Novel Diagnostic and Prognostic Biomarkers in Biliary Tract Cancer

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Abstract

Introduction: The worldwide incidence of biliary tract carcinoma (BTC; tumours of the bile ducts and gall-bladder) continues to rise with the only potentially curative treatment remaining surgical resection or transplantation, possible in only a minority of patients. Late presentation and a paucity of effective treatments mandate the development of techniques for early lesion detection.

Areas Covered*:* This article reviews currently available biomarkers for the diagnosis and prognosis of BTC, as well as recently published studies describing novel serum, bile and urinary biomarkers.

Expert Opinion: The incorporation of novel analysis techniques, such as digital image analysis and fluorescence *in situ* hybridization, into existing management algorithms may enhance the accuracy of brush cytology taken at the time of therapeutic endoscopy. However, a key goal is the discovery of reliable non-invasive biomarkers with high sensitivity and specificity. Recent advances in gene sequencing and expression, clonal evolution and tumour heterogeneity in other cancers should advance understanding of BTC tumour biology and facilitate biomarker discovery.

Keywords: Cholangiocarcinoma; Biliary Tract Cancer; Gallbladder Cancer; Biomarkers; Diagnosis; Prognosis

1. Introduction

Biliary tract carcinoma (BTC) comprises tumours of the gallbladder and bile ducts, the commonest of which is cholangiocarcinoma (CCa), an adenocarcinoma arising from the epithelium of the biliary tract and which is predominantly diagnosed in the 7th decade of life [1]. CCa affects 1-2 per 100,000 in the UK population [2, 3] although the UK and worldwide incidence continues to rise with associated increasing mortality rates [3-6].

CCa can usually be considered to be sporadic but certain recognised predisposing factors have been identified, including primary sclerosing cholangitis (PSC; CCa occurs in up to 40% of PSC patients), prolonged or recurrent biliary infection, liver fluke infection (particularly in areas where Opisthorchis viverrini is endemic [7] cirrhosis, Caroli disease, the presence of choledochal cysts and hepatolithiasis, and carcinogen exposure (dioxins, nitrosamines, alcohol and thorotrast). BTC is also more prevalent in diabetics and smokers [1, 4]. CCa may occur via an adenoma-dysplasia-carcinoma sequence, although CCa tumour biology remains poorly understood [8, 9]. A number of mutations exist in tissue derived from CCa specimens, including abnormalities in known oncogenes and tumour suppressor genes; however, the frequency of such mutations is difficult to accurately estimate and this information, to date, remains clinically unusable [10-12].

Indeterminate biliary strictures present a diagnostic challenge to the investigating clinician. Many pathologies share clinical and radiological findings with the differentiation of BTC, PSC and autoimmune cholangiopathy remaining particularly difficult [13-15]. Endoscopic retrograde cholangiopancreatography (ERCP) can be utilized following cross-sectional radiological techniques for lesions assessment and biopsy with sensitivity for malignancy of 9-57% [16-19]. Endoscopic ultrasound is a further well-established technique that can be used in conjunction with fine needle aspiration (FNA) for the visualization and sampling of gallbladder, hilar and extrahepatic lesions, as well as peri-hilar lymph nodes and vessels with a sensitivity for CCa of appproximately 75% [20]. Novel techniques such as peroral single operator cholangioscopy (e.g. Spyglass, Boston Scientific Corp, Natick, Massachusetts, USA) can have improved diagnostic accuracy as compared to standard ERCP [21] and has a specificity and sensitivity for visually directed biopsies of 98% and 49%, respectively [22]. Further novel techniques, such as Methylene blue, which

can successfully differentiate malignant lesions [23] and narrow-band imaging (NBI), which enhances the vascular pattern of the mucosal surface and can effectively differentiate tumour margins [24, 25], have also been utilised to augment the visualization of mucosa during cholangioscopy but are not routinely available.

The majority of BTCs involve the main extrahepatic bile duct in the perihilar region (60-70%), whereas the remainder tend to arise within the common bile duct (CBD) distal to the cystic duct. Only very infrequently do tumours arise in either smaller bile ducts within the liver parenchyma (intra-hepatic) or within the gall-bladder [26]. CCa cells tend to insidiously infiltrate and spread along the biliary tract, so that patients often have minimal clinical symptoms until late presentation, usually with cholestasis and evidence of locally advanced or metastatic disease on imaging [27-29]. The only chance for long-term survival remains radical resective surgery with negative pathological margins (or, in highly selected patients, liver transplantation). These strategies remain possible in only a minority of selected patients and result in post-resection five year survival rates of up to 54%, depending on site and severity of disease [30]. Non-surgical strategies currently provide only limited success in prolonging survival for those with advanced disease. Five-year survival rates in unresectable patients remain under 10% [30, 31] with a median survival of usually less than 12 months following treatment with cisplatin- and gemcitabine-based chemotherapy regimens [32].

