Special Report

Biomarkers in Medicine

Potential diagnostic and prognostic biomarkers for cholangiocarcinoma in serum and bile

Cholangiocarcinoma (CCA) is a devastating malignancy that is difficult to treat because of its insensitivity to conventional therapies and the inability to detect early tumor formation. Novel molecular techniques have enabled the use of serum and bile markers for CCA diagnosis and prognosis. Herein, we summarize the principal characteristics of serum and bile markers of CCA. Biomarkers such as interleukin-6, matrix metalloproteinases, serotonin (5-hydroxytryptamine) and bile acids have shown promise for improving CCA diagnosis. Several markers such as CYFRA 21-1, MK-1 and C-reactive protein were recently shown to be effective for CCA prognosis.

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Cholangiocarcinoma (CCA) is a devastating malignancy, and the number of cases is currently increasing worldwide [1]. CCA represents ~3% of all gastrointestinal malignancies, but it is the second most common hepatic malignancy after hepatocellular carcinoma (HCC). The incidence of CCA varies widely in different geographic regions; the highest number of cases occurs in southeast Asia while the lowest number occurs in Australia [2]. CCA is diagnosed based on clinical presentation, ultrasonography, computed tomography, magnetic resonance imaging, endoscopic retrograde cholangiopancreatography, percutaneous transhepatic cholangiography, endoscopic ultrasound, fine-needle aspiration, positron emission tomography and tumor markers. Although there have been advances in the diagnosis and management of CCA, these cancers remain challenging to treat because of their insensitivity to conventional therapies and diagnosticians' inability to detect early tumor formation. Novel targets for diagnostic and prognostic approaches are urgently needed. Various studies conducted over many years have attempted to identify biomarkers in the serum or bile with adequate CCA diagnostic accuracy. These would also be useful for population screening and for the surveillance of disorders for which individuals may be at risk. Herein, we discuss the current diagnostic and prognostic biomarkers for CCA in the serum and bile.

Biomarkers for diagnosis

Carbohydrate antigen 19-9 and carcinoembryonic antigen

In clinical practice, the most widely studied and used tumor marker is carbohydrate antigen 19-9 (CA19-9), which is often elevated in CCA [3,4]. In a retrospective study of 208 patients with sclerosing cholangitis including 14 patients with CCA, CA19-9 showed 79% sensitivity and 98% specificity in CCA diagnosis, with a cut-off value of 129 U/ml [5]. However, CA19-9 is nondiagnostic for patients who are antigen-negative. In addition, CA19-9 levels have been found to be elevated in other conditions such as pancreatic cancer and gastric cancer. A recent study showed that in CCA,

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CA19-9 had 52.9% sensitivity and 94.1% specificity [6], while another study showed 74% sensitivity, 82% specificity and 78% accuracy of CA19-9 in CCA diagnosis [7]. A systematic review discussed the diagnostic performance of CA19-9 for CCA, in which 1264 CCA patients and 2039 controls were analyzed. The authors found 72% diagnostic sensitivity, 84% specificity, 4.93 positive likelihood ratio, 0.35 negative likelihood ratio, 15.10 diagnostic odds ratio and an area under the receiver operating characteristic curve (AUC) of 0.83 [8]. Furthermore, carcinoembryonic antigen (CEA) is a common marker used in clinical practice. Although CEA is often elevated in CCA [3,4], it is mainly used to detect colorectal cancer. Only ~30% of patients with CCA showed elevated CEA levels [9,10]. Moreover, a study showed that evaluation of CEA level alone was neither sensitive nor specific for CCA diagnosis [11].

