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MANAGEMENT OF THE CIRRHOTIC PATIENT (NS REAU AND A CARDENAS, SECTION EDITORS)

Screening for Hepatocellular Carcinoma and Cholangiocarcinoma: Can Biomarkers Replace Imaging?

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Abstract Treatment of liver and biliary tract cancer is most effective for early and localized disease. Effective screening methods for hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) will lead to early detection and treatment, and thus improvement in survival. Patients at risk of developing HCC and CCA will benefit the most from effective surveillance strategies. In this review, we provide an update on the current status of HCC and CCA surveillance and describe the recent efforts on biomarker development.

Keywords Early detection · Surveillance · Diagnosis · Liver cancer · Bile duct cancer

Introduction

Based on the most recent data from 2007 to 2011, the United States (US) Surveillance, Epidemiology and End Results (SEER) program reports an age-adjusted incidence rate of 7.9 per 100,000 people per year for liver and intrahepatic bile duct cancer, with a corresponding death rate of 5.8 per 100,000 people per year, resulting in a relatively high mortality ratio of 73 %. Examination of trends in incidence from the SEER database shows that the age-adjusted incidence rate per year is rising and almost doubled between 1992 and 2011 [1]. Similar trends have been observed in Canada, Australia, New Zealand, and Western Europe [2]. Further, despite the recent decrease in new cases in Japan, the Philippines, and China, East Asia has the highest overall incidence rate in the world [2]. After East Asia, sub-Saharan Africa has the next highest incidence of hepatocellular carcinoma (HCC) in the world, but due to the limited medical and research infrastructure in this region, data on trends in incidence is very limited [3].

Treatment of HCC and cholangiocarcinoma (CCA) is most effective for patients with early stage, localized disease while treatment of advanced liver cancer is merely palliative in nature. Unfortunately, in the US, only 20 to 30 % of HCC and CCA cases are diagnosed with early stage disease [4, 5]. In Taiwan and Japan, where there are comprehensive nationwide programs for early diagnosis and surveillance of individuals at risk for HCC from chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, approximately 70 % of HCCs are diagnosed at very early or early stages, resulting in 5-year survival estimates of 50–70 % [6••]. In the US, CCA is often diagnosed in advanced stages when disease is incurable [7]. Thus, national and international efforts to reduce the burden or morbidity and mortality from HCC and CCA need to focus on identification of the population at risk and aggressive surveillance using effective screening tools.

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The emerging evidence suggests that biomarkers are optimally used as a complement, rather than a replacement for imaging studies. Biomarkers uniquely may be elevated before there is any evidence of imaging abnormalities and may also guide consideration of what the primary tumor type is, such as HCC versus CCA, or mixed HCC-CCA. Cancer biomarker development is described by Pepe and colleagues as a five-phase process in which each stage is characterized by study design application (Fig. 1) [8•, 9]. In this review, we provide an update on the current status of liver and biliary cancer surveillance and describe recent efforts that are taking advantage of novel technologies in next-generation DNA sequencing, genome-wide methylation and proteomic studies, and advances in metabolomics to usher in a new era of biomarker development. The novel biomarkers in development will hopefully result in substantial improvements in both surveillance and diagnosis of HCC and CCA. Biomarkers discussed in this review, their current stage in biomarker development, and their clinical applicability are summarized in Table 1 for HCC and in Table 2 for CCA.

Hepatocellular Carcinoma

HCC is the most common malignancy of the liver. Current practice guidelines from the American Association for the Study of Liver Diseases (AASLD) recommend surveillance for HCC in patients with an expected risk of HCC exceeding 1.5 % per year or 0.2 % per year in patients with chronic HBV. Based on these recommendations, surveillance for HCC should be offered to all patients with cirrhosis and to hepatitis B carriers who have a family history of HCC, are Asian-born males 40 years or older, are Asian females 50 years or older, or African-born individuals 20 years or older. Individuals with immune active chronic HBV and those with coinfection with HCV or HIV or who have other chronic liver diseases should also be enrolled in surveillance programs [10, 11].

