Biomarkers for the Diagnosis of Cholangiocarcinoma: A Systematic Review

Gyem Tshering,¹ Palden Wangyel Dorji,¹ Wanna Chaijaroenkul,¹ and Kesara Na-Bangchang^{1,2*}

¹Chulabhorn International College of Medicine, Thammasat University, Rangsit Center, Klong Luang, Pathum Thani, Thailand; ²Center of Excellence in Pharmacology and Molecular Biology of Malaria and Cholangiocarcinoma, Chulabhorn International College of Medicine, Thammasat University, Rangsit Center, Klong Luang, Pathum Thani, Thailand

Abstract. Cholangiocarcinoma (CCA), a malignant tumor of the bile duct, is a major public health problem in many Southeast Asian countries, particularly Thailand. The slow progression makes it difficult for early diagnosis and most patients are detected in advanced stages. This study aimed to review all relevant articles related to the biomarkers for the diagnosis of CCA and point out potential biomarkers. A thorough search was performed in PubMed and ScienceDirect for CCA biomarker articles. Required data were extracted. A total of 46 articles that fulfilled the inclusion and had none of the exclusion criteria were included in the analysis (17, 22, 3, 4, and 1 articles on blood, tissue, bile, both blood and tissue, and urine biomarkers, respectively). Carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA), either alone or in combination with other biomarkers, are the most commonly studied biomarkers in the serum. Their sensitivity and specificity ranged from 47.2% to 98.2% and 89.7% to 100%, respectively. However, in the tissue, gene methylations and DNA-related markers were the most studied CCA biomarkers. Their sensitivity and specificity ranged from 58% to 87% and 98% to 100%, respectively. Some articles investigated biomarkers both in blood and tissues, particularly CA19-9 and CEA, with sensitivity and specificity ranging from 33% to 100% and 50% to 97.7%, respectively. Although quite a number of biomarkers with a potential role in the early detection of CCA have been established, it is difficult to single out any particular marker that could be used in the routine clinical settings.

INTRODUCTION

Rationale. Cholangiocarcinoma (CCA) is a highly malignant tumor found in the epithelial cells lining the bile duct.^{1–3} This type of cancer imposes a major public health concern in Southeast Asian countries, particularly Thailand.⁴ The highest incidence is found in the Khon kaen province, Northeastern Thailand, where the age-standardized annual incidence rates are 36.3 and 87.7 per 100,000 population in females and males, respectively.⁵ Infection with the liver fluke, *Opisthorchis viverrini*, which is present in *Koi pla* (a raw freshwater cyprinoid fish meal of the people in the region) together with the consumption of nitrosamine are considered the main risk factors that lead to the cancer development.^{6–9}

The worldwide incidence of CCA has increased to 18% of all liver cancers during the past 30–40 years.¹⁰ As per the United States Vital Statistics databases, in the United States alone, the incidence of CCA increased markedly between 1973 and 1997 with an estimated annual percent change of 9.11% (95% confidence interval (CI): 7.46–10.78).¹¹ The trend in incidence of the two types of CCA, that is, intrahepatic CCA (ICC) and extrahepatic CCA (EHC) may differ. For instance, between 1990 and 2008, ICC increased from 0.43 to 1.84 per 100,000 population/year in males and from 0.27 to 1.51 in females in England and Wales, whereas EHC in males declined from 0.78 to 0.51 and from 0.62 to 0.39 in females;¹² however, the overall incidence of CCA is still on the rise throughout the world.¹⁰ The prognosis of this malignancy is poor because of its silent clinical characteristics and limited therapeutic measures.

