CCA patients.

6. Implications for Personalized Medicine and Future Directions

The persistence of the FGF/FGFR signaling pathway remains an active focal point to identify therapeutic opportunities within the context of CCA. Although targeted FGFR2 inhibitors have improved outcomes for a minority of patients, the majority of CCA cases (approximately 85%) lack actionable tumor-specific alterations in *FGFR2*. Our conceptual framework, which highlights the dual impact of pleiotropic GVs associated with FGF/FGFR signaling—acting as both tumorigenic signals and immune shields—, provides a critical foundation to personalized therapeutic strategies.

6.1. Personalized Therapeutic Strategies based on Germline Vulnerability

Understanding the patient-intrinsic germline profile of each individual patient allows for the discovery of therapeutic vulnerabilities that may not be apparent from standard somatic tumor profiling alone.

6.1.2. Overcoming Germline-Driven Immune Evasion (The "Shield")

The identification of GVs that modulate the TME offers strategies for optimizing immunotherapy responses. The "shield" function of these variants highlights how they can suppress anti-tumor immunity via the combined influence of FGF/VEGF signaling components.

• **Targeting FGF/VEGF Axis to Enhance Immunotherapy:** Combined stimulation by FGF2 and VEGFA in the TME has been shown to induce T cell exhaustion by upregulating inhibitory checkpoints (PD-L1, CTLA4, TIM3) and suppressing cytotoxic effector molecules (IFNγ, GZMB). Interestingly, inhibiting the FGF/VEGF axis can reverse this immunosuppression. For instance, inhibition of FGFR signaling has been shown to restore tumor cell responsiveness to IFNγ stimulation

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in certain models. This suggests a rational combination strategy involving FGFR inhibitors (or anti-angiogenic agents targeting VEGFA-VEGFR) combined with immune checkpoint blockade (e.g., anti-PD-L1/CTLA4 antibodies) to break the germline-conditioned "shield" and maximize anti-tumor immunity.

• **Germline Biomarkers for Immunotherapy Response:** GVs influencing innate and adaptive immune cell function in genes such as *CTLA4, RBM20, FRAS1, NKG2D, TLRs, HLA* could serve as predictive biomarkers to stratify patients who are most likely to respond to immune checkpoint inhibition, therefore improving response rates and potentially overcoming pharmacological resistance.

6.2. Future Directions and Clinical Translation

To fully realize the potential of germline insights, future research should overcome current limitations and focus on translational efforts:

• **Development of Mechanism-Guided Pleiotropic Gene Variant Risk Score:** Moving beyond analyses focused solely on single-gene associations, the intricate and pleiotropic nature of CCA pathogenesis requires the development of a comprehensive risk assessment framework. CCA is a complex malignancy, and its pathogenesis involves the interplay between genetic predisposition and pleiotropic factors. Based on the exploration of dual impact GVs—acting as both signals promoting tumorigenesis and as shields protecting tumors from anti-tumor immune responses—we propose a conceptual framework for a Mechanism-Guided Pleiotropic Gene Variant Risk Score. The gene variants compiled in this work, which link polymorphic genetic variants to direct or pleiotropic influences on FGF/FGFR signaling pathways, could be foundational for this risk score. We provide this compilation as the MGP-Gene Panel, which clinical NGS-based diagnostics can employ for rigorous CCA association studies. While the concept of polygenic risk scoring is not yet sufficiently established for routine clinical adoption, these scores are crucial for integrating the impact of multiple PGVs into predictive models. Such integration is essential for advancing patient risk stratification and early diagnosis in CCA management.

• **Mechanistic Dissection of Resistance:** The mechanistic basis underlying resistance and poor prognosis to current targeted therapies in CCA still remains largely unknown. Future studies should investigate whether specific dual-impact GVs contribute to the emergence of acquired drug resistance, potentially by sustaining alternative signaling pathways, like cross-talk with EGFR or WNT signaling. Altogether, this will facilitate the development of novel combination therapies designed to overcome CCA treatment resistance.

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