Currently Used Biomarkers: AFP, AFP-L3, and DCP

Worldwide, the commonest modalities used for surveillance for HCC in at-risk individuals are liver ultrasound and serum alpha fetoprotein (AFP) measurement. AFP is a glycoprotein produced by fetal liver and yolk sac; serum levels are high in utero but normalize to adult values rapidly after birth. High serum AFP values have been shown to occur in different cancers, including HCCs and germ cell tumors. The use of AFP as a screening modality has been controversial, particularly in low- to medium-incidence regions, but is widespread in high-incidence countries, where it is incorporated into most national and regional guidelines. The main objection to the use of AFP is its low sensitivity of only 20–30 % for the detection of HCC at an early stage, when it is most amenable to curative treatment. The sensitivity of AFP rises to 50–60 % for the detection of intermediate to advanced stage disease [12–14]. Proponents of the use of AFP argue that while most studies evaluating the performance of AFP have evaluated it in the cross-sectional setting, experienced practitioners typically follow and act on trends and variations in AFP levels, rather than individual measurements alone [15••]. Further, the combination of AFP with ultrasound improves the likelihood of detection of the more diffuse or infiltrating HCCs that do not develop as distinct nodules.

In addition to the AFP, other serum markers in clinical use for HCC surveillance include the lens culinaris binding subfraction of AFP (AFP-L3%) and the des gamma carboxyprothrombin (DCP). AFP-L3 is a glycosylated variant of AFP abnormally increased in HCC. DCP, also known as protein induced by vitamin K absence II (PIVKA-II), is a form of prothrombin generated when there is an acquired defect in

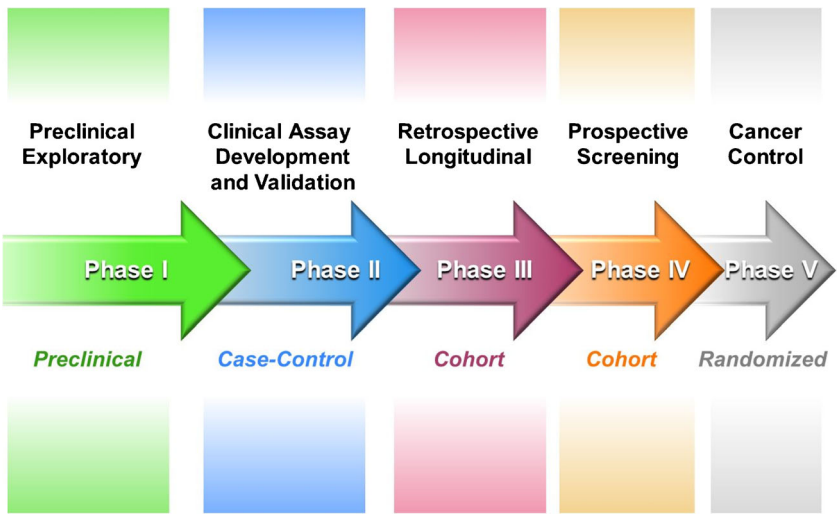

Fig. 1 Phases of cancer biomarker development (concept from Pepe et al. [9])

Table 1 Currently used and novel biomarkers for surveillance of HCC

Biomarker	Source	Phase of biomarker development[a]	Application	Suggested cutoffs and clinical utility
AFP	Serum	5	Risk stratification[b] Diagnosis[b] Prognosis[b]	10.9 ng/mL for early detection [1] 59 ng/mL may be optimal for HCV-infected patients [2] Practice is moving towards looking at AFP measurement trends over time and using models for risk stratification
AFP-L3%	Serum	2	Risk stratification[b, c] Diagnosis[b] Prognosis[b]	1.7 % [1]
DCP	Serum	2	Risk stratification[b, c] Diagnosis[b] Prognosis[b]	125 mAU/mL [3]
OPN	Plasma	2	Early detection	91 ng/mg [4]
GP73	Serum	2	Early detection Prognosis	150 μg/L [5]
Dickkopf-1	Serum	2	Early detection Prognosis	2.153 ng/mL [6••]
Axl	Serum	2	Early detection	14.05 ng/mL [7]
Micro-RNA	Serum, plasma or urine	2	Diagnosis Prognosis	NA
DNAJB1-PRKACA	Tissue and potentially serum	1	Diagnosis of fibrolamellar HCC subtype	NA

