Diagnostic and prognostic biomarkers in cholangiocarcinoma

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Abbreviations: CCA, cholangiocarcinoma; iCCA, intrahepatic cholangiocarcinoma; pCCA, perihilar cholangiocarcinoma; dCCA, distal cholangiocarcinoma; PSC, primary sclerosing cholangitis; eCCA, extrahepatic cholangiocarcinoma; CA19-9, carbohydrate antigen 19-9; EVs, extracellular vesicles; cfDNA, cell-free DNA; cfRNA, cell-free RNA; miR, microRNA; lncRNA, long non-coding RNAs; AUC, area under the ROC curve; HCC, hepatocellular carcinoma; CEA, carcinoembryonic antigen; IL-6, interleukin 6; CTCs, circulating tumour cells.

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Highlights
• Currently available diagnostic biomarkers for CCA are inaccurate.
• Potential new biomarkers in liquid biopsies may include nucleic acids, proteins and metabolites, extracellular vesicles and circulating tumour cells.
• New omics technologies facilitate the profiling and analysis of disease specific signatures and are powerful sources of biomarkers.
• Novel promising biomarkers for the diagnosis of CCA need to be validated in large international cohorts of patients, including appropriate control groups of individuals/patients.
ABSTRACT
The high mortality rate of cholangiocarcinoma (CCA) is due, in part, to the lack of non-invasive approaches able to accurately detect this silent tumour at early stages, when therapeutic options can be potentially curative or may at least increase the overall survival of patients. The fact that the majority of CCA tumours are not linked to any known aetiological factor highly compromises the monitoring of patients at risk for tumour development and also their early diagnosis. Combination of clinical/biochemical features, imaging techniques and analysis of non-specific tumour biomarkers in serum are commonly used to help in the diagnosis of CCA, but tumour biopsy is usually required to confirm the diagnosis. Moreover, no prognostic biomarkers are currently used in the clinical setting, deserving more innovative research, and international validation and consensus. Important efforts have been made in the last years to identify new accurate non-invasive biomarkers, by using innovative techniques and high-throughput omics technologies. This review summarises and discusses the advances in the investigation of novel diagnostic and prognostic biomarkers in CCA and envisions the future directions in this field of research.

Key words: biliary cancer; biomarker; CCA prognosis; early diagnosis; omics.

1. Introduction
Cholangiocarcinoma (CCA) comprises a highly aggressive and heterogeneous group of biliary malignancies that can originate at any site of the biliary tree, and account for ~15% of all primary liver cancers. Its incidence is increasing worldwide and currently represents ~2% of all cancer-related deaths per year. According to the anatomical localization, CCAs are classified into intrahepatic (iCCA), perihilar (pCCA) and distal (dCCA). The aetiology of the majority (~80%) of CCAs is unknown, but there are several risk factors that may predispose to its development, including age, obesity, diabetes, inflammatory liver diseases (primary sclerosing cholangitis [PSC], hepatolithiasis, cirrhosis), infectious agents (Opisthorchis viverrini, Clonorchis sinensis, hepatitis B [HBV], hepatitis C [HCV], human immunodeficiency virus [HIV]), drugs/toxins (alcohol, smoking, thorotrust, nitrosamines, asbestos, oral contraceptive pills, etc), and congenital disorders (choledochal cysts, Caroli disease, congenital hepatic fibrosis).

The diagnosis of CCA is usually conducted by a combination of clinical, biochemical, radiological, and histological information. Different imaging techniques may be used for the diagnosis of each CCA subtype: ultrasound (US), computed tomography (CT), magnetic resonance imaging/magnetic resonance cholangiopancreatography (MRI/MRCP) and positron emission tomography (PET) for iCCA, MRCP for pCCA and dCCA, percutaneous transhepatic cholangiography for pCCA, and endoscopic retrograde cholangiopancreatography or endoscopic ultrasound for dCCA. Histological analysis is mandatory to confirm the diagnosis.
and can provide valuable information for the clinical management of patients, but it is not always recommended due to the location of the tumours and the risk of peritoneal seeding. Moreover, the serum levels of non-specific tumour biomarkers, such as carbohydrate antigen 19-9 (CA19-9), are currently measured to help in the diagnosis of CCA, but these are unreliable due to their low sensitivity and specificity, particularly in early stages of the disease. As such, most patients with CCA are diagnosed late, when the disease is in an advanced stage, and when therapeutic options are reduced, resulting in dismal prognosis. However, in the small proportion of patients in which tumours are detected in early stages (~35%), surgical resection of the tumour or liver transplantation (in cirrhotic livers) can be potentially curative or, at least, significantly increase the overall survival of patients. Therefore, there is an urgent need to identify accurate non-invasive biomarkers for the diagnosis of these tumours, and thus increase the number of potential resectable cases.

In recent years, new innovative studies have been conducted in the quest for accurate biomarkers for the early diagnosis of CCA and also to predict prognosis, risk of relapse after surgery, and select the best therapeutic regimen(s) for patients. These strategies involve “omics” approaches in blood, bile, urine, extracellular vesicles (EVs) and tissues, and have resulted in promising candidates that can change the current paradigm.

2. Non-invasive biomarkers

2.1. Circulating nucleic acids

Circulating nucleic acids can be found in most biofluids and comprise fragments of genomic DNA (cell-free DNA [cfDNA]) and RNA (cfRNA; typically microRNAs [miRs], but also long non-coding RNAs [lncRNAs]). Whether actively exported or originated from dying cells, circulating nucleic acids embody potential diagnostic and/or prognostic tools for human disease, including CCA.

Cell free DNA
cfDNA was first shown to reflect changes in cancer aggressiveness and tumour size in the late 70’s, highlighting its potential as a diagnostic and/or prognostic biomarker. The ability to screen for mutations in cfDNA is particularly appealing when compared to the primary tumour, as it more accurately reflects the overall mutational pattern of these heterogeneous tumours. This concept was recently validated in CCA; plasma samples from CCA patients with known tumour genomic background were screened for 31 oncogenic mutations in KRAS, NRAS, BRAF, and PIK3CA genes by multiplex digital PCR. For each patient, the exact mutations in the tumour were also found in the plasma. Of note, the cfDNA from patients with CCA tumours, but wild type for the 31 studied mutations, was used as negative control and strongly supported the data. These results suggest that the use of cfDNA screening in patients with...
CCA may be helpful in order to determine the mutational characteristics of the primary tumours and to guide potential mutation-based therapeutic interventions. In this regard, an integrative genomic characterization of cfDNA, primary tumours, and metastases of iCCA patients with FGFR2 mutations and with acquired resistance to BGJ398, a pan-FGFR inhibitor, revealed de novo point mutations in the FGFR2 kinase domain that were detected in cfDNA.\textsuperscript{10} Despite the small sample size, given that FGFR2 mutations are found exclusively in iCCA and account for ~10% of the diagnosed cases,\textsuperscript{11} screening for these alterations in cfDNA may represent an important approach to guide clinical decisions.