At present, the treatments available for CCA are surgical resection, liver transplantation, and adjuvant therapy (radiation, chemotherapy). Surgical resection is currently the only intervention with the possibility of cure. However, all the

patients diagnosed with CCA cannot undergo surgery. They require full evaluation for resectability. Evaluations should be based on physiological suitability, tumor location, hepatic lobar atrophy, hepatic ductal extension, and many other factors.¹³ Another invasive method is liver transplantation. Despite high 5-year survival rates, liver transplantation has received contradictory views from many authors mainly because of high recurrence rates.¹⁴ It has been suggested that liver transplantation should be reserved only for early stage CCA.¹⁵ Chemotherapy is one of the adjuvant therapies to surgery. It has been reported to provide inconsistent results in improving survival, both in resected and unresected patients. Previously, fluorouracil either alone or in combination with methotrexate, leucovorin, cisplatin, or mitomycin were the most studied. However, survival rates were not as good as surgery.¹⁶ Use of gemcitabine-based combination regimens showed increased responses of about 22–50%.¹⁷ Studies using gemcitabine in combination with cisplatin showed increased progression free survival and overall survival.¹⁸ Radiation is another adjuvant therapy that is recommended post-surgery. It is most commonly used as combination of external beam irradiation and brachytherapy with Iridium-192. Retrospective studies have demonstrated that radiation significantly increased 5-year survival rates.^{19,20}

However, radiotherapy and chemotherapy provide inconsistent cure rates, and surgical resection at early stage is the only curative treatment at present.²¹ Nevertheless, the disease has a slow progression which makes it difficult for early diagnosis and hence most patients are detected only in advanced or metastatic stages.²²

One possible solution for the poor prognosis and delayed treatment would be to establish reliable biomarkers that are specific to the early stages of the disease. If such specific biomarkers are recognized and standardized, then the disease can be detected at an early stage and necessary treatments can be implemented. Several studies have been conducted to explain the pathogenesis of CCA and also to discover and establish the biomarkers that can be used in

*Address correspondence to Kesara Na-Bangchang, Center of Excellence in Pharmacology and Molecular Biology of Malaria and Cholangiocarcinoma, Chulabhorn International College of Medicine, Thammasat University, Rangsit Center, Pathum Thani 12120, Thailand. E-mail: kesaratnu@yahoo.com

diagnosing CCA.²³ A number of genes have been reported to be overexpressed, whereas some were under-expressed in biological samples obtained from CCA patients.²⁴ Some of the cell surface proteins such as selectins, integrins (ITGA6 and ITGAV), and cadherins (E-Cadherin) have also been widely investigated in the pathophysiology of several cancers. The protein sets identified provide a rich resource of potential circulating markers and targets for imaging and therapeutics for cancers because they play a pivotal role in cancer invasion and metastasis.^{25,26} The protein sets identified provide a rich resource of potential circulating markers and targets for imaging and therapeutics for cancers.²⁵ TNF- α , one of the most important pro-inflammatory cytokines, has been shown to be increased in chronic infections and reinfections of *O. viverrini*.^{27,28} It is reported to promote the invasiveness of CCA via the TNF- α receptor 2.²⁹ Serum biomarkers such as carbohydrate antigen 19-9 (CA19-9) and cancer antigen 125 (CA125) are being applied in most routine laboratory tests for CCA diagnosis.^{30,31} Nevertheless, their wide ranges of sensitivity (50–90%) and specificity (54–98%) have been reported.^{30,32} Overexpression of oxysterol binding protein-like 8 (OSBPL-8) was reported in *O. viverrini* and *N*-nitrosodimethylamine -induced CCA hamster model.³³