The lack of effective treatments for advanced disease mandates the development of techniques for early lesion detection. Whilst efforts to discover effective serum or bile biomarkers for early detection are ongoing, the relative rarity of the disease and the frequent presence of cholestasis and cholangitis which can confound bile assays, have so far limited discovery efforts.

This review assesses currently available biomarkers for the assessment of BTC, as well as reviewing recently published studies describing novel serum, bile and urinary biomarkers. A literature search was undertaken utilising PubMed and Embase search engines and the keywords cholangiocarcinoma; biliary tract cancer/carcinoma; gallbladder cancer/carcinoma; biomarkers; diagnosis; and prognosis entered in various combinations.

2. Serum Biomarkers

Serum samples are easy to obtain and patient-acceptable, and therefore remain the most frequently utilized method of identifying biomarkers for CCa.

2.1. Established Serum Biomarkers

2.1.1. Carbohydrate Antigen 19-9 (CA19-9)

CA19-9, or sialylated Lewis (a) antigen, remains the most widely used biomarker for the diagnosis of CCa. Despite this, it cannot be detected in ~7% of the population who are Lewis (a) antigen negative (lacking the requisite fucosyltransferase for the production of CA19-9 [33, 34]) and is elevated in some other upper gastrointestinal malignancies (e.g. stomach and pancreatic adenocarcinoma), primary biliary cirrhosis, cholestasis, cholangitis and in smokers [35]. Indeed, 10% of patients under investigation for BTC (based upon radiological and clinical features) are found to have raised CA19-9 levels, but are subsequently found to have benign disease [36]. Serum bilirubin levels are also an independent predictor of serum CA19-9 levels: following intervention for cholestasis (with a subsequent drop in serum bilirubin), serum CA19-9 significantly decreases in tandem [36], thus limiting its specificity in patients presenting with cholestasis or cholangitis. However, if serum CA19-9 levels remain raised after intervention for cholestasis, this is strongly predictive (although not diagnostic) of malignant disease [37]. Depending upon the cut-off value used, CA19-9 has been shown to have a sensitivity range of 78-89% and a specificity range of 67-87% for the diagnosis of BTC [30, 36, 38, 39]. The use of higher serum CA19-9 cut-off levels decreases sensitivity, only allowing diagnosis of CCa to be made at an advanced stage of disease [33]. Further studies have attempted to use serum CA19-9 evaluation to differentiate between PSC-related and CCa-related strictures, and demonstrate wide-ranging but generally low sensitivity and specificity [31, 33, 40]. Further, the absolute value of CA19-9 may be affected by the specific assay used, with one study showing that different commercial assays deliver different results, potentially affecting the ability of CA19-9 to discriminate between malignant and benign disease [41]. One method for improving the diagnostic accuracy of CA19-9 may be to combine it with other biomarkers, such as bile-derived galectin-3-binding protein (LGALS3BP)/mac-2-binding protein levels, leading to significantly improved

diagnostic accuracy over CA19-9 alone (area under ROC curve 0.75; p<0.001) [42]. The sensitivity and specificity of CA19-9 also improves when combined with radiological imaging or endoscopic evaluation, particularly CT (computed tomography), MRI (magnetic resonance imaging), MRCP (magnetic resonance cholangiopancreatography) or ERCP (endoscopic retrograde cholangiopancreatography) [33]. At present, a combination of CA19-9 and MRI/MRCP or ultrasound represents the most effective, cost-efficient and acceptable technique for screening and follow-up of BTC.

2.1.2. Carcinoembryonic Antigen (CEA)

CEA is a glycosyl phosphatidyl inositol (GPI)-cell surface anchored glycoprotein that may be involved in cell adhesion. It is produced during foetal development, but its production tends to cease before birth and therefore levels in adults are generally low or non-existent, although they can be raised in smokers. It remains a useful biomarker in colorectal cancer, but is only raised in approximately 30% of patients with BTC [39, 43, 44]. On its own it has a limited role as a biomarker for BTC, but its clinical usefulness improves when used in combination with other markers [33].

2.1.3. Carbohydrate Antigen 125 (CA125)

CA125 is a protein encoded by the MUC16 gene and is a large membrane-associated glycoprotein with a single transmembrane domain. It is commonly employed for the detection of ovarian cancer, where it has a well-established role as a biomarker, but it is less useful for the detection of CCa where it is only elevated in 40–50% of CCa patients. When elevated, it may also indicate the presence of peritoneal involvement [6].

2.2. Novel Serum Biomarkers

Identification of novel serum markers to supplement or replace currently utilised biomarkers remains crucial. However, studies to date have not been able to demonstrate a suitable biomarker with high diagnostic accuracy. Some of the currently reported serum biomarkers for BTC are summarised below (see Table 1).