[a] See Fig. 1 for phases of biomarker development

[b] Clinically available for this indication

[c] FDA approved for this indication

posttranslational carboxylation. These assays are routinely used in Japan and other Asian countries and are approved for use in risk stratification for HCC by the US Food and Drug Administration. AFP-L3 and DCP have been shown to increase sensitivity and specificity of AFP in diagnosing HCC [12, 16, 17].

A number of models have been developed which integrate the results of biomarker assays with different patient clinical and laboratory characteristics to enhance the diagnostic accuracy of biomarkers in specific patient settings. The GALAD model by Johnson et al. includes gender, age, AFP-L3, AFP, and DCP as predictors of risk for HCC in the setting of chronic liver disease. This model has been validated and has shown consistency in stratifying patients based on risk of HCC [18•]. A similar algorithm, known as BALAD-2, was developed by the same group for accurate prognostication of patients with HCC [19]. El-Serag et al. recently developed another model using platelet count, alanine aminotransferase (ALT), age, and AFP to predict risk for HCC in patients with HCV-induced cirrhosis. In general, patients with high AFP who also had decreased platelet count, decreased ALT, or increased age showed higher risk of HCC. Their model-predicted probability levels showed a very close relationship with raw frequencies of HCC that only deviated in risks higher than 90 % where there is no practical significance. This model is yet to be tested on other populations with liver disease due to causes other than HCV and in populations outside of the Veterans Affair (VA) Health system where males predominate [20•]. The recently published ADRESS model can be used for predicting HCC probability in patients with cirrhosis using the variables of age, diabetes, race, cirrhosis etiology, sex, and severity of liver dysfunction. Although no biomarkers are included in the model, it can possibly stratify patients with cirrhosis into groups based on their potential surveillance program benefit [21•]. Finally, AFP and DCP biomarkers also have utility in combination with clinical factors for predicting recurrence of HCC after liver transplantation [22–24].

Ultrasound

Ultrasound is a widely used tool for both diagnosis and surveillance of various liver and cholestatic diseases and is currently established for surveillance of early-stage HCC. AASLD Guidelines recommend HCC surveillance with liver ultrasound every 6 months [11]. Ultrasound offers clinicians a safe, non-invasive, and low cost real-time imaging tool that is widely available. However, ultrasound efficacy is operator dependent and its performance in detecting lesions, especially smaller ones, is impaired. This becomes even more problematic given the increasing prevalence of obesity-related fatty liver

Table 2 Currently used and novel biomarkers for surveillance of CCA

Biomarker	Source	Phase of biomarker development[a]	Application	Suggested cutoffs and clinical utility
CA19-9	Serum	2	Diagnosis[b] Prognosis[b]	129 U/mL [8•, 9]
CEA	Serum	2	Diagnosis[b]	5.2 ng/mL [10]
CYFRA 21-1	Serum or plasma	2	Diagnosis Prognosis	1.5 ng/mL [11]
MUC5AC	Serum or bile	2	Diagnosis Prognosis	Serum 10.5 ng/mL [12] Bile 6.25 ng/mL [13] Serum-to-bile ratio 0.85 [13]
Conventional cytology	Bile duct brushings or fine needle aspiration	4	Diagnosis[b]	Benign Equivocal (atypical or suspicious) Malignant
FISH assay (UroVysion™)	Bile duct brushings	4	Diagnosis[b]	Negative: 2 copies of each probe Positive: ≥5 cells with polysomy (>2 copies of at least 2 probes excluding cells with tetrasomy) Equivocal: ≥10 cells with trisomy (3 copies of a single probe) or ≥10 cells with tetrasomy (4 copies of each probe). Tetrasomy can represent replicating cells [14, 15••, 16].
PB FISH assay	Bile duct brushings	3	Diagnosis[b]	Negative: 2 copies of each probe Positive: ≥5 cells with polysomy (>2 copies of at least 2 probes excluding cells with tetrasomy) Equivocal: ≥10 cells with trisomy (3 copies of a single probe) or ≥10 cells with tetrasomy (4 copies of each probe). Tetrasomy can represent replicating cells [14, 15••, 16].
Methylated BMP-3	Bile	1	Early detection	NA