There have also been extensive studies on the differential expression of proteins in CCA cells compared with normal cells.³⁴ This included studies of secreted proteins that are directly accessible in the biological fluids of CCA patients.^{35,36} Another type of molecule that is directly accessible in biological fluids are the autoantibodies (AABs). Their presence in the serum samples from patients of various malignancies has been reported.^{37,38} Because circulating AABs are produced in large quantities against even small amounts of antigens, they could be of significant diagnostic values. However, so far, there has been limited information on the application of AABs as biomarkers especially with regard to primary liver tumors such as CCA. Despite the fact that there are quite a large number of studies both in vitro and ex vivo with attempts to establish biomarkers that can be used for the diagnosis of CCA and/or differentiation of CCA from other resembling tumors, the results from these studies cannot be applied to clinical practice. This is mainly because of their insufficient sensitivity and/or specificity, or inconsistency of results in subsequent studies. Considering all the lapses, relevant articles on the investigation of the biomarkers for the diagnosis of CCA were reviewed and analyzed in the present study to point out the potential biomarkers that can be applied in clinical settings. Information on the sensitivity and specificity of the proposed biomarkers were included in the analysis because the diagnosis accuracy is determined by these two parameters.

METHODS

A literature search was performed on PubMed and ScienceDirect on March 9, 2017. The search terms used were “cholangiocarcinoma,” “bile duct cancer,” “Klatskin tumor,” “diagnosis,” and “markers.” No other search conditions were applied. All articles obtained from the two databases were checked for duplication. The remaining articles were initially screened as per the inclusion criteria based on the content of the abstract section. The criteria for inclusion of the articles in this systematic review were as follows: 1) studies of which

CCA are the main subject, 2) studies on biomarkers related to CCA diagnosis, 3) prospective or retrospective studies, 4) in vitro, in vivo in animal models, or clinical studies, and 5) full published research articles. The exclusion criteria included at least one of the following criteria: 1) articles in languages other than English, 2) articles on other cancers with inclusion of CCA, 3) case reports, 4) review articles, or 5) unavailability of information on sensitivity and specificity.

Data extraction from all articles was performed by two independent researchers. When conflicting opinions arose, the decision was sought from a higher professional level personnel and the decision was considered final. The information extracted included the following: name of first author, year of publication, study type, study objective, sample size, sample characteristics, type of biomarkers measured, and results. For the evaluation of performance of the diagnostic biomarkers, the sensitivity and specificity and/or receiver operating characteristic (ROC) values (if available) were also extracted.

RESULTS

Study selection. A total of 652 articles related to CCA diagnosis published up to March 9 were retrieved from PubMed and ScienceDirect, of which five were duplicates. Through initial screening of the abstract, 561 articles were excluded from the analysis as they did not fulfill the inclusion criteria. The remaining 86 articles were downloaded for full text assessment. Forty-four articles were excluded from the analysis; 14 articles did not study CCA as the main subject, nine articles did not provide clear results and interpretations, and 21 articles did not provide information on the test performance (sensitivity and specificity). Finally, a total of 41 articles^{39–84} fulfilled all the inclusion criteria and had none of the exclusion criteria for the review (Figure 1). We also did a manual search of relevant articles in the references of the included articles but did not get any additional relevant ones.

Study characteristics. The characteristics of the articles included in the systematic review are summarized in Table 1 with detailed information in Supplemental Table 1. Of the 46 articles, 17, 22, 3, 4, and 1 articles, respectively, were related to the investigation of biomarkers in the blood (serum or plasma), tissue, bile, both blood and tissue, and urine samples.

Biomarkers in the blood. A number of potential blood biomarkers for diagnosis and differentiation of CCA from other similar types of tumors were reported. These included carbohydrate antigen (CA), carcinoembryonic antigen (CEA), micro-RNA (miRNA), exostosin1 (EXT1), cathepsin B to cystatin C ratio, heat shock protein (HSP70) related, and angiotensin-2 (Angpt-2).