2.2.1. Interleukin-6 (IL-6)

IL-6 is a major inflammatory cytokine, whose high serum levels have been shown to correlate well with CCa tumour burden, both pre- and post-surgical resection [45]. However, high values can also be found in patients with associated inflammatory processes, such as cholestasis and cholangitis, as well as in hepatocellular carcinoma (HCC), metastatic liver lesions, and some benign liver pathologies [35]. Combining its use with CA19-9 (or other biomarkers) may improve its diagnostic accuracy. One group of authors demonstrated that at a cut-off level of 25.8 pg/mL, IL-6 had a diagnostic sensitivity and specificity of 73% and 92% respectively (PPV and NPV 83% and 87%, respectively), when utilized to differentiate between 26 CCa, 26 HCC and 23 healthy patients [46]. Serum levels were shown to correlate closely with tumour burden and also to decrease after treatment with photodynamic therapy. Similarly, the sensitivity and specificity for serum IL-6 (cut-off value 0.18 ng/mL) in 45 CCa patients was 71% and 90% for the differentiation of CCa and benign liver disease (n=10), whereas the specificity for differentiating from 25 other hepatic tumours (15 HCC, 15 metastatic liver cancer) was only 26.7% [47]. A combination of leucine-rich alpha-2 glycoprotein 1 (LRG-1), CA19-9 and IL-6 was also shown to be capable of discriminating CCa from benign biliary pathology with high diagnostic accuracy [48].

2.2.2. Mucins

Mucins are cell-specific, high molecular weight, heavily O-glycosylated glycoproteins expressed by ductal and glandular epithelia. They can be found in either transmembrane or secreted forms and have roles in epithelial protection, cellular adhesion and signal transduction. CCa neoplasms are commonly mucin-producing adenocarcinomas in which abnormal expression of various mucins, including mucin1 (MUC1) and mucin5AC (MUC5AC), has been demonstrated and linked to poor outcomes. Importantly, MUC5AC expression has been confirmed in serum from CCa

patients utilising various techniques including agarose gel electrophoresis, ELISA (enzyme-linked immunosorbent assay) and immunoblotting, but was not found in tissue derived from hepatocellular carcinomas (HCC). MUC5AC had a sensitivity and specificity of 71% and 90%, respectively, (area under ROC curve 0.841) for the diagnosis of CCa (as compared to gastrointestinal cancer patients, benign hepatic pathology and healthy controls) and may therefore be able to differentiate between tumour types [49, 50]. Further, those CCa patients with higher MUC5AC levels were shown to have poorer outcome (median survival 158 vs. 297 days).

A further study investigated serum MUC5AC and bile MUC4 using real-time PCR and western blotting in 72 patients with biliary obstruction (39 CCa, 7 PSC) [51]. Serum MUC5AC was again raised in 44% of CCa patients (but not in patients with other malignancies), whilst bile MUC4 (collected at ERCP) was present in 27% of CCa cases, but not in the other malignancies. Further, those patients with either raised serum MUC5AC or bile MUC4 had significantly worse prognosis (median survival 6.8 vs. 17.6 months).

Serum MUC5AC was similarly measured in 179 CCa patients, where it was significantly associated with blood group A, tumours over 5cm in size and advanced disease, as well as poorer prognosis, as compared with patients not expressing serum MUC5AC (median survival, 127 vs. 329 days; $P < 0.001$) [52]. Those patients expressing serum MUC5AC also had a 2.5 times increased risk of death ($p<0.001$).

In further support for the potential role of mucins in CCa biology, MUC1 positivity was present in 65.8% of tumours obtained from 34 patients with intrahepatic CCa, 51 with extrahepatic CCa, 11 with gallbladder cancer and 14 patients with pancreatic adenocarcinoma, where it was associated with poor differentiation ($p=0.002$), T status ($p=0.003$) and poor patient survival ($p=0.015$) [53]. In the same study, MUC5AC was associated with advanced tumours ($p=0.013$) and MUC6 with well-differentiated CCa ($p=0.006$).

2.2.3. CYFRA21-1

CYFRA21-1 is a soluble fragment of cytokeratin 19, the intermediate filament protein that forms a crucial part of the epithelial cell cytoskeleton and is therefore constitutively expressed in many epithelial cells. It has been shown to be elevated in many different tumours including cervical, oesophageal and breast, but has a particular biomarker role in non small-cell lung carcinoma [54, 55].