Cell free non-coding RNA
Throughout the last decade, cfRNAs, particularly miRs, have been envisioned as promising disease biomarkers due to their abundance and stability in biofluids. In comparison to other putative biomarkers, such as proteins or metabolites, miRs are less resistant to degradation and/or modification and can be easily detected and amplified. In the context of CCA, few studies have investigated the circulating miR profiles in patients (Figure 1), but two meta-analyses have suggested their overall potential diagnostic value.\textsuperscript{12,13} Data analysis on both meta-analyses pinpointed miRs as promising tools for CCA diagnosis, with pooled sensitivities of 0.83 and 0.76, and specificities of 0.79 and 0.91, respectively. In both studies, pooled area under the ROC curve (AUC) was ~0.9. Further, bile was the biological fluid showcasing the highest diagnostic efficiency (AUC of 0.95), followed by serum (0.913), tissue (0.846) and urine (0.745).\textsuperscript{12}

Circulating miR-21 is one of the most well-characterized miRs in terms of its potential as a biomarker for CCA. Increased serum and plasma levels of miR-21 were already shown to allow for the differential diagnosis between patients with iCCA and healthy controls,\textsuperscript{5} with an AUC of 0.91 in serum and 0.94 in plasma.\textsuperscript{14} More recently, serum miR-21 levels were also found to positively correlate with tumour stage (TNM criteria) and poor survival. Indeed, miR-21 serum levels decreased after tumour resection, highlighting the value of circulating miR-21, not only as a diagnostic tool, but also as a putative prognostic biomarker.\textsuperscript{15} However, the levels of this miR, considered an onco-miR,\textsuperscript{16} are also found elevated in serum of patients with hepatocellular carcinoma (HCC)\textsuperscript{17-20} and other cancers,\textsuperscript{21-24} probably limiting its specificity to discriminate between tumours, particularly primary liver tumours. Serum miR-26a levels have also been found increased in patients with CCA and, similarly to miR-21, they positively correlate with clinical stage, metastasis, tumour differentiation status and poor survival. In terms of its diagnostic value, serum miR-26a yielded an AUC value of 0.90 (sensitivity: 84.8%; specificity: 81.8%) in distinguishing CCA from healthy controls.\textsuperscript{5} Decreased serum levels of miR-106a in CCA patients appear to also act as a predictor of poor prognosis, while signalling a higher likelihood of lymph node metastasis.\textsuperscript{25} miR-150 represents another potential CCA diagnostic biomarker, although apparently contradictory results have been published. A
microarray study using plasma from patients with CCA reported reduced miR-150 levels in CCA compared with healthy individuals and patients with PSC.\textsuperscript{26} In contrast, a different study highlighted miR-150 as being upregulated in iCCA compared with controls without cancer (AUC: 0.764; sensitivity: 80.6%; specificity: 58.1%).\textsuperscript{5} Of note, the above study also showed that the combination of miR-150 with CA19-9 improved the diagnostic accuracy of both biomarkers. Indeed, it is now apparent that merging different miRs into a panel of biomarkers can offer greater sensitivity and specificity. For instance, a miR profile of 8 plasma miRs (483-5p, 505-3p, 874, 885-5p, 320b, 92b-3p, 1275, 1307-3p) was shown to associate with iCCA, regardless of the degree of tumour differentiation.\textsuperscript{27} The levels of miR-192 were also shown to be increased in serum from patients with \textit{O. viverrini}-related CCA compared with healthy controls (AUC: 0.803; sensitivity: 74%; specificity: 71%), positively correlating with lymph node metastasis and poor survival.\textsuperscript{28} Of note, in this study, the authors also reported increased serum levels of both miR-21 and miR-150, further supporting their potential diagnostic value, while miR-26a was found downregulated, conflicting with previous data and thus rising some questions regarding its diagnostic accuracy for all forms of the disease.

Since PSC is a well-established risk factor for CCA development, some studies have evaluated and compared the serum miR profile between patients with isolated PSC and those with PSC-derived CCA. For instance, increased serum levels of miR-222 (AUC: 0.71) and miR-483-5p (AUC: 0.70) were found in CCA patients compared with PSC patients. The combination of both miRs increased the AUC to 0.77, suggesting that they could help in the monitoring of PSC patients for early CCA detection.\textsuperscript{29} In another study, a panel composed of 5 serum miRs (26a, 30b, 122, 126 and 1281) was markedly different in patients with CCA compared with PSC patients,\textsuperscript{30} further underlining their potential diagnostic value. Nevertheless, it is worth mentioning that these studies did not specifically include CCA patients with a PSC background, which would be of great importance in order to find accurate diagnostic biomarkers for PSC patients who might be at risk for CCA development.

Cholangiocytes have a pivotal role in bile formation, regulation and transport. In turn, circulating bile miRs may also embody a key diagnostic and/or prognostic value in CCA. miRs have already been profiled in bile samples from patients with CCA through high-throughput PCR miRNA microarray, with miR-9 being highlighted as a potential diagnostic biomarker (AUC: 0.98; sensitivity: 88.9%; specificity: 100%).\textsuperscript{31} Furthermore, the levels of miR-150-5p were found lower in bile from patients with CCA compared with healthy individuals.\textsuperscript{26} On the other hand, the levels of miR-412, -640, -1537 and -3189 were found increased in bile from patients with PSC-derived CCA compared to those with isolated PSC, allowing the differential diagnosis of these two diseases with an AUC value of ~0.8. Of note, combination of miR-1537 with CA19-9 provided a higher diagnostic value when compared solely with CA19-9.\textsuperscript{30}

Some studies have also explored the potential of urinary miRs as biomarkers for CCA (Figure 1). For instance, urinary levels of miR-192 and miR-21 have been found markedly increased
in patients with *O. viverrini*-related CCA compared with healthy individuals, and the combination of these two miRs increased its diagnostic value, when comparing with each one used alone (AUC: 0.85; sensitivity: 81.8%; specificity: 71.4%).