Carbohydrate antigen and CEA were two of the most commonly studied biomarkers in the serum. Carbohydrate antigen, particularly CA19-9, is an antigen found in the normal epithelial cells of many organs including biliary ducts.⁸⁵ Elevated levels of CA19-9 are found to correlate with not only the tumor mass but also with the degree of tumor differentiation.⁸⁶ Carcinoembryonic antigen is a glycoprotein produced by the gastrointestinal tract.⁸⁷ Carcinoembryonic antigen level has been shown to correlate with tumor metastasis.⁸⁸ Several articles investigated the application of the two biomarkers, either alone⁷² or a combination of the two,⁶⁷ and even in combination with other relevant biomarkers^{53–56,66,75} for the

FIGURE 1. Summary flowchart of literature search.

diagnosis of CCA or differentiation of CCA from other similar tumors. Carbohydrate antigen-S27, studied as a diagnostic marker, was found to be significantly higher in CCA cases compared with controls ($P < 0.001$).⁷² In a study that investigated the accuracy of the combination of CA19-9 and CEA in the diagnosis of CCA in patients with primary sclerosing cholangitis (PSC), median levels of the biomarkers were found to be higher in the histologically proven CCA patients than that in other patients.⁶⁷ In terms of sensitivity and specificity, however, CA and CEA provided wide ranges of sensitivity (47.2–98.2%) and specificity (89.7–100%).

Micro-RNA is a small noncoding RNA that plays an important role in regulating protein expression.⁸⁹ It has been shown to be involved in the development and metastasis of many kinds of human cancers.^{90–92} Because of its presence in the early stages of many cancers, it has often been used as diagnostic indicators of those cancers.^{93–95} A study that tried to characterize miRNA in the serum of ICC patients found out that miRNAs (miR-21, miR-34c, miR-200b, and miR-221) showed a 2.18–3.79 fold increase in the tumor cells compared with non-tumor cells.⁴⁵ In another study that evaluated the diagnostic utility of miRNA in differentiating PSC and CCA, upregulation of miR-222 and miR-483-5p was demonstrated in CCA compared with PSC ($P < 0.0011$ and $P = 0.0013$).⁸⁰ Yet, in another study that determined the diagnostic value of miR-26a in CCA, it was found to be highly expressed in CCA patients compared with healthy controls ($P < 0.01$). The sensitivity and specificity were 84.8% and 81.8%, respectively.⁸¹

Exostosin1 is one of the five genes encoding the exostosin family, and its mutations are believed to be responsible for

inherited heredity disorders.⁹⁶ The plasma level of EXT1 was shown to be significantly higher in the CCA patients than healthy subjects ($P < 0.05$). Its sensitivity and specificity were 91.7% and 50%, respectively.⁵⁰

Cathepsin B is a lysosomal cysteine protease that is detected in many cancers.⁹⁷ It is regulated by cysteine protease inhibitors, namely cystatin C and stefin A.⁹⁸ Measurement of cathepsin B to cystatin C ratio has been suggested to be a beneficial tool for diagnosis of cancers because an imbalance between the proteinases and their inhibitors is correlated with tumor progressions.^{99,100} The ratio was reported to be significantly higher in patients with CCA than healthy subjects ($P = 0.005$). Its sensitivity and specificity were 63% and 100%, respectively.⁶⁰

HSP70 is a stress response protein,¹⁰¹ and its overproduction can lead to increased resistance of the cancer cells against apoptosis-inducing agents such as tumor necrosis factor- α .¹⁰² The highest titers of plasma antibodies against HSP70 were found in CCA patients compared with the cholangitis group ($P < 0.05$) and the healthy controls ($P < 0.001$). Sensitivity and specificity were 70.97% and 82.61%, respectively.⁶⁸

Angpt-2 is an antagonistic ligand to the tyrosine kinase Tie2 of the Angpt/Tie2 system.¹⁰³ Because Angpt-2 is strongly expressed in the vasculature of many tumors, it is hypothesized to promote tumor progressions along with other cytokines.¹⁰⁴ Evaluation of serum Angpt-2 as potential biomarkers for diagnosis of CCA provided a satisfactory result. Median Angpt-2 levels were found to be highest in CCA patients compared with PSC and patients with bile duct stones ($P = 0.01$). Sensitivity and specificity were 74% and 94%, respectively.⁷⁷

TABLE 1

Summary of sensitivity and specificity of biomarkers for the diagnosis of cholangiocarcinoma (CCA) based on the research articles included in the study