Serum CYFRA21-1 levels can differentiate intrahepatic CCa from benign liver disease and HCC, and have prognostic value [56]. In 55 patients who had undergone operative intervention for intrahepatic CCa, as compared to 90 patients with benign liver pathology, the AUC was higher for CYFRA21-1 than CEA or CA19-9 (0.901, 0.779 and 0.794, respectively). It had a sensitivity and specificity of 74.7% and 92.2% for CCa at a cut-off value of 2.7 ng/mL. Furthermore, the 3-year recurrence-free survival rates were significantly worse in CCa patients with high serum CYFRA21-1 values (25.0% vs. 76.2%) and CYFRA21-1 was found to be independently associated with recurrence and death following surgery on multivariate analysis [56, 57]. It has subsequently been postulated that raised CYFRA21-1 serum levels may represent a high burden of circulating tumour cells and that performing local operative resections in the presence of raised CYFRA21-1 may therefore prove futile [56].
However, another study group evaluated CYFRA21-1 in 66 patients with BTC (6 PSC-related), 39 patients with benign biliary disease and 19 patients with PSC to show that at a cut-off value of 1.5 ng/mL, CYFRA21-1 had a sensitivity and specificity of 56% and 88%, respectively, as compared to 79% and 78% for CA19-9 (cut-off value 37 IU/mL), concluding that it was less useful than CA19-9 [58]. A higher cut-off value for CYFRA21-1 (3 ng/mL) improved specificity, but decreased sensitivity (97% and 30% respectively). Combining CYFRA21-1 and CA19-9 (cut-off values 1.5 ng/mL and 37 IU/mL, respectively) led to a sensitivity and specificity of 45% and 96% and a high level of CYFRA21-1 (>3.0 ng/mL) was a strong predictor of reduced median survival (2 vs. 10 months; $p<0.001$) [58].

2.2.4. Trypsinogen

Tumour-associated trypsinogens 1 and 2 are the precursor zymogen forms of the enzyme trypsin and are encoded by the same genes as pancreatic trypsinogen. A recent study demonstrated that serum trypsinogen-2 (PRSS2) was capable of differentiating between CCa and PSC in 84 patients referred for liver transplantation or other hepatic surgery more effectively than CA19-9, when assessed by time-resolved immunofluorometric assay with areas under the ROC curve (AUC) of 0.804 and 0.613, respectively [59]. Trypsinogen-2 was also more effective at differentiating between PSC with and without CCa (AUC 0.759) and results were unaffected by the presence of other inflammatory conditions (e.g. inflammatory bowel disease) or high serum bilirubin levels. This potentially enhances the specificity of PRSS2 for the

detection of CCa, which frequently presents with cholangitis and cholestasis.

2.2.5. Receptor Binding Cancer Antigen 1 (RCAS1)

RCAS1 is a membrane protein that may participate in suppression of cell proliferation and induction of apoptotic cell death. Raised levels of serum RCAS1 were found in 73.9% of BTC patients (a higher percentage than CA19-9 or CEA), with a specificity of 96%, and it was suggested that it may aid in monitoring recurrence [60-62]. Its levels were shown to reduce to within normal limits following surgical resection, were not affected by the presence of cholestasis and remained elevated in some patients where CA19-9 was normal, thereby providing a potential complementarity.

2.2.6. Dickkopf-related protein 1 (DKK1)

DKK1, a secreted protein with cysteine-rich regions involved in multiple cellular processes, via its inhibitory effect on the WNT signaling pathway, has been implicated in both metastatic spread and local invasion in several tumour types. Shi *et al* showed that DKK1 expression was raised in CCa cell lines, as well as sera and tissues from patients with CCa [63]. DKK 1 expression was also associated with elevated matrix metalloproteinase 9, VEGF-C and metastasis to hepatic hilar lymph nodes. Further, DKK1 was associated with poorer overall survival and time to recurrence on multivariate analysis, even in patients with low risk of recurrence ($p<0.05$). DKK1 down-regulation via siRNA decreased cell migration and invasiveness, as well as down-regulating MMP9 and VEGF-C expression.

2.2.7. Other Biomarkers

Proteomic analysis of serum samples is now readily available. Tolek *et al* analyzed serum proteomes from six CCa patients to identify 36 CCa-associated proteins, with α1β-Glycoprotein (A1BG) and afamin (AFM) detected at consistently different levels in CCa patients, as compared to ten healthy controls [64]. A1BG and AFM were subsequently validated for their diagnostic and prognostic potential in 64 CCa patients where the A1BG/AFM ratio was capable of diagnosing CCa with 84.4% sensitivity and 87.5% specificity. Furthermore, an elevated post-surgical ratio was associated with involved resection margins and poorer outcome.

Surface enhanced laser desorption/ionization (SELDI) analysis of serum from 56 CCa patients, 49 patients with other hepatobiliary disease, 269 patients with other cancers

and 53 healthy controls demonstrated three specific peaks that were significantly lower in CCa samples as compared to controls [65]. The peaks were all identified as variants of transthyretin (TTR or prealbumin), a largely hepatically-derived 55-kDa protein which functions as a serum and cerebrospinal fluid carrier of thyroxine and thyroid hormones. TTR serum levels were subsequently assessed and confirmed to be decreased in CCa. Combining it with CA19-9 gave a sensitivity and specificity of 98.2% and 100.0% respectively, for differentiating CCa from benign disease. However, TTR levels are also reduced in liver disease, malnutrition and acute inflammation, as well as other cancers such as ovarian, cervical and endometrial. Vascular endothelial growth factor (VEGF), a platelet-derived growth factor crucial for the stimulation of vasculogenesis and angiogenesis has been implicated in the proliferation and metastasis of a variety of solid tumours. Levels of serum VEGF were significantly higher in 29 patients with extrahepatic CCa and 19 patients with pancreatic cancer than in 25 patients with benign biliary pathologies (CBD stones, PSC and cholangitis) undergoing ERCP (0.97 ng/mL ($p=0.0016$) and 0.66 ng/mL ($p<0.001$) vs. 0.28 ng/mL, respectively) [66]. In contrast, bile concentrations of VEGF were similar between the three groups.