Overall, since most data on circulating nucleic acids as diagnostic and prognostic biomarkers in CCA have resulted from proof-of-concept studies, larger and international evaluations are eagerly awaited to validate their potential clinical value.

### 2.2. Cytokines/proteins

CA19-9 and carcinoembryonic antigen (CEA) are the most widely clinically-used biomarkers to help in the diagnosis and/or monitoring of CCA, but there are large differences in sensitivity and specificity among the different published studies, limiting their diagnostic and prognostic value. For CA19-9, the most recent data resulting from a large meta-analysis described a pooled sensitivity and specificity of 72% and 84%, respectively, regarding the distinction between CCA and healthy controls or patients with benign biliary disease. Similarly, the diagnostic sensitivity and specificity of CEA range from 42% to 85% and 70% to 89%, respectively. Increased serum levels of CEA and CA19-9 have been proposed as an indicator of reduced overall survival in resectable or inoperable CCAs. However, while an important number of studies describe CEA and CA19-9 as independent prognostic markers, prognostic cut-off values vary significantly between reports and large meta-analyses are still lacking. Nonetheless, when elevated in CCA, CA19-9 was further suggested as biomarker to monitor response to chemotherapy and predict outcome in CCA patients.

Other promising circulating diagnostic and prognostic biomarkers (Table 1) include cytokeratin-19 fragment (CYFRA 21-1), matrix metalloproteinase-7 (MMP-7) and osteopontin. CYFRA 21-1 is elevated in patients with iCCA compared to patients with benign biliary diseases (sensitivity: 75.6%; specificity: 96.2%), presenting superior diagnostic values than CA19-9 and CEA. In addition, serum levels of CYFRA 21-1 correlate with disease stage, and represent an independent predictor of impaired relapse-free and overall survival. MMP-7 serum levels are also elevated in patients with CCA compared to patients with benign biliary (sensitivity: 75%; specificity: 78%), but its prognostic relevance is still unclear. Circulating osteopontin, a secreted glycol phosphoprotein, is also elevated in CCA patients compared to healthy controls or patients with PSC. It is important to note that high pre- and postoperative osteopontin levels were associated with reduced overall survival after tumour resection.

Circulating cytokines have also been proposed as diagnostic and prognostic biomarkers in CCA patients. The pro-inflammatory cytokine interleukin 6 (IL-6), secreted by CCA cells is found elevated in serum of patients with CCA compared to healthy individuals (sensitivity: 73%; specificity: 92%), and was further proposed as marker for therapy monitoring. Other potential biomarker candidates reported to diagnose CCA are S100A6, DKK1, KL-6-
Mucin\textsuperscript{52} and SSP411\textsuperscript{53}. However, larger studies are warranted to investigate/confirm their diagnostic and/or prognostic relevance in CCA.

2.3. Metabolites
Metabolomics or metabolic profiling, defined as the analysis of low molecular weight metabolites (<1,500 Da) in biological samples, is a promising approach for the identification of potential biomarkers useful in the diagnosis and prognosis of different diseases, including different types of cancers, like CCA.\textsuperscript{4} Due to the large number of molecules present in biological specimens, powerful bioinformatics tools for data mining and visualization are used to present the results in a comprehensive way (Figure 2).

Cancer cells present profound alterations in their metabolism,\textsuperscript{54} which represent an opportunity for diagnosis and monitoring. The analysis of the metabolome in body fluids (blood/serum/plasma, bile or urine) is emerging as a new diagnostic strategy in cancer, since changes in metabolites may reflect, at least partly, what is happening in tumour cells. However, the identification of specific metabolites is a challenging goal due to the presence of many confounding factors, including age, gender, diet, underlying liver diseases, concomitant diseases, drugs and others. To date, only a reduced number of studies have investigated the usefulness of metabolites in body fluids in the diagnosis of CCA.

In bile, the analysis of bile acid concentration and composition in patients with biliary tract cancer (iCCA, pCCA or extrahepatic CCA [eCCA]), biliary tract stones and healthy controls showed a reduction in the proportion of secondary bile acids in patients with CCA compared with those with biliary tract stones and healthy individuals.\textsuperscript{55} This finding was associated with an alteration of bile acid transport that could explain the exposure of the bile duct epithelium to cocarcinogenic bile acids.\textsuperscript{56} Another study analysed bile compounds in patients with CCA, PSC and benign biliary diseases and showed that changes in phosphatidylcholines, bile acids and lipids were able to discriminate CCA from other conditions (sensitivity: 88.9%; specificity: 78.1%).\textsuperscript{57} The analysis of metabolites in bile of patients with CCA, HCC, non-malignant liver diseases and healthy individuals\textsuperscript{58} showed a decrease in glycine- and taurine-conjugated bile acids, phospholipids and cholesterol in patients with CCA compared to control groups and, to a certain extent, also to HCC patients. In contrast, another study that analysed the metabolites in bile of patients with inoperable pCCA or dCCA and non-malignant biliary diseases without cholestasis, including PSC, found increased levels of glycine-conjugated bile acids and phosphatidylcholines in patients with CCA, and constructed models that were able to discriminate CCA patients from those with non-malignant biliary diseases (sensitivity: 80%; specificity: 95%).\textsuperscript{59} From all these studies, bile acid species\textsuperscript{60} are positioned as some of the metabolites with potential as biomarkers. However, future studies should confirm in larger and international cohorts of patients the usefulness of the determination of bile acids and phospholipids in bile for the diagnosis of biliary tumours.
Other studies have demonstrated that the analysis of metabolites in serum could help in the diagnosis of CCA. Serum analysis in two independent Chinese cohorts of patients with CCA identified several metabolites useful in the early diagnosis of this tumour, as well as to distinguish iCCA from eCCA. In particular, an increase in serum 21-deoxycortisol and bilirubin levels and a decrease of lysophosphatidylcholines LPC(14:0) and LPC(15:0) levels were found in patients with CCA compared with healthy individuals.\textsuperscript{61} Moreover, the combination of the 4 candidate biomarkers was useful for distinguishing CCA from healthy controls with high accuracy (99%). A recent study analysing the metabolomics profile in biopsy-proven patients with iCCA, HCC, PSC and healthy individuals has demonstrated that specific changes in serum concentrations of certain metabolites can help in the early and differential diagnosis of these diseases. Several metabolites presented higher diagnostic values for iCCA vs the other groups under study, with superior AUC than that found for CA19-9.\textsuperscript{62} An algorithm combining 6 metabolites; three sphingomyelins (SMs), two phosphatidylcholines (PCs) and one ceramide (Cer) – SM(42:3), SM(43:2), PC(O-16:0/20:3), PC(O-18:0/18:2), SM(d18:2/16:0) and Cer(d18:1/16:0) –accurately differentiated iCCA from HCC (AUC: 0.9; sensitivity: 80%; specificity: 90%). Another algorithm that combined PC(34:3) and histidine accurately differentiated PSC from iCCA (AUC: 0.990; sensitivity: 100%; specificity: 70%). These interesting data, however, should be confirmed in patients with CCA arising from PSC. Remarkably, all of these results were successfully validated in another independent cohort of patients.