Interleukin-6

IL-6 is an inflammatory cytokine produced by cholangiocytes in the presence of inflammatory stimuli. It is also secreted by CCA cells and participates in their mitogenic signaling and survival [12,13]. IL-6 upregulates the potent antiapoptotic Bcl-2 protein, myeloid cell leukemia sequence 1 (Mcl-1) via an AKT-dependent mechanism [12]. Mcl-1 is integral to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) resistance in CCA [14]. Consequently, IL-6 inhibition reduces Mcl-1 expression and enhances TRAIL-mediated apoptosis [9]. IL-6 also acts through a mechanism that is dependent upon the signal transducer and activator of transcription (STAT) to enhance Mcl-1 expression [15]. Suppressor of cytokine signaling 3 (SOCS-3) regulates the IL-6/STAT-3 signaling pathway in a feedback manner, and epigenetic silencing of SOCS-3 facilitates sustained IL-6/STAT-3 signaling, resulting in enhanced Mcl-1 expression [16]. This information suggests several approaches for the treatment of the inflammatory subtypes of CCA. For example, neutralizing antibodies against IL-6 have been used in human clinical trials, and the results suggest that they may be effective therapeutics for treating CCA [17,18]. Another study showed that serum IL-6 level is a promising marker, as 73% sensitivity and 92% specificity were determined with a cut-off of 25.8 pg/ml. The positive predictive value and negative predictive value were 83 and 87%, respectively [19]. However, elevated levels of IL-6 are also observed in HCC and other benign biliary lesions. *In vitro* studies identified IL-6 as an autocrine growth factor in CCA cell lines [13,20]; moreover, IL-6 is elevated in the serum of patients with CCA and decreases sharply after resection [21].

Mucins

Serum mucin (MUC) represents another group of promising biomarkers composed of *O*-glycosylated proteins. Biliary MUC4 and serum MUC5AC are tumor-associated mucins that may be useful in diagnosing CCA. Western blotting was used to detect the biliary MUC4 protein in 27% of CCA and 29% of primary sclerosing cholangitis cases, but not in other benign and malignant biliary diseases. Serum MUC5AC was detected in 44% of CCA patients and was negatively correlated with survival [22]. MUC5AC showed 71% sensitivity, 90% specificity, 90% positive predictive value and 69% negative predictive value for CCA diagnosis [23]. Additionally, the tumor marker CA-125, also known as MUC16 since 2000, may be elevated in CCA but is not specific to this disease. MUC16 may be increased in other gastrointestinal or gynecological malignancies or in cholangiopathies [24]. Specifically, an antibody against M11, a subtype of MUC16 known as MUC16/M11, recognizes the CA-125 epitope expressed in a mucin-like glycoprotein [25–27]. Increased MUC16/M11 levels are correlated with poor survival in patients with intrahepatic CCA with mass-forming type tissues, and are representative of an independent prognostic factor of poor survival [28].

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are zinc-dependent proteases that are required for a number of physiological processes, including tissue modeling and repair, embryonic development and angiogenesis [29,30]. Overexpression of MMPs is thought to play an important role in tumor invasion by excessively breaking down the extracellular matrix [31]. MMP-7 and MMP-9 have been reported to be frequently expressed in the tumor tissues of patients with CCA [32]. A prospective study was conducted to investigate whether serum levels of MMP-7 were diagnostic for CCA. This study showed an AUC of 0.84 (95% CI: 0.78–0.91), with 75% sensitivity and 78% specificity. Additionally, elevated levels of serum MMP7 were generally correlated with early and late stages of CCA. Thus, MMP7 was concluded to be a 'potential good marker' of CCA [33].

Serotonin

Serotonin (5-hydroxytryptamine [5-HT]) is a well-known neurotransmitter that modulates neural signaling in a wide range of neuropsychological activities. Since its discovery as a mediator of liver regeneration, serotonin has gained considerable attention in liver physiopathology [34]. In contrast to its beneficial effects in enhancing hepatic growth, serotonin has been shown to be negatively correlated with various liver diseases. Given its role in liver regeneration, it is not unexpected

that serotonin may act as a promoter of malignancy. In a study investigating the impact of serotonin on CCA [35], the expression of TPH-1, the rate-limiting enzyme of serotonin synthesis, was found to be elevated in both CCA cell lines and tumor biopsies from 48 patients; in contrast, cell lines and patients showed downregulation of the serotonin-degrading enzyme. Accordingly, serotonin secretion was elevated in cell suspensions and in bile samples from patients with CCA, but not in samples from patients with intrahepatic lithiasis. Although the addition of serotonin had only a small proliferative effect on starved CCA cell lines, depletion of the molecule by a tryptophan 5-hydroxylase inhibitor markedly suppressed the proliferation of CCA cells *in vitro* and in a subcutaneous xenograft model. Taken together, these results suggest that serotonin in CCA is not only an inconsequential byproduct but also is actively produced by cancerous cells to promote their growth.