Li *et al* investigated levels of serum M2-PK, the dimeric form of the pyruvate kinase isoenzyme M2, which is responsible for energy production within the glycolytic pathway by catalyzing the dephosphorylation of phosphoenolpyruvate to pyruvate. M2-PK is the predominant isoform found within proliferating cells, such as fibroblasts, embryonic cells and stem cells and has been implicated in the metabolism of various tumour types. In 115 patients with CCa, 85 with benign disease and 120 healthy blood donors, both M2-PK and CA19-9 were significantly higher in those with CCa and M2-PK was more sensitive and specific than CA19-9 for the diagnosis of malignancy (84.2% vs. 68.4% and 90% vs. 75%, respectively) [67].

Jamnongkan *et al* investigated oxidized alpha-1 antitrypsin, an oxidative stress indicator for many pathological states, in opisthorchis-related CCa tissue and serum to demonstrate that patients with high tissue levels had a poorer prognosis [68]. Serum levels of oxidized alpha-1 antitrypsin were also significantly raised in patients with heavy infections, advanced periductal fibrosis and CCa, as compared to healthy controls ($p<0.001$) and raised serum levels had an OR for CCa of 22.0, potentially representing a promising serum marker in areas where opisthorchis infection is

endemic. Further, SMAD7, involved in epithelial-mesenchymal transition has been found to be elevated in CCa tissue and elevated expression associated with lymph node metastsis, perineural invasion and survival [69].

3. Bile Biomarkers

Bile provides a rich source of potential CCa biomarkers as it is more local to the tumour than serum. However, its collection remains an invasive process with significant potential complications and its use as a screening tool is therefore limited. The following reviews putative biomarkers identified within bile.

3.1. Insulin-like Growth Factor-1 (IGF1)

Human CCa tissues express IGF1, a 70 amino acid peptide hormone similar in molecular structure to insulin and which plays an important role in growth and anabolic processes, thereby also having a potentially crucial role in tumour development and proliferation [70]. IGF1 levels measured in bile from patients with biliary obstruction undergoing ERCP were up to 20 times higher in patients with extrahepatic CCa (n=29) than those with pancreatic cancer (n=19) or benign biliary abnormalities (bile duct stones, PSC, cholangitis; n=25) (mean, 84.6 nmol/L vs. 5.8 nmol/L vs. 4.1 nmol/L; $p<0.001$ with an area under the ROC curve of 1.0) [66]. Serum IGF-1 levels were also measured, but were similar between the three groups.

3.2. Elastase

Gene expression analysis, 2D electrophoretic analysis and mass spectrometry of fasting bile samples from patients undergoing ERCP or transhepatic catheterisation for biliary obstruction from malignant or benign causes (CCa or ductal stones) demonstrated up-regulation of elastase mRNA, protein expression, and elastase activity in CCa compared to gallstone patients. The specific form was found to be elastase 3B, a pancreas-derived 29kDa serine protease, similar to classical pancreatic elastase 1.

Biliary amylase levels were utilized to correct for the presence of pancreaticobiliary reflux and bile elastase/amylase ratios were significantly raised in CCa patients as compared to gallstone patients (0.214+/-0.045 vs. 0.023+/-0.005, respectively; $p<0.001$) with an area under the ROC curve of 0.877. Moreover, the ratio had a sensitivity and specificity of 82% and 89% respectively, for differentiating malignant and benign causes of biliary obstruction (cut-off value 0.065) [71].

3.3. Mini Chromosome Maintenance (MCM)

MCM is the process of initiation and elongation of DNA replication and MCM replication proteins have been used as markers of dysplasia for the diagnosis of cervical and bladder cancer. Immunofluorometric analysis of bile sediment samples demonstrated that levels of MCM5, a protein which plays a role in transition from the G0 to G1/S phases of the cell cycle, were significantly more sensitive than brush cytology for the diagnosis of CCa (66 % vs. 20%, respectively; p=0.004) with a comparable positive predicted value (97% vs. 100%, respectively; p=ns) in 60 patients with biliary strictures of indeterminate aetiology [72].