An integrated analysis of the transcriptome and metabolome in surgically resected tumour tissue of patients with iCCA and HCC showed specific profiles of genes and metabolites that could be useful in the diagnosis of iCCA.\textsuperscript{63} In addition, the integration of genomics, transcriptomics and metabolomics in tumour tissue has also been proposed for the stratification of molecular subtypes of iCCA and HCC with similar prognosis.\textsuperscript{64}

### 2.4. Extracellular vesicles

In terms of minimal invasive biomarkers EVs became of particular interest during the last years. EVs can be found in all body fluids including blood,\textsuperscript{65} saliva,\textsuperscript{66} urine\textsuperscript{67} and bile.\textsuperscript{68} Commonly, EVs include two main subclasses that can be differentiated according to their size and biogenesis.\textsuperscript{69} According to the generally accepted nomenclature, larger EVs (also called microvesicles [MVs]) roughly range from 100 to 1000 nm in size and directly bud from the plasma membrane of their parental cell, whereas small EVs (also called exosomes) are considerably smaller (below 100 nm) and originate from accumulated intraluminal vesicles within the endomembranous system, forming so called multivesicular bodies (MVBs). The fusion of the MVBs with the plasma membrane results in the release of exosomes into the extracellular space.\textsuperscript{70,71}
EVs contain a variety of biomolecules including lipids, nucleic acids and proteins/antigens, and act as physiological mediators of cell communication. Furthermore, EVs amount and content reflect the pathobiological state of the cells they originate from. EVs have been shown to support the generation of tumour stroma during CCA development by inducing the differentiation of mesenchymal stem cells to fibroblasts, thus preparing their own tumour niche. Proteomic profiling of EVs derived from human serum has revealed promising candidate proteins (FCN2, ITIH4, FIBG) for the differential diagnosis of early-stage CCA and PSC patients (AUC: 0.96; sensitivity: 0.88; specificity: 0.88). Importantly, some of the identified serum EV protein biomarkers allowed the accurate and early diagnosis of CCA (AMPN, VNN1 and PIGR; AUC: 0.88, 0.88 and 0.84, respectively) and HCC (LG3BP, PIGR and A2MG; AUC: 0.90, 0.84 and 0.80, respectively) compared to healthy individuals, as well as the differential diagnosis of iCCA and HCC (FIBG, A1AG1, VTDB; AUC: 0.89, 0.85 and 0.82, respectively), which currently comprises a major challenge nowadays. Of note, the combinations of some of these biomarkers increased their diagnostic accuracy. Moreover, the analysis of the surface antigen composition of serum EVs allowed to diagnose CCA from healthy individuals and other cancer entities with up to 90% sensitivity, but was unable to differentiate between CCA and HCC. Additionally, a correlation between CCA/HCC tumour burden and EV levels specific for those cancer entities was observed, highlighting the prognostic value of EVs, especially in terms of early detection of small tumours. The concentration of EVs per se in bile and serum was found increased in patients with CCA and pancreatic carcinoma, discriminating malignant from non-malignant pancreatobiliary diseases with 100% sensitivity in bile and 47% in serum. Another study used a combined approach comprising EV isolation followed by microRNA content profiling. In particular, a miRNA panel (miRs 191, 486-3p, 1274b, 16, and 484) showed good diagnostic values for CCA diagnosis compared to non-malignant biliary diseases (sensitivity: 67%; specificity: 96%). An overview of the conducted studies summarizing their diagnostic capability for EV-based CCA diagnosis can be found in Table 2.