[a] See Fig. 1 for phases of biomarker development

[b] Clinically available for this indication

disease and cirrhosis in North America, Europe, and Asia. Regenerative nodules in cirrhotic livers can be confused with neoplastic masses, leading to additional diagnostic test that are more invasive, have increased costs and can unnecessarily increase patient anxiety. Together, these limitations raise a fundamental problem in relying solely on ultrasound for surveillance of HCC.

A meta-analysis on surveillance performance for detecting HCC on cirrhotic patients via ultrasound reported a pooled sensitivity of 94 %, specificity of 94 %, and a summary receiver operator curve (ROC) plot of 98 %. However, these numbers were for detecting HCC at any stage of disease. When only looking at studies that reported detection of early HCC as defined by Milan criteria (one nodule <5 cm or three nodules each <3 cm and no gross vascular invasion), ultrasound had a pooled sensitivity of 63 %. Adding AFP for detection of early-stage HCC increased pooled sensitivity to only 69 %. Studies that conducted surveillance in 6-month intervals had a pooled sensitivity for detecting early-stage HCC of 70 % while studies that conducted surveillance on 12-month intervals had a pooled sensitivity of 50 % [25]. In contrast to the use of ultrasound in the setting of research studies, it has been shown that the performance of ultrasound is not as impressive in routine clinical use, with only 44 % sensitivity for the detection of HCC. However, in combination with AFP, sensitivity was 66 % [26]. Once a new nodule is identified by ultrasound, a diagnostic algorithm is proposed for definitive non-invasive diagnosis using cross-sectional multiphasic contrast imaging with CT or MRI [27].

New Biomarkers for HCC

Osteopontin

Osteopontin (OPN) is a phosphoprotein that is measurable in plasma and has been shown to be increased in patients with different types of malignancy including HCC [28]. A recent meta-analysis comparing the diagnostic potential of OPN to AFP showed that OPN had comparable biomarker characteristics to AFP suggesting its utility as a potential biomarker for HCC [29]. Moreover, OPN levels have shown to be increased up to 12 months prior to HCC diagnosis, making OPN a potential candidate biomarker for early detection of HCC [30, 31]. However, data on the use of OPN in combination with AFP or other biomarkers is limited.

Golgi Protein 73

Golgi Protein 73 (GP73) is a transmembrane glycoprotein normally found within the Golgi complex. Increased expression of GP73 has been reported in liver disease, particularly in serum of patients with HCC. Wang et al. recently reported that in HCC cells, GP73 is upregulated in response to the inflammatory modulator IL-1β through induction of the transcription factor epithelium-specific ETS (ESE)-1, which in turn directly binds to and transcriptionally activates GP73. These findings potentially provide a link between inflammation and GP73 activation in the development of HCC [32]. GP73 can be assayed using Western immunoblotting or enzyme-linked immunosorbent assay (ELISA). Initial studies using Western immunoblotting suggested that GP73 has high accuracy in detecting HCC, with better sensitivity, specificity, and accuracy than AFP, and some ELISA-based studies have been positive; however, in general, most studies using ELISA had negative results [33–37]. It has been suggested that GP73-specific antibodies may interfere with the ELISA test. In addition to aiding in surveillance, GP73 has been evaluated as a biomarker for prognosis of patients with HCC. In a study by Bao et al., increased GP73 expression in HCC tissue samples as compared to non-cancer liver tissue samples was associated with more advanced disease and poor prognosis after surgical resection [38].