3.4. Other Biomarkers

Relative bile fibronectin concentrations (absolute fibronectin level divided by concentration of bile acids) measured in 62 gallstone patients with cholangitis, 5 with benign strictures and 28 with CCa were significantly different between the groups; and with a cut-off value of 40 ng/μmol, its sensitivity and specificity for the diagnosis of CCa was 57% and 79%, respectively [73]. However, its increase in patients with cholangitis and cholestasis may limit its usefulness for the diagnosis of CCa.

Shen *et al* recently compared bile protein expression profiles from patients with cholangitis and CCa, utilising a classic 2D gel and MS approach, identifying 97 differentially expressed protein spots derived from 49 different genes, 38 of which were up-regulated in CCa bile [74]. One of these proteins, spermatogenesis-associated protein 20 (SPATA20), was confirmed as being significantly upregulated in bile from CCa patients, and had a sensitivity and specificity for CCa diagnosis of 90.0% and 83.3% respectively. Alternate methods for bile sample preparation for proteomic analysis have also been employed, including prefractionation to enrich glycoproteins, leading to detection of CEA-related cell adhesion molecule 1, CA125, MUC2 and Galectin-3-binding protein (LGALS3BP), a highly glycosylated protein implicated in tumour development [57].

Dhar *et al* measured plasma and bile M2-PK levels in 88 patients with BTC, 79 with benign biliary diseases, and 17 healthy controls. Sensitivity (90.3%) and specificity (84.3%) of bile M2-PK for malignancy were significantly higher than those for plasma M2-PK and serum CA19-9 [75].

Alpini *et al* demonstrated that serotonin metabolism is dysregulated in CCa and that increased serotonin can be found in bile from CCa patients, where it may have

implications for CCa cell growth [76]. The same group went on to show that mRNA and protein expression of the dopamine synthesis enzymes tyrosine hydroxylase and dopa decarboxylase were increased in CCa cell-lines and human biopsies [77]. Similarly, monoamine oxidase A (MAOA) was assessed in CCa and non-malignant controls to show that its expression correlated with differentiation, invasion and survival and that the MAOA promoter was hypermethylated upstream of the start codon in CCa samples and cell-lines. Further, IL-6 decreased MAOA expression [78] and may also upregulate progranulin expression and secretion via the ERK1/2/RSK1/C/EBPβ in CCa cell-lines and tissues where it increased CCa growth by an Akt-dependant mechanism [79].

4. Urinary Biomarkers

Urine also provides an easy and acceptable source for analysis of biomarkers, although its use in CCa remains limited to date. Metabolic profiling of small waste products in urine may, in the future, be able to identify certain patterns indicative of metabolic changes taking place in those with CCa. Proteomic analysis of urine via capillary electrophoresis mass spectrometry recently assessed the distribution of 42 peptides (most being fragments of interstitial collagens of extra-renal origin) to correctly identify 35 of 42 CCa patients and 64 of 81 PSC and benign pathology patients with a sensitivity of 83% and a specificity of 79% [80]. Further, all of those with CCa in addition to PSC were correctly identified.

5. Conclusion

Currently, the most widely utilized biomarkers for diagnosis of CCa include serum CA19-9, CA125 and CEA. However, CA19-9 levels are altered in a number of benign conditions, as well as in other upper abdominal malignant conditions. Further, approximately 7% of the population is Lewis negative and do not produce CA19-9, and it can also be low in up to 50% of patients with CCa. Similarly, CA125 and CEA are not consistently elevated in CCa patients and can be raised in a variety of both benign and malignant conditions. Thus, the need for better diagnostic and prognostic biomarkers in biliary tract carcinoma remains. A number of novel serum and biliary biomarkers have therefore been recently proposed. Models that combine multiple markers appear to significantly improve the diagnostic accuracy of existing markers such as CA19-9 and the identification of a suitable biomarker panel may provide a more viable means of success than any individual marker. However, further studies are required to replicate initial findings and investigate their applicability in a clinical setting.

The difficulty of obtaining bile for population screening and the lack of effective (both specific and sensitive) serum biomarkers means that suitable biomarkers for early detection and population screening are lacking. Alternate sources of biomarkers, including bile-rich gastric fluid via nasogastric aspiration or capsule endoscopy may ultimately prove to be a better technique. Further, a better understanding of tumour biology and the influence of various hormones and systemic factors may be necessary before effective biomarkers are found.

Possible future techniques for novel biomarker discovery include proteomic and gene expression profiling and the use of combinations of biomarkers to aid both diagnosis and choice of treatment regimen. However, proteomic analysis of bile and serum retains inherent problems in that they have very large dynamic ranges of expression with relatively few abundant proteins making up most of the protein content and contain contaminants that necessitate a significant degree of sample processing for their removal prior to analysis.