IGF-1

IGF-1 and IGF-1R were detected in biopsies from 18 patients with CCA. Three CCA cell lines, including HuH-28, TFK-1 and Mz-ChA-1, also expressed IGF-1 and IGF-1R [36]. The biliary IGF-1 concentration was 15–20-fold higher in extrahepatic CCA patients than in patients with pancreatic cancer or benign biliary disorders [37]. The only drawback of this method was the need for an invasive procedure to evaluate biliary IGF-1 levels. IGF-1 showed 82% sensitivity and 89% specificity [38].

Minichromosome maintenance

The expression levels of minichromosome maintenance (MCM) are increased in various malignant human tumors, including prostate cancer, colorectal cancer and neuroblastoma. Kim *et al.* examined the transcriptional regulation of MCM7 in CCA development and found that MCM7 transactivation may alter the transcriptional regulation of carcinogenic target genes, leading to CCA [39]. MCM replication proteins, particularly MCM5, showed a sensitivity of 66% compared with 20% for brush cytology in the detection of CCA [40]

Serum total sialic acid

Serum levels of total sialic acid (TSA) have been reported to be higher in cell lines undergoing neoplastic transformations, as well as in CCA [41]. The mean value of serum TSA in 89 CCA patients (2.75 ± 0.67 mM) was significantly higher than that in 38 benign hepatobiliary disease patients (2.33 ± 0.69 mM) and 43 healthy controls (1.89 ± 0.46 mM). The AUCs of CCA versus benign hepatobiliary diseases and CCA versus controls were 0.6699 and 0.8558, respectively [42]. Kongtawelert *et al.* also reported that the concentration of serum TSA in 69 CCA patients was significantly higher than that in 59 HCC patients, 37 cirrhosis patients, 61 chronic hepatitis patients and 50 healthy blood donors [43].

Tumor type M2 pyruvate kinase

Tumor type M2 pyruvate kinase (TuM2-PK) concentrations were also found to be significantly increased in patients with CCA. Moreover, the diagnostic performance of TuM2-PK was higher than that of CA19-9, with 84.2% sensitivity and 90% specificity compared with 68.4% sensitivity and 75% specificity, respectively [44]. Immunohistochemistry for TuM2-PK was performed in 19 CCA patients, and showed that TuM2-PK expression may enhance tumor cell invasion and promote lymph node metastasis of CCA [45].

TGF-β

TGF-β, a secreted homodimeric protein, belongs to a large family of pleiotropic factors that transmit signals via heterotetrameric complexes of type I and type II serine/threonine kinase receptors. Inhibition of TGF-β receptor in the liver and skeletal muscle significantly reduced the extent of hepatic fibrosis in a thioacetamide-induced liver fibrosis model [46]. Expression of TGF-β1 was observed in specimens from 47.4% of the CCA cases and was significantly correlated with lymph node metastasis, distant metastasis and tumor recurrence [47]. TGF-β expression was detected in pairs of CCA and normal bile duct tissues adjacent to the tumor in 47 specimens. Thirty-six specimens (76.6%) expressed higher levels of TGF-β in tumor tissues than in normal tissues. Furthermore, TGF-β expression was related to clinical stage and lymph node and liver metastases [48].

Bile acids

The concentration and composition of bile acids in patients with CCA was compared with that in patients with biliary tract stones or no biliary disease, and the levels of total bile acid, lithocholic acid and deoxycholic acid were significantly lower in CCA patients [49]. Bile homeostasis was analyzed using a simple nuclear magnetic resonance approach, and bile from patients with CCA exhibited significant changes in glycine-conjugated and taurine-conjugated bile acids, phospholipids, cholesterol and urea [50]. Profiles from CCA patients were clearly different from those of controls and HCC patients.