Dickkopf-1

Dickkopf-1 (DKK1) is a secretory antagonist of the Wnt pathway that is normally expressed in embryonic tissue and has been shown to be upregulated in HCC tissues and increased in the serum of HCC patients as compared to cirrhotic and non-cirrhotic controls [39]. Furthermore, DKK1 had greater sensitivity and specificity than AFP in a Chinese cohort of HCC compared to patients with chronic HBV and cirrhosis controls. Further, the use of DKK1 and AFP together achieved better accuracy for detecting HCC than either test alone [40]. Indeed, DKK1 (cutoff of 500 pg/mL) and AFP (cutoff of 20 ng/mL) showed better diagnostic performance than AFP combined with DCP or with OPN [41]. However, a small Australian cohort showed no significant difference in DKK1 serum levels between HCC patients and age-matched, cirrhotic, non-cirrhotic, and HBV controls [42].

Recent data suggests that HCC patients with increased serum DKK1 may have poorer overall and relapse-free survival than patients with low DKK1 [43, 44]. Moreover, Sunagazaka et al. have suggested that DKK1 may be a potential biomarker for diagnosis of HCC with stem cell features [45]. Serum levels of DKK1 may have a role in detecting early HCC in patients with negative AFP levels and in defining prognosis for patients with HCC, but further validation using larger cohorts in other populations is needed to confirm the utility of DKK1 in all populations at risk of developing HCC.

Axl

Axl is a receptor tyrosine kinase that has been shown to be overexpressed in many different cancer types, including HCC, and to predict poor survival of patients with breast cancer, lung cancer, and mesothelioma. Binding of the extracellular domain (ECD) of Axl to its ligand, growth arrest-specific protein 6, leads to the phosphorylation of downstream oncogenic signaling molecules. Proteolytic processing of the ECD results in the release of an 80-kDa soluble variant of Axl (sAxl) that can be detected in serum. Release of sAxl has been shown to reflect the levels of total Axl in HCC cell lines. A study comparing serum levels of sAxl in patients with HCC to healthy and cirrhotic controls reported a significant increase in patients with HCC, specifically in patients with early-stage disease. Patients with breast, ovarian, or colorectal cancers and patients with liver metastasis of colorectal cancer showed no changes in serum sAxl levels [46]. Also, there was no apparent difference between serum sAxl levels of healthy controls and cirrhosis patients. Using cutoffs of 14.05 ng/mL for Axl and 20 ng/mL for AFP, serum Axl outperformed AFP in detecting very early HCC and discriminating very early HCC from liver cirrhosis [46].

Micro-RNAs and Long Non-coding RNAs

With the recent advances in microarray and next-generation sequencing technology, micro-RNAs (miRNAs) and long non-coding RNAs (lnc-RNAs) have been shown to have both diagnostic and therapeutic potential for many diseases including cancer. Increased micro-RNA 21 (miR-21) serum levels have been demonstrated in patients with HCC as compared to chronic hepatitis and normal controls. Although serum miR-21 levels have shown high sensitivity, its specificity is low limiting its effectiveness as a sole marker in diagnosing HCC [47]. The micro-RNA-200 family has recently been studied in HCC specimens and cirrhosis liver specimens and showed significant downregulation, especially of miR-200a and miR-200b [48]. Serum micro-RNA 101 (miR-101) and micro-RNA 18a (miR-18a) have also been shown to be increased in HCC patients, making these markers potentially useful for surveillance of HCC in patients with HBV [49] [50]. There are a large number of other miRNAs and lncRNA under evaluation as biomarkers for HCC; however, their utility has not been completely elucidated and the potential of these newer markers remains to be fully explored.

Fibrolamellar HCC

Due to the lack of recognition of patient risk and consequent absence of surveillance, HCCs occurring in non-cirrhotic livers are typically diagnosed at later stages of disease than

those developing in patients with underlying cirrhosis. Fibrolamellar HCC is a subtype of HCC that usually develops in non-cirrhotic livers and is characterized by highly metastatic behavior, including a propensity to lymph node metastases. Fibrolamellar HCC is traditionally identified by its hypointense central scar on T2-weighted MRI and radiating septa, central calcifications, or necrosis. On contrast-enhanced MRI, arterial hyperenhancement of the lesion minus the central scar is characteristic [51]. Most notably, fibrolamellar HCCs have recently been shown to bear a characteristic fusion protein DNAJB1-PRKACA which potentially has significant diagnostic and therapeutic relevance [52•, 53•, 54, 55]. Serum AFP is usually not elevated in fibrolamellar HCC, despite the fact that most fibrolamellar HCCs are diagnosed at very advanced stages [56].