6. Expert Opinion

Current biomarkers for the diagnosis of BTC have limited sensitivity and specificity, with particular difficulty in distinguishing PSC-related and malignant strictures, potentially contributing to late diagnosis with subsequent high mortality rates. The incorporation of novel analysis techniques, such as digital image analysis (DIA) and fluorescence *in situ* hybridization (FISH), into management algorithms may enhance the sensitivity of samples taken at ERCP, whilst maintaining the high specificity of brush cytology, which can be difficult to interpret, particularly in patients with PSC [81, 82]. CCa has been associated with mutations in several oncogenes and up to 80% of tumour cells have been shown to exhibit chromosomal aneuploidy [83]. FISH and DIA can therefore be used to assess for the presence of these DNA abnormalities in brush cytology. They have been shown to improve the overall sensitivity for detecting CCa [82] and in PSC, where confirmation of CCa is particularly challenging, presence of polysomy is highly suggestive of CCa [84]. Similarly, techniques capable of assessing DNA-ploidy, such as flow cytometry, may improve the sensitivity of brush cytology [85]. However, such techniques require further validation and their routine use has only been adopted by a few centres [86]. Similarly, models that combine multiple biomarkers may improve the diagnostic accuracy of existing markers such as CA19-9 and the identification of a suitable biomarker panel that can be incorporated into treatment pathways may provide good diagnostic accuracy in the future.

A key goal in BTC is the discovery and validation of reliable, easily and consistently measurable biomarkers with good sensitivity and specificity. Statistical modelling of a biomarker signature in pancreatic cancer suggests that targeting 85% specificity would be acceptable in an enriched screening population with a cancer risk of <1%, results which may be applicable to specific at-risk groups in BTC (e.g. PSC) [87]. A better understanding of the underlying tumour biology of BTC would facilitate biomarker development. Various molecular processes have been described as being initiated during chronic cholestasis and associated local inflammation, such as that found during PSC, including release of various pro-inflammatory factors such as IL-6 and -8, tumour necrosis factors, tumour growth factors and platelet-derived growth factors into the local environment, which may ultimately lead to DNA damage, apoptotic escape mechanisms and initiation of pro-oncological pathways, such as p53,

p73, Smad4, K-ras, KIT, Bcl-2, survivin, cadherins and matrix metalloproteinase 7 [88-90]. Ultimately, such mechanisms may drive the development of BTC. Further, in patients with PSC, various cell-cycle and apoptotic control mechanisms, including those influenced by p53, Bcl-2, Bax and COX-2 pathways all played crucial roles in the pathogenesis of 128 BTCs (70 extrahepatic, 42 intrahepatic, 16 gallbladder) combined in a tissue microarray [91]. There was also differential expression of p16, p53 and Bcl-2 between intra- and extrahepatic CCa. p16 levels have subsequently been identified as having prognostic significance [91-94], as have the expression levels of epithelial-mesenchymal transition related genes, previously implicated in malignant invasion and poor prognosis [95] and HER3 of the receptor protein tyrosine kinase family [96]. Further, a specific isoform of p53 (Δ133p53) has now been identified as being associated with shorter overall survival than alternate p53 isoforms [97].

However, many of these pathways are crucial to multiple tumour types and little is known of the specific tumour biology underlying BTC development and progression. Comparison of intrahepatic CCA and HCC (n=11 and 24, respectively) tumours demonstrated specific chromosomal gains in CCa (20q, 5p, 7q, and 13q) as compared to HCC (gains in1q and loss of 4q, 10q and 13q) [98]. Similarly, in 50 BTC tumours, gains in 1q, 8q and 20q and losses in 5q, 8q, 9p and 18q were consistently found in early and advanced tumours with loss of 9p being the commonest abnormality (78% T1/2 and 68% T3/4) and the types of gains altered as per the presence or absence of lymph node (gains of 5p and 19q13 and losses of 6q14-q16) or distant metastatic disease (7p12-p14 ($P<0.003$), 7p21-pter ($P<0.007$) and 7q31 ($P<0.01$)) [99]. Andresen *et al* utilized an epigenetic technique to identify that various proteins involved in signaling and oncogenic pathways, including cysteine dioxygenase type 1 (CDO1), serine/threonine-protein kinase DCLK1, secreted frizzled-related protein 1 (SFRP1) and zinc finger and SCAN domain containing 18 (ZSCAN18) had high methylation frequencies in 93 CCa samples, as compared to being unmethylated in 43 control samples, with at least one of these four biomarkers being present in 87% of samples with a specificity of 100% [100].

microRNAs (miRNAs) are small non-coding RNAs (approximately 22 nucleotides) involved in regulating the expression of multiple genes and multiple cellular processes (including cell cycle, proliferation, apoptosis and migration) under both physiological and pathological conditions. This may also hold true for the

development and progression of BTC [101, 102] and their expression may be epigenetically regulated by hormonal factors involved in tumour cell growth, such as IL-6 [103, 104]. Certain miRNAs, such as miR-421, miR-2 and miR-26a, are capable of acting in a pro-oncogenic fashion by down-regulating the effects of genes involved in tumour suppression such as FXR (farnesoid X receptor), arsenic resistance protein-2, PTEN (phosphatase and tension homolog) and glycogen synthase kinase, and have been shown to be upregulated in CCa [105-108]. miRNA-25, which may help in regulating apoptotic pathways by protecting cholangiocytes from tumour necrosis factor and programmed cell death protein 4, is over-expressed in CCa [109, 110]. Other miRNAs, such as miR-320, miR-204 and miR-29b which attenutate pro-apoptotic pathways, have been shown to be down-regulated [111, 112]. miRNA-21 and -200b are over-expressed in CCa and may also contribute to tumour progression by promoting resistance to chemotherapy [113].