2.5. Circulating tumour cells

Due to the difficulty in obtaining a histological or cytological diagnosis in biliary-pancreatic cancers, circulating tumour cells (CTCs) are of great interest. CTCs are released by primary tumours into the bloodstream at a concentration of about 10^6 CTCs/g of tumour/day and, although at very low concentrations, may be a route for metastasis of some solid neoplasms. Few studies have evaluated the presence of CTCs as a diagnostic tool for CCA, although there are emerging data in other cancers such as glioblastoma multiforme, hepatocellular carcinoma, and pancreatic, breast, and colorectal cancers. A number of technologies have been developed to isolate and identify CTCs from peripheral blood, including enzyme-linked immunosorbent spot (ELISPOT) assay, real time PCR, flow cytometry, and
immunocytochemistry, and automated or semi-automated systems (CellSearch, CellSpotter, or iChip). To date, the only tool approved by the US Food and Drug Administration for the detection of CTCs is the CellSearch System, a semi-automated platform for the preparation and subsequent capture of CTCs using epithelial cell specific EpCAM antibodies, prior to labelling with immunofluorescent markers. To further strengthen the discriminating capability of this tool, cells are also sorted for their positivity to DAPI, cytokeratins 8/18 (markers of hepatocytes), and 19 (marker of cholangiocytes in the liver) and their negativity for CD45 (marker of leukocytes).\textsuperscript{84} This system has the disadvantage that, apparently, few CCA tumours (10-20\%) have significant elevation of EpCAM expression.\textsuperscript{85} Using this system, the prevalence of CTCs in blood from large cohorts of patients with metastatic carcinomas was 36\% (≥2 CTCs per 7.5 mL of blood) compared to 0.3\% in healthy and nonmalignant disease subjects.\textsuperscript{86} A different technology, based on a microfluidic platform capable of separating CTCs from peripheral whole blood samples using EpCAM-coated microposts to differentiate between epithelial cells and blood leukocytes (the 'CTC-chip'), was developed and the efficiency of CTC capture determined in clinical specimens was 65-71\% and the sensitivity and specificity were close to 100\% in the wide variety of solid-organ cancers tested.\textsuperscript{87,88} A further refinement of this technology is the CTC-iChip, which has been used to sort very rare CTCs from patients suffering from prostate cancer.\textsuperscript{89} To date, the number of studies suggesting that circulating CTCs are associated with poor prognosis in patients with advanced CCA are scarce.\textsuperscript{84,90,91} However, it remains unclear whether they have any diagnostic role in CCA. The first pioneering study on biliary-pancreatic cancers study was conducted in patients using a reverse transcriptase-PCR approach to detect CEA in blood samples; this study outlined a correlation between increased CEA expression and hematogenous dissemination and worst prognosis.\textsuperscript{92} In addition, it was also reported, using the CellSearch System, that only 25\% of patients with biliary tract cancer had elevated CTCs (>2 per 7.5 ml of blood),\textsuperscript{84} suggesting the possibility of using CTCs as non-invasive biomarkers to predict the outcome of patients.\textsuperscript{93} Of note, the presence of CTCs has been shown to correlate with higher tumour extent and lower overall survival in patients with CCA.\textsuperscript{90} Moreover, an elevation of CTCs count over baseline, together with other circulating markers, predicted a worse overall and disease-free survival in patients with CCA.\textsuperscript{94} From a biological point of view, it was hypothesized that CTCs in the bloodstream could be putatively sustained by the interaction of the tumour cells with immune cells and CD105\(^+\) CD14\(^+\) myeloid fibroblasts, and be responsible for the metastatic spread of CCA.\textsuperscript{81} CTCs have also been isolated from portal venous blood in patients with hepatobiliary-pancreatic malignancies.\textsuperscript{95} Although mainly focusing on patients with pancreatic ductal adenocarcinoma, portal vein derived CTCs were also examined in a small subgroup of CCA patients. Blood samples were obtained by direct intraoperative venipuncture during pancreaticoduodenectomy. CTCs were isolated by fluorescence-activated cell sorting (CD44\(^+\), CD147\(^+\), EpCAM\(^+\), CD45\(^−\)) and characterized for mRNA expression and
acetylated chromatin encoding K-RAS exon 12 mutation (K-RAS mut). K-RAS mut mRNA was detected at low levels and with high variability in CCA patients. The authors hypothesize that K-RAS mut gene expression may be a useful indicator for aggressive adenocarcinoma CTCs. These cells might retain malignant potential in portal venous blood even after successful tumour resection with high risk for disease recurrence and metastatic progression. In line with this, another study was conducted to examine KRAS mutation-positive CTCs in the portal venous blood of patients with hepatobiliary-pancreatic malignancies, including a small subgroup of patients with dCCA. The CTCs isolated from the portal circulation were shown to be highly proliferative and resistant to apoptosis. CTCs recruited multiple immune cell types, including myeloid fibroblasts suggesting that CTC survival inside the portal venous circulation is supported by their interactions with immune cells within multi-cell type clusters that could represent a source of local recurrence and metastatic progression.

3. Biomarkers in tumour tissue
Biomarkers in tumour tissue may be of particular value for resected CCAs, as they could predict prognosis (i.e., overall survival and tumour recurrence) (Table 3) and response to potential adjuvant therapies. In this regard, specific genomic and transcriptomic signatures have already been identified. High genomic heterogeneity was reported in CCA, with the most prevalent alterations related to DNA repair (TP53), growth pathways (KRAS, BRAF, SMAD4, FGFR2, PTPN3, KMT2C, ARID1A, PBRM1 and BAP1) and developmental pathways that significantly impact the cancer growth, such as Notch and Wnt signalling pathways (NOTCH1, NICD, WNT7B and WNT10A). Noteworthy, FGFR2 gene fusions, usually found in 5.5% to 13.6% patients with iCCA, deserve special attention since they are pharmacologically targetable and are specifically found in iCCA tumours, while being absent in any other liver malignancy, harbouring also a diagnostic value. IDH1 and IDH2 gene mutations are also frequently found in non-infectious CCA, mainly in iCCA, accounting for 4.9% to 36% of the cases. These alterations were also described in CCA cells and correlated with their methylation status. In a high-throughput screening of several cancer cell lines, including 17 biliary tract cancer cells, IDH-mutant iCCA cells exhibited good response to dasatinib, a multi-tyrosine kinase inhibitor, which was also shown to increase apoptosis and tumour regression in IDH-mutant xenografts. Consequently, a clinical trial is now being conducted to evaluate the therapeutic efficacy of dasatinib in IDH-mutant advanced iCCA (NTC02428855). Other clinical trials are also evaluating the clinical efficacy of IDH inhibitors for CCA (NCT02989857; NCT02381886). Particular etiological and/or risk factors for CCA may indeed select specific mutations that allow cancer development, progression and evolution. In this regard, mutations in TP53 seem to be highly frequent (58%) in CCA arising from patients with HBV. Therefore, the mutational genomic analysis and gene expression profiles of CCA may allow a precise
stratification of patients, paving the path for personalized therapy.\textsuperscript{101} Mutations in KRAS (12-16\%) and TP53 (13-20\%) have been associated with worse prognosis, i.e., lower overall survival and higher tumour recurrence than mutations in IDH1/2 or undetermined, in 2 large and independent cohorts of patients with iCCA undergoing tumour resection.\textsuperscript{103,117} The analysis of the transcriptome of iCCA tumours revealed two distinct types of iCCA: the “inflammation type”, which is mainly characterized by the increased expression of inflammatory-related genes and the “proliferation type”, which shows the worst outcome and is characterized by the activation of oncogenes.\textsuperscript{104} Further, a panel of 36 biomarkers that related with disease outcome was identified in a mRNA microarray from patients with surgically-resected iCCA,\textsuperscript{118} which strongly associated with poor survival. Moreover, mutations on KRAS/BRAF genes were directly linked to patients’ poor prognosis, along with an increased expression of human epidermal growth factor receptor 2 (HER2), which was completely absent in CCA with good prognosis.