Biomarkers	Sensitivity (%)	Specificity (%)	ROC	References
Biomarkers in the blood				
miR-21	–	–	0.94	47
miR	84.8	81.8	0.723	92
miR (miR-1281, miR-126, miR-26a, miR-30b, and miR-122)	32–68	88–93	–	84
miR200c	–	–	0.74	91
miR-483-5p			0.77	
miR-194			0.74	
EXT1	91.7	50	0.74	50
Tu M2-PK	84.2	90	0.924	53
CA19-9	68.4	75	0.693	
Serum AFP, CA19.9, CA125, and CEA	90	90	0.94	54
TTR and CA19-9	98.2	100	–	55
CEA, CA19-9, CYFRA, and MMP7	92	95.8	0.64	56
Serum cathepsin B to cystatin C ratio	63	100	≥ 0.06	60
CCA-CA and ALP combined	86.36	91.67	–	66
CEA	53.3	86.3	–	67
CA19-9*	60	91	–	
Plasma autoantibodies against HSP70, ENO1, and RNH1	70.97	82.61	–	68
Serum CA-S27	87.5	58.8	0.822	72
Combined AFP–, CA242+, and CA19-9+	47.2	89.7	–	75
A1BG/AFM ratio	84	87.5	0.886	76
Serum Angpt-2	74	94	0.85	77
Biomarkers in tissues/bile				
miR	67	96	–	107
miR	95	100	0.995	108
miR(miR-412, miR-640, miR-1537, and miR-3189)	50–67	89–92	–	84
CDO1, SFRP1	87	100	0.924	39
ZSCAN18 and DCLK1				
CDO1 and CNRIP1	85	98	0.944	40
SEPT9 and VIM				
STIP1	8/85.3/36	100/44.2/80.2	0.496/0.699/0.611	41
SerpinH1	64/93.3/68	93.9/90.9/94.8	0.819/0.945/0.891	
14-3-3Sigma†	71.1/86.8/71.1	84.8/97.5/93.1	0.75/0.967/0.869	
AGR3	–	–	0.801	43
HAAH and HoxB7	73	80	–	46
Human pancreatic cancer fusion 2 (HPC2) (a mAb)	80	68	–	48
N-cadherin	58	73	–	
p53	47	90	–	49
Ki-67	42	80	–	
CEA	63	73	–	
CA19-9	16	82	–	
CAM5.2	16	100	–	
ICL	42	98	–	
BMP3	58	100	0.83	51
CLDN4, HOXB7, TMSB4, and TTR	90	100	0.98	52
WFA	87.4	92.1	0.93	57
L1CAM	66	93	0.82	58
Bile M2-PK	50	100	0.73	61
IGF2BP3, HOXB7, and NEK2	77.8	87.2	–	62
BGN, IGFBP5, and CLDN4	–	–	0.987	63
Claudin-4	96.6	92.3	0.962	64
Claudin-7 genes	86.2	100	0.981	
αvβ6 integrin	86	100	–	65
Bile HSP27 and HSP70	90	100	0.940	69
S100A9	92.6	75	0.894	70
CCTy	80.8	77.8	0.860	
p38δ	89.1	90.7	–	74
Villin	100	–	–	78
Mammaglobin	–	100	–	
TUBB3 (both cases)‡	50	100	–	79
Biomarkers in both blood and tissues/bile				
CEA	33	85	–	42
CA19-9	63	50	–	
CA19-9	78	67	0.79	44

(continued)

TABLE 1
Continued

Biomarkers	Sensitivity (%)	Specificity (%)	ROC	References
slgG4	100	87.1	0.981	45
CEA	68	81.5	0.76	73
CA19-9	66.7	97.7	0.76	
Biomarkers in urine				
Urine proteomics	83	79	0.87	59