Cholangiocarcinoma

Cholangiocarcinoma (CCA) can be subclassified into intrahepatic (iCCA), perihilar (pCCA), and distal cholangiocarcinomas (dCCA) based on their anatomic location. Patients with perihilar or distal CCA commonly present with symptoms of biliary tract obstruction such as jaundice, pale stools, dark urine, or pruritus, while the clinical presentation of iCCA is non-specific. Patients with advanced iCCA may present with weight loss, malaise, abdominal discomfort, jaundice, hepatomegaly, night sweats, or a palpable liver mass [57]. However, patients with early iCCAs usually have no symptoms; thus, it is not uncommon for early iCCAs to be diagnosed incidentally during workup of other diseases. Moreover, it has been shown that iCCA incidence rate is increasing although prognosis remains poor [7].

Established risk factors for CCA include primary sclerosing cholangitis (PSC), choledochal cystic disease, hepatobiliary liver flukes, and Caroli's disease, but account for less than a third of CCA cases. Although there is lack of prospective data, patients with PSC have a lifetime prevalence of CCA of approximately 5–10 % and a 5-year survival rate of less than 10 % and may benefit from cancer surveillance [58, 59, 60•]. Surveillance guidelines for early detection of sporadic CCA are yet to be established. This is primarily due to the rarity of cholangiocarcinoma and the lack of data on at-risk populations that would benefit from surveillance. Risk factors for intrahepatic cholangiocarcinoma are similar to those for HCC but with weaker associations. These risk factors include cirrhosis, chronic viral hepatitis, obesity, diabetes, and excessive alcohol use [61]. The recent AASLD guidelines on PSC discussed surveillance but did not make explicit recommendations due to a lack of evidence [58].

In 2011, Razumilava et al. recommended surveillance of PSC patients with annual MRI and MRCP or ultrasound and CA19-9. If dominant strictures are found, they recommend ERCP with epithelial brushings using conventional cytology and the UroVysion™ Fluorescence in situ hybridization (FISH) assay and more recently developed pancreatobiliary FISH assay [62]. Needless to say, these surveillance recommendations are both invasive and costly, but are currently the most effective strategy for detecting early CCA.

An important consideration in discussions of the feasibility of surveillance for CCA is the concept of aerodigestive cancer or pan-cancer assays. Aerodigestive cancers are the cancers arising from the lungs and upper respiratory tract ranging through the GI tract to the rectum, which have the potential to shed cells that are collected in the digestive tract; these include squamous cell head and neck cancer, lung cancer, esophageal cancer, gastric cancer, CCA, pancreas cancer, small intestinal cancer, and colon cancer. While most of these cancers are relatively uncommon and screening for the individual cancers would not be justified based on their low incidence, the high incidence of colon cancer justifies stool- or blood-based screening. If it is possible to identify specific biomarkers that are characteristic of the other cancer sites, they can potentially be effectively incorporated into a multi-cancer detection panel.

Imaging modalities of utility in the surveillance of PSC patients for CCA and the diagnosis and follow-up of CCA patients include MRI/MRCP, ERCP, EUS, and PET-CT scanning. As with HCC, these modalities are critical for accurate assessment of disease presence and severity and they are complemented but not usurped by the advances in diagnostic biomarker technology. Increasingly, biomarkers are providing clues to tumor heterogeneity and allowing individualization and personalization of therapy for CCA, such as by the identification of isocitrate dehydrogenase (IDH) 1 and 2 mutations and fibroblast growth factor receptor 2 (FGFR2) fusions [63–66]. Imaging, particularly MRI/MRCP, endoscopic retrograde cholangiopancreatography (ERCP), and endoscopic ultrasound (EUS) also serves as a guide and tool for sampling tumors for tissue through cytology brushing, core needle, or fine needle aspiration biopsies. While PET-CT scanning has not proved to be of substantial utility in HCC, it has been shown to be of utility in the staging of CCA and identification of postsurgical recurrence and distant metastases.