Furthermore, 73 studies based on immunohistochemistry analysis of 4,126 CCA patients were combined in a meta-analysis that allowed the identification of 77 prognostic biomarkers in CCA patients that underwent surgical resection.\textsuperscript{119} The results from this meta-analysis indicated that fascin, EGFR, mucin 1 (MUC1), MUC4 and p27 are independently associated with overall survival in resected CCA patients. Furthermore, the analysis of 53 patients with biliary tract cancer who underwent tumour resection revealed 39 transcriptomic prognostic biomarkers, all of them related with T-cell activation and immune response. For instance, the expression levels of cytotoxic T-lymphocyte antigen 4 (CTL4) and forkhead box P3 (FOXP3) correlated with recurrence-free survival, suggesting an enrichment in T regulatory cells in the tumour microenvironment.\textsuperscript{120} Moreover, high IL-33 tissue expression correlated with favourable prognosis in patients with iCCA or pCCA,\textsuperscript{121} but data on circulating IL-33 levels were lacking. In parallel, individual case reports have further described Granulocyte-colony stimulating factor (G-CSF)-expressing CCAs and suggested monitoring circulating G-CSF to diagnose disease relapse after radical tumour resection.\textsuperscript{122,123} Differential miR expression profiles were also identified in CCA tumour tissue compared to non-tumour liver tissue. Several miRs were already shown to be deregulated in CCA\textsuperscript{6,7} and interestingly, the oncomiR miR-21 has arisen as a promising biomarker in tumour tissue. Remarkable, in two different studies, miR-21 was found to be overexpressed in CCA tumour tissue, regardless of its aetiology, and provided a 95\% sensitivity and 100\% specificity in the differential identification of CCA and normal bile duct specimens or non-tumour liver tissue, respectively.\textsuperscript{124,125} The expression of miR-21 in tumour tissue positively correlated with the clinical stage at diagnosis and with the tumour differentiation status and, more importantly, increased levels of miR-21 in iCCA were directly associated with poor overall and progression-free survival.\textsuperscript{5,126} High miR-21 expression levels were also evident in CCA cell lines compared with non-malignant cholangiocytes,\textsuperscript{127} while experimental inhibition of miR-21
was shown to dampen CCA growth in vivo.\textsuperscript{5,128} It is worth mentioning that miR-21 expression is also increased in tumour tissue from patients with HCC, compared to healthy individuals.\textsuperscript{129-131} Specific tissue miRNA expression profiles may also be used with diagnostic purposes since distinct miR signatures were associated with different subtypes and histological grade of \textit{O. viverrini}-induced iCCA.\textsuperscript{132} Furthermore, a panel of 7 miRs were found differentially expressed in tumour tissue of patients with CCA and pancreatic adenocarcinoma, further proposing that different miR expression tissue profiles may also allow the differential diagnosis of tumours with similar clinical presentations.\textsuperscript{133}

4. Summary and future perspectives
The early and accurate non-invasive diagnosis of CCA remains a major challenge. This is particularly important in order to increase the number of patients eligible for surgical tumour resection or liver transplantation, which are the only potential curative options nowadays. For this purpose, it is of pivotal importance to develop novel diagnostic strategies as well as to determine the unknown aetiologies of the majority of CCAs, which is key for monitoring patients at risk and early diagnose tumour development. Several novel approaches have been recently investigated in the search of non-invasive biomarkers for CCA, including CTCs, EVs, miRNAs and metabolites, and multiple potential biomarkers have been described. However, the most promising biomarkers (single or clusters) need to be internationally validated in large biopsy-proven cohorts of patients, with appropriate control groups. Moreover, future studies should investigate the accuracy of potential candidate biomarkers for all types of CCA, or for specific subgroups associated with known risk factors. In this regard, the European Network for the Study of Cholangiocarcinoma (ENS-CCA: www.enscca.org / www.cholangiocarcinoma.eu), a pan-European and multidisciplinary collaborative group, represents an ideal platform for these types of validation studies, for the generation of consensus statements, and for accelerating the translation of biomarkers into the clinics.

FIGURE LEGENDS

\textbf{Figure 1.} Circulating miRs up- (red arrows) or down- (blue arrows) regulated in the serum, bile and urine of patients with CCA, comparing with primary sclerosing cholangitis (PSC) (in grey) or with healthy individuals (in black). Corresponding references are indicated in square brackets.

\textbf{Figure 2.} Flow diagram of a typical metabolite profiling workflow. Step 1 consists in the selection of patients and is followed by sample preparation depending of the types of metabolites to measure. The analysis of metabolites can be carried out by different techniques (GC-MS, gas-chromatography coupled to mass spectrometry; LC-MS, liquid chromatography coupled to mass spectrometry; NMR, nuclear magnetic resonance) and includes a step of
data pre-processing. Finally, statistical analyses are performed and appropriate representations are selected to visualise the results.

References


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Table 1. Cytokines/proteins as circulating biomarkers for biliary cancer.

<table>
<thead>
<tr>
<th>Protein/ Cytokine</th>
<th>Source</th>
<th>Levels</th>
<th>Comparison</th>
<th>SEN (%)</th>
<th>SPE (%)</th>
<th>AUC</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP7</td>
<td>Serum</td>
<td>Up</td>
<td>CCA (n=44) vs. benign biliary tract disease (n=36)</td>
<td>75</td>
<td>78</td>
<td>0.730</td>
<td>[46]</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>Serum</td>
<td>Up</td>
<td>CCA (n=80) vs. healthy controls (n=42)</td>
<td>88</td>
<td>100</td>
<td>0.964</td>
<td>[47]</td>
</tr>
<tr>
<td>IL-6</td>
<td>Serum</td>
<td>Up</td>
<td>CCA (n=26) vs. healthy controls (n=23)</td>
<td>73</td>
<td>92</td>
<td>0.875</td>
<td>[49]</td>
</tr>
<tr>
<td>S100A6</td>
<td>Serum</td>
<td>Up</td>
<td>CCA (n=29) vs. healthy controls (n=22)</td>
<td>86</td>
<td>91</td>
<td>0.909</td>
<td>[50]</td>
</tr>
<tr>
<td>DKK1</td>
<td>Serum</td>
<td>Up</td>
<td>iCCA (n=37) vs. healthy controls (n=50)</td>
<td>76</td>
<td>100</td>
<td>0.872</td>
<td>[51]</td>
</tr>
<tr>
<td>SSP411</td>
<td>Serum</td>
<td>Up</td>
<td>CCA (n=35) vs. &quot;cholangitis (n=13) and healthy controls (n=23)&quot;</td>
<td>90</td>
<td>83</td>
<td>0.913</td>
<td>[53]</td>
</tr>
</tbody>
</table>