Many solid tumours, including BTC, are characterized by RNA, DNA and chromosomal abnormalities and the initial work on gene expression, branched clonal evolution and tumour heterogeneity in other tumour types mandates further proteomic, metabolomic and genetic analysis of BTC tumours to aid understanding of tumour biology and facilitate biomarker develoment [114].

7. Highlights Box

1. Sensitivity and specificity of commonly utilized biomarkers remain inadequate for the diagnosis of patients with BTC, and the search for novel biomarkers remains crucial for the early detection of lesions.

2. Combinations of multiple biomarkers may improve the diagnostic accuracy of existing markers such as serum CA19-9.

3. Novel techniques, such as DIA and FISH, could be incorporated into existing management algorithms to enhance the accuracy of brush cytology.

4. The difficulty and invasiveness of obtaining bile for population screening mean that efforts should focus on the identification of an appropriate serum biomarker, unless alternate ways of obtaining bile for analysis (e.g. bile-rich gastric fluid via nasogastric aspiration or capsule endoscopy) prove effective.

5. Techniques for novel biomarker identification include proteomic and gene expression profiling; however, proteomic analysis of both bile and serum remains difficult due to the large dynamic ranges of expression with relatively few abundant proteins making up most of the protein content.

6. Improved understanding of tumour biology may allow targeted analysis of serum and bile samples for effective biomarkers.

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		No. Samples			Performance		
Biomarker/Combination	**Sample Type**	**BTC**	**Benign/ Cancer Controls**	**Healthy Controls**	**Sensitivity**	**Specificity**	**Reference**
IL6	Serum	26	26	23	73%	92%	[46]
IL-6	Serum	45	40	10	71%	90%	[47]
IL6/LRG1/CA19-9	Serum	31	13	-	AUC 0.98		[48]
MUC5AC	Serum	179	122	74	62.6%	96.9%	[50]
MUC5AC	Serum	169	60	30	71%	90%	[49]
MUC5AC	Serum	39	33	-	44%	96%	[51]
CYFRA21-1	Serum	55	90	-	74.7%	92.2%	[56]
CYFRA21-1	Serum	66	58	-	56%	88%	[58]
CYFRA21-1/CA19-9	Serum	66	58	-	45%	96%	[58]
PRSS2	Serum	38	46	-	AUC 0.804		[59]
RCAS1	Serum	23	72	35	73.9%	96.2%	[60]
RCAS1	Serum	31	107	-	74.4%	91.0%	[61]
A1BG/AFM ratio	Serum	64	4	20	84.4%	87.5%	[64]
TTR/CA19-9	Serum	56	322	53	98.2%	100%	[65]
VEGF	Serum	29	44	-	AUC 0.744		[66]
M2-PK	Serum	115	85	120	84.2%	90.0%	[67]
MAC-2BP/CA19-9	Bile	26	52	0	AUC 0.75		[42]
IGF1	Bile	29	44	-	AUC 1.000		[66]
Elastase	Bile	22	28	-	82%	89%	[71]
MCM5	Bile	27	75	-	66%	N/A	[72]
Fibronectin	Bile	28	67	-	57%	79%	[73]
SPATA20	Bile	35	23	47	90%	83.3%	[74]
M2-PK	Bile	88	79	17	90.3%	84.3%	[75]
42 Peptides	Urine	42	81	-	83%	79%	[80]

Table 1. A summary of recent novel biomarker studies for the diagnosis and assessment of patients with BTC (BTC- Biliary tract cancer; AUC- Area under ROC curve; MAC-2BP- MAC-2 binding protein; CA19-9- Carbohydrate antigen 19-9; IL-6- Interleukin-6; LRG1; MUC5AC- Mucin5AC; CYFRA21-1- Soluble fragment of cytokeratin 19; PRSS2- Trypsinogen-2; TTR- Transthyretin; RCAS1- Receptor binding cancer antigen 1; A1BG- α1β-Glycoprotein; AFM- Afamin; VEGF- Vascular endothelial growth factor; IGF1- Insulin-like Growth Factor-1; MCM5- Mini chromosome maintenance protein 5; SPATA20- Spermatogenesis-associated protein 20; M2PK- M2 isotope of pyruvate kinase).