Currently Used Biomarkers: CA19-9 and CEA

Carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA) are both glycoproteins measurable in serum for which there are clinically available assays. CA19-9 and CEA are the most studied biomarkers for diagnosis of CCA. Current data suggests these markers by themselves cannot be used as the sole criteria for diagnosis of CCA but rather have utility as adjunctive markers in the context of patient factors and with the aid of multiphasic cross-sectional MRI or CT imaging, ERCP with brush cytology and/or forceps biopsy, or percutaneous or EUS-guided biopsy. In patients with a biliary

stricture, an increased serum CA19-9 may support the diagnosis of CCA, particularly if the levels are persistently high after drainage of biliary obstruction. However, a negative test does not exclude CCA. A cutoff of 129 U/mL maximizes the utility of CA19-9 for detecting CCA in patients with PSC [67–69]. Moreover, it has been suggested that the serum CA19-9 is also effective for staging of disease since very high levels (CA19-9 >1000 U/mL) may be associated with metastatic disease [70]. In a recently developed clinical staging system for pCCA, CA19-9 is a key differentiation variable for advanced disease [71]. CEA has demonstrated poor sensitivity and specificity for diagnosing CCA, but used in combination with CA19-9, the two markers show better marker diagnostic utility than either biomarker alone in differentiating CCA from benign biliary diseases [72].

ERCP Sampling by Brush Cytology, Intraductal Biopsy, and FISH Assay

Although invasive and expensive, ERCP is invaluable for visualizing and sampling the bile duct epithelium in the setting of strictures suspicious for malignancy. The sensitivity of conventional brush cytology for diagnosis of CCA ranges from 6 to 88 % due to heterogeneity between studies in terms of categorization of equivocal results into positive and negative diagnoses and in terms of the populations under study (some studies include patients with masses on imaging while others only enroll PSC patients under surveillance). Furthermore, conventional cytology interpretation is difficult due to the overlapping nature of the cellular features seen in benign and malignant biliary strictures [73••].

In fluorescent in situ hybridization (FISH), fluorescently labeled DNA probes are hybridized to cytology preparations in order to detect aneuploidy in cells from biliary stricture brushings or fine needle aspirations. Up until recently, most studies have used the UroVysion™ FISH probe set which was originally optimized for detection of urothelial cancers [74, 75] (FISH result and interpretations are summarized in Table 2). Multiple reports show that in up to 60 % of patients with negative standard cytology, UroVysion™ FISH can confirm the diagnosis of CCA [76, 77•, 78, 79]. In a meta-analysis of patients with PSC, FISH polysomy showed 51 % sensitivity and 93 % specificity [80]. Due to the influence of pretest probability on biomarker performance, it is important not to use FISH in settings where the suspicion of cancer is very low, such as in patients with biliary stone disease. FISH trisomy 7 is sometimes seen and is usually non-specific, although in PSC it may identify patients who eventually will progress to invasive cancer [81, 82]. Eaton et al. have shown that FISH is useful in identifying PSC patients with multifocal polysomy, a subgroup of patients who were more likely to develop CCA and thus could benefit from close surveillance [81]. In a cohort of patients with PSC and equivocal biliary cytology (atypical or suspicious), patients with FISH polysomy were 76 % more likely to develop a pancreaticobiliary tract malignancy within 2 years of cytology findings ($P<0.001$). Most of these cancers turned out to be CCAs (27 were CCA, 2 pancreatic adenocarcinomas, and 1 gallbladder cancer) [79]. Tetrasomy is typically associated with biliary stone disease and not with malignancy.

A new pancreatobiliary FISH (PB FISH) panel has been optimized for diagnosing malignancy in pancreaticobiliary brushings. Initial results suggest that PB FISH provides an approximately 20 % improvement in sensitivity over UroVysion FISH™, reaching sensitivities of up to 77 % with a specificity of 96 % for detection of malignancy in pancreatobiliary strictures [83]. Further validation of these initial results is forthcoming.