SEN, sensitivity; SPE, specificity.
### Table 2. Overview of potential EV biomarkers for CCA diagnosis.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Source</th>
<th>Method</th>
<th>Controls</th>
<th>SEN</th>
<th>SPE</th>
<th>AUC</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCN2, ITIH4, FIBG</td>
<td>Serum EVs</td>
<td>Proteomics</td>
<td>PSC</td>
<td>92-100</td>
<td>81</td>
<td>0.88-0.96</td>
<td>[74]</td>
</tr>
<tr>
<td>EpCAM⁺ ASGPR1⁺ CD133⁺</td>
<td>Serum EVs</td>
<td>FACS</td>
<td>Healthy</td>
<td>90</td>
<td>50</td>
<td>0.82</td>
<td>[75]</td>
</tr>
<tr>
<td>Total amount</td>
<td>Serum EVs</td>
<td>NTA</td>
<td>Non-malignant bile duct stenoses</td>
<td>47</td>
<td>80</td>
<td>0.81</td>
<td>[76]</td>
</tr>
<tr>
<td>Total amount</td>
<td>Bile EVs</td>
<td>NTA</td>
<td>Non-malignant bile duct stenoses</td>
<td>100</td>
<td>100</td>
<td>0.10</td>
<td>[77]</td>
</tr>
<tr>
<td>miR-191 miR-486-3p miR-1274b</td>
<td>Serum EVs</td>
<td>miR arrays</td>
<td>Non-malignant bile duct stenoses</td>
<td>67</td>
<td>96</td>
<td>-</td>
<td>[78]</td>
</tr>
</tbody>
</table>

AUC, area under (ROC) curve; EV, extracellular vesicles; FACS, fluorescence-activated cell sorting; miR, microRNA; NTA, nanoparticle tracking analysis; SEN, sensitivity; SPE, specificity; PSC, primary sclerosing cholangitis.