Alpha fetoprotein

Serum alpha fetoprotein (AFP) has been widely used in clinical practice as a marker of HCC. Some sera from CCA patients are positive for AFP. Recently, Li *et al.*

evaluated serum AFP, CA19-9, CA125 and CEA for diagnosing CCA. They reported that the level of AFP in the serum of CCA patients was significantly lower than that of HCC patients ($p < 0.01$). However, the value of AUC did not fulfill the criteria to support AFP as a practical diagnostic biomarker of CCA [51]. Levels of AFP, CEA, CA19-9, CA242, and CA50 were measured in the serum of 45 CCA patients and 76 HCC patients. AFP was a sensitive indicator to distinguish CCA from HCC, but its specificity was low. However, using a combination of AFP and CA242 markers, the sensitivity, specificity, and accuracy for diagnosing CCA were 88.9, 89.7 and 78.5%, respectively [52].

Biomarkers for prognosis

The probability of the long-term survival of CCA patients depends on early diagnosis and, in cases with localized disease, the feasibility of surgical resection [53,54]. Patients not suitable for surgery generally face rapid disease progression with a survival rate of only a few months [53]; thus, prognostic markers are urgently needed to identify CCA patients with poor prognosis who may benefit from more aggressive surgical strategies [55].

With the exception of the factors discussed above, including mucins, CA19-9, and CEA, few studies have evaluated prognostic markers in patients with CCA [56,57]. Other markers such as CYFRA 21-1, MK-1 and C-reactive protein (CRP) have been reported as prognostic markers in patients with CCA. The 3-year recurrence-free survival rates for patients with high and low concentrations of CYFRA 21-1 are 25 and 76%, respectively [58]. In addition, the expression of MK-1, a tumor-associated antigen encoded by the GA733–2 gene, is found in 79% of gallbladder carcinomas. MK-1 expression is observed in ~90% of well-differentiated tubular adenocarcinomas, but in only ~10% of those that are poorly differentiated. Multivariate analysis has shown that MK-1 expression is an independent prognostic marker that significantly correlates with increased overall survival [59]. Therefore, MK-1 may be used as a prognostic marker for CCA. Gerhardt *et al.* found that elevated CRP may be an independent predictor of poor survival. In CCA patients, CRP levels may increase because of the complicated tumor-induced structure and cholangitis development [60]. Generally, increased CRP levels in malignant disease are an inflammatory response to tumor invasion [61]. A lower inflammatory state is correlated with a better prognosis. Saisho *et al.* found that CRP <1.0 mg/dl is a favorable prognostic factor in patients with biliary tract cancers receiving chemotherapy [62].

Conclusion

Although a number of new serum and bile biomarkers have been recently proposed for CCA, further confirmation is needed to support their applicability in the clinical setting. Some biomarkers appear to be very promising, including MMPs, serotonin, and bile acids, but these biomarkers are useful for discriminating between benign and malignant biliary conditions only in patients who have undergone bile drainage. As a prognosis biomarker, CRP may be a good candidate. Thus, verification of these biomarkers in serum and bile samples is required, as it is only through such validation that measurements of serum and bile markers can be confirmed to be effective for the surveillance of CCA.

Future perspective

Some biomarkers are very promising, including metalloproteinase, serotonin and bile acids, in discriminating between benign and malignant biliary conditions. CRP seems to be a good prognosis biomarker in CCA patients. These biomarkers need further significant investigation and confirmation, which could provide an effective surveillance for CCA.

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Executive summary

- We summarize the principal characteristics of potential serum and bile markers for cholangiocarcinoma in this mini review.
- Most of markers for cholangiocarcinoma patients are elevated in serum or bile except bile acids.
- MMPs, serotonin, CRP and bile acids are very promising in discriminating between benign and malignant biliary conditions.
- The level of MMPs could reflect the tumor stage and CRP is a practical prognostic biomarker to reflect therapy effectiveness.

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