New Biomarkers for CCA

CYFRA21-1 Fragment of Cytokeratin 19

In the liver, hepatocytes differentially express cytokeratins 8 and 18 while cholangiocytes express cytokeratins 7 and 19. Cytokeratin 19 can be used to discriminate iCCA from HCC in hepatic masses. However, it is not effective in differentiating tumoral tissue from benign cholangiocytes. CYFRA21-1 is a soluble serum fragment of cytokeratin 19 and a potential marker for detecting CCA via serum electrochemiluminescent immunoassay (ECLIA). A retrospective study comparing CYFRA21-1 to CEA and CA19-9 in patients with histologically confirmed bile tract cancers showed that CYFRA21-1 had better sensitivity and specificity for detecting iCCA than any of the other biomarkers. Maximal Youden's indexes were used to determine the cutoff for each of the biomarkers studied (3.27 mg/mL, 76.53 U/mL, and 2.70 U/mL for CYFRA21-1, CA19-9, and CEA, respectively). In a prospective study of patients with PSC, CYFRA21-1 at a cutoff of 1.5 ng/mL was more specific than CA19-9 [84]. Moreover, CYFRA21-1 was an effective predictor of poor prognosis and recurrence after tumor resection [85]. The utility of CYFRA21-1 for early detection of CCA in patients with PSC is unknown; further studies are required to investigate its performance in this setting.

Mucin 5AC

Mucin 5AC is a glycoprotein mucus component that is rarely secreted from non-malignant biliary tract epithelial cells. Ruzzenente et al. compared serum mucin 5AC (MUC5AC) levels of patients with malignant biliary obstruction to patients with benign biliary obstruction and healthy controls. MUC5AC enhanced the diagnostic accuracy of CA19-9 and CEA. Within patients with malignant disease, high levels of MUC5AC were also associated with poor prognosis [86].

Measurement of the serum-to-bile MUC5AC ratio resulted in a higher accuracy in detecting CCA from benign biliary disorders [87]. Current data on MUC5AC is preliminary and should be validated prospectively to ensure effectiveness and test potential cutoffs.

Methylated Bone Morphogenic Protein 3

Recently, Kisiel et al. reported increased promoter methylation of the known tumor suppressor methylated bone morphogenic protein 3 (BMP-3) in CCA cell lines and resected tumor tissue but not in immortalized cholangiocytes or in matched benign bile tract epithelium. Further studies to validate these results in a larger cohort and in patients with PSC are now needed to determine this biomarker's clinical applicability to detect early cholangiocarcinoma [88]. The need for implementing invasive techniques to acquire bile has been a fundamental limitation to the clinical use of bile biomarkers as surveillance for CCA. However, with the availability of the recently approved multi-target stool DNA testing system (Cologuard®) for screening for colorectal polyps and cancer, it is now possible to imagine the potential addition of novel aerodigestive cancer site-specific assays to a stool DNA-based test, leading to further population-based bile duct cancer detection in a much more feasible and non-invasive fashion [89].

Conclusion

In summary, biomarkers and imaging studies are inherently complementary in the surveillance and diagnosis of hepatobiliary malignancies. Continued advances in the use of both modalities, particularly the ability to combine modalities by using molecular marker binding in imaging studies, holds promise for substantially improving screening for HCC and CCA in the near future. There is a major gap in the availability of appropriately and prospectively collected sample repositories of cohorts of case and control patients with liver and biliary cancers to facilitate the evaluation of novel biomarkers. Efforts to establish these key sample repositories, for example through the US National Cancer Institute Early Detection Research Program, should be intensified.

Compliance with Ethics Guidelines

Conflict of Interest Maria E. Lozada and Roongruedee Chaiteerakij declare no conflict of interest. Dr. Roberts has received research grants from Wako Diagnostics and Inova Diagnostics, developers of serum biomarker tests. He has a current grant from Gilead Sciences, outside the submitted work.

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