### Table 3. Tumour tissue prognostic biomarkers for biliary cancer.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Expression (high/low)</th>
<th>Method</th>
<th>Overall survival</th>
<th>Recurrence-free survival</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRAS</td>
<td>Kirsten rat sarcoma viral oncogene homolog</td>
<td>High</td>
<td>TES / WES</td>
<td>Decreased</td>
<td>Decreased</td>
<td>[117]</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumor protein 53</td>
<td>Low</td>
<td>TES / WES</td>
<td>Decreased</td>
<td>Decreased</td>
<td></td>
</tr>
<tr>
<td>PROM 1</td>
<td>Prominin-1 /CD133</td>
<td>High</td>
<td>IHC</td>
<td>Decreased</td>
<td>–</td>
<td>[134]</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective Tissue Growth Factor</td>
<td>High</td>
<td>IHC</td>
<td>Increased</td>
<td>–</td>
<td>[135]</td>
</tr>
<tr>
<td>VIM</td>
<td>Vimentin</td>
<td>High</td>
<td>IHC</td>
<td>Decreased</td>
<td>–</td>
<td>[136]</td>
</tr>
<tr>
<td>DKK1</td>
<td>Dickkopf WNT Signaling Pathway Inhibitor 1</td>
<td>High</td>
<td>IHC / PCR</td>
<td>Decreased</td>
<td>–</td>
<td>[51]</td>
</tr>
<tr>
<td>SOX 2</td>
<td>SRY-Box 2</td>
<td>High</td>
<td>IHC</td>
<td>Decreased</td>
<td>–</td>
<td>[137]</td>
</tr>
<tr>
<td>SOX17</td>
<td>SRY-Box 17</td>
<td>Low</td>
<td>PCR</td>
<td>Decreased</td>
<td>–</td>
<td>[138]</td>
</tr>
<tr>
<td>MUC1</td>
<td>Mucin 1, Cell Surface Associated</td>
<td>High</td>
<td>PCR</td>
<td>Decreased</td>
<td>–</td>
<td>[139]</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Diff 1</td>
<td>Diff 2</td>
<td>Diff 3</td>
<td>Ref.</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>PTPN14</td>
<td>Protein Tyrosine Phosphatase, Non-Receptor Type 14</td>
<td>Low</td>
<td>ISH</td>
<td>Decreased</td>
<td>–</td>
<td>[5]</td>
</tr>
<tr>
<td>Inc RNA AFAP1-AS1</td>
<td>AFAP1 Antisense RNA 1</td>
<td>High</td>
<td>PCR</td>
<td>Decreased</td>
<td>–</td>
<td>[140]</td>
</tr>
<tr>
<td>Inc RNA PANDAR</td>
<td>Promoter Of CDKN1A Antisense DNA Damage Activated RNA</td>
<td>High</td>
<td>PCR</td>
<td>Decreased</td>
<td>–</td>
<td>[141]</td>
</tr>
<tr>
<td>CEACAM 6</td>
<td>Carcinoembryonic Antigen Related Cell Adhesion Molecule 6</td>
<td>High</td>
<td>PCR / IHC</td>
<td>Decreased</td>
<td>[142]</td>
<td></td>
</tr>
<tr>
<td>CD151</td>
<td>Cluster of differentiation 151</td>
<td>High</td>
<td>PCR / IHC</td>
<td>Decreased</td>
<td>Decreased</td>
<td>[143]</td>
</tr>
<tr>
<td>C-met</td>
<td>MET Proto-Oncogene, Receptor Tyrosine Kinase</td>
<td>Low</td>
<td>PCR / IHC</td>
<td>Increased</td>
<td>Increased</td>
<td>[143]</td>
</tr>
<tr>
<td>BECN1</td>
<td>Beclin 1</td>
<td>High</td>
<td>PCR</td>
<td>Increased</td>
<td>Increased</td>
<td>[144,145]</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal Transducer And Activator Of Transcription 3</td>
<td>High</td>
<td>PCR / IHC</td>
<td>Decreased</td>
<td>Decreased</td>
<td>[146]</td>
</tr>
<tr>
<td>CAPN4/CAPNS 1</td>
<td>Calpain Small Subunit 1</td>
<td>High</td>
<td>PCR / IHC</td>
<td>Decreased</td>
<td>Decreased</td>
<td>[147]</td>
</tr>
<tr>
<td>SOX9</td>
<td>SRY-Box 9</td>
<td>High</td>
<td>IHC</td>
<td>Decreased</td>
<td>–</td>
<td>[148]</td>
</tr>
<tr>
<td>CDH1</td>
<td>E-cadherin</td>
<td>Low</td>
<td>IHC</td>
<td>Decreased</td>
<td>–</td>
<td>[136,149]</td>
</tr>
<tr>
<td>FASCIN/FSCN1</td>
<td>Fascin Actin-Bundling Protein 1</td>
<td>High</td>
<td>IHC</td>
<td>Decreased</td>
<td>–</td>
<td>[136]</td>
</tr>
<tr>
<td>S100A4</td>
<td>S100 Calcium Binding Protein A4</td>
<td>High</td>
<td>IHC</td>
<td>Decreased</td>
<td>–</td>
<td>[150]</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
<td>High</td>
<td>IHC</td>
<td>Decreased</td>
<td>–</td>
<td>[151]</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
<td>High</td>
<td>IHC</td>
<td>Decreased</td>
<td>–</td>
<td>[152]</td>
</tr>
<tr>
<td>MUC4</td>
<td>Mucin 4, Cell Surface Associated</td>
<td>High</td>
<td>IHC</td>
<td>Decreased</td>
<td>–</td>
<td>[153]</td>
</tr>
<tr>
<td>MUC16 /CEA 125</td>
<td>Mucin 16, Cell Surface Associated</td>
<td>High</td>
<td>IHC</td>
<td>Decreased</td>
<td>–</td>
<td>[154]</td>
</tr>
<tr>
<td>CD44</td>
<td>Cluster of differentiation 44</td>
<td>High</td>
<td>IHC</td>
<td>Decreased</td>
<td>–</td>
<td>[155]</td>
</tr>
<tr>
<td>FBXW7</td>
<td>F-Box And WD Repeat Domain Containing 7</td>
<td>Low</td>
<td>IHC</td>
<td>Decreased</td>
<td>Decreased</td>
<td>[156]</td>
</tr>
<tr>
<td>CDKN1B/p27</td>
<td>Cyclin Dependent Kinase Inhibitor 1B</td>
<td>Low</td>
<td>IHC</td>
<td>Decreased</td>
<td>Decreased</td>
<td>[157]</td>
</tr>
<tr>
<td>CCND1</td>
<td>Cyclin D1</td>
<td>High</td>
<td>IHC</td>
<td>Decreased</td>
<td>Decreased</td>
<td>[158]</td>
</tr>
<tr>
<td>HDGF</td>
<td>Heparin Binding Growth Factor</td>
<td>High</td>
<td>IHC</td>
<td>Decreased</td>
<td>–</td>
<td>[152]</td>
</tr>
<tr>
<td>KRT103</td>
<td>Keratin103</td>
<td>Low</td>
<td>IHC</td>
<td>Increased</td>
<td>–</td>
<td>[159]</td>
</tr>
<tr>
<td>HDAC1</td>
<td>Histone Deacetylase 1</td>
<td>High</td>
<td>IHC</td>
<td>Decreased</td>
<td>Decreased</td>
<td>[160]</td>
</tr>
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<td>NOTCH4</td>
<td>Notch4</td>
<td>High</td>
<td>IHC</td>
<td>Decreased</td>
<td>–</td>
<td>[161]</td>
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<tr>
<td>PTP4A3/PRL3</td>
<td>Protein Tyrosine Phosphatase Type IVA, Member 3</td>
<td>High</td>
<td>IHC</td>
<td>Decreased</td>
<td>–</td>
<td>[162]</td>
</tr>
<tr>
<td>AKT1</td>
<td>AKT Serine/Threonine Kinase 1</td>
<td>High</td>
<td>IHC</td>
<td>Increased</td>
<td>–</td>
<td>[163,164]</td>
</tr>
<tr>
<td>MTOR</td>
<td>Mechanistic Target Of Rapamycin Kinase</td>
<td>High</td>
<td>IHC</td>
<td>Increased</td>
<td>–</td>
<td>[163,164]</td>
</tr>
<tr>
<td>SMAD7</td>
<td>SMAD Family Member 7</td>
<td>High</td>
<td>IHC</td>
<td>Decreased</td>
<td>Decreased</td>
<td>[165]</td>
</tr>
<tr>
<td>FOXC2</td>
<td>Forkhead Box C2</td>
<td>High</td>
<td>IHC</td>
<td>Decreased</td>
<td>Decreased</td>
<td>[166]</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Protein/MiRNA</th>
<th>Description</th>
<th>Detection Method</th>
<th>Expression</th>
<th>Change</th>
<th>Reference</th>
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<tbody>
<tr>
<td>SKP2</td>
<td>S-Phase Kinase Associated Protein 2</td>
<td>High</td>
<td>IHC</td>
<td>Decreased</td>
<td>[167]</td>
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<tr>
<td>CTL4</td>
<td>Cytotoxic T-Lymphocyte Antigen 4</td>
<td>High</td>
<td>mRNA microarray</td>
<td>Decreased</td>
<td>[120]</td>
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<tr>
<td>IL-33</td>
<td>Interleukin 33</td>
<td>High</td>
<td>PCR / ISH</td>
<td>Increased</td>
<td>[121]</td>
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<tr>
<td>MIR21</td>
<td>MicroRNA 21</td>
<td>High</td>
<td>ISH / PCR</td>
<td>Decreased</td>
<td>[5, 126]</td>
</tr>
</tbody>
</table>

IHC, immunohistochemistry; ISH, *in situ* hybridization; PCR, polymerase chain reaction; TES, targeted exome sequencing; WB, western blot; WES, whole-exome sequencing.