A1BG = α 1 β -glycoprotein; AFM = afamin; AFP = alpha-fetoprotein; AGR3 = anterior gradient3; ALP = alkaline phosphatase; BGN = biglycan; BMP3 = bone morphogenetic protein 3; arbohydate antigen; CAM = anticytokeratin; CCTy = chaperonin-containing TCR1; CDO1 = cysteine dioxygenase type 1; CEA = carcinoembryonic antigen; CLDN4 = claudin 4; CNRIP1 = cannabinoid receptor interacting protein 1; CYFRA = cytokeratin-19 fragment; DCLK1 = doublecortin-like kinase 1; EXT1 = exostosin1; HAAH = human aspartyl beta-hydroxylase; HoxB7 = homeobox B7; HSP27 = heat shock protein27; HSP70 = heat shock protein70; ICL = intracytoplasmic lumina; IGF2BP3 = mRNA of IGF-II mRNA-binding protein; IGFBP5 = insulin-like growth factor-binding protein 5; Ki-67 = antigen Ki-67; L1CAM = L1 cell adhesion molecule; M2-PK = M2 pyruvate kinase; mAb = monoclonal antibody; miR = micro-RNA; MMP7 = matrix metalloproteinase; NEK2 = serine/threonine kinase; p53 = tumor protein; ROC = receiver operating characteristic; S100A9 = S100 calcium-binding protein A9; SEPT9 = septin 9; SFRP1 = secreted frizzled-related protein 1; slgG4 = serum immunoglobulin G4; STIP1 = stress-induced phosphoprotein1; TMSB4 = thymosin beta 4; TTR = transthyretin; Tu M2-PK = tumor type M2 pyruvate kinase; TUBB3 = tubulin beta-3; VIM = vimentin; WFA = *Wisteria floribunda* agglutinin; ZSCAN18 = zinc finger and SCAN domain containing 18.

* Sensitivity and specificity for differentiation of confirmed CCA vs. primary sclerosing cholangitis (both CEA and CA19-9).

† The sensitivity, specificity, and ROC values are for ICC vs. bile duct adenoma, ICC vs. ductular reaction, and ICC vs. nonmalignant bile duct lesions, respectively.

‡ For both peripheral CCA vs. hepatocellular carcinoma and peripheral CCA vs. peribiliary gland hamartomas.

Biomarkers in the tissues/bile. The potential biomarkers in the body tissues and in the bile reported from various studies included gene methylations and DNA-related markers, miRNAs, human aspartyl beta-hydroxylase (HAAH) and homeobox B7 (HoxB7) mRNA, CA19-9 and CEA, and p38 δ mitogen-activated protein kinase (MAPK). Alterations at the molecular level, either genetic or epigenetic aberrations, can play important roles in cancer development and progression.^{105,106} As such, loss of expression of essential tumor suppressor genes could lead to tumor development. Because these abnormalities are seen in the early stages of tumorigenesis, they could be used as markers for early detection of the tumors.¹⁰⁷⁻¹⁰⁹ In a study applying an epigenome-wide approach to identify suitable DNA methylation-based biomarkers, the proportions of samples with expression of CDO1, SFRP1, ZSCAN18, and DCLK1 genes were higher in primary tumors (77%, 59%, 54%, and 44%, respectively) compared with the nonmalignant controls (0%, 19%, 0%, and 38%, respectively).³⁹ In a study using biliary brush specimens, for four of 13 candidate genes, that is, CDO1, CNRIP1, SEPT9, and VIM 3, the proportions of samples with protein methylations were significantly higher (47%, 67%, 67%, and 33%, respectively) in tumor samples compared with non-malignant controls (none of the genes were methylated).⁴⁰ Yet, in another study assessing methylation of the bone morphogenetic protein 3, the methylation of this protein was found to be higher in CCA tissues compared with nonmalignant samples ($P < 0.003$).⁵¹ The overall sensitivity and specificity ranged from 58% to 87% and 98% to 100%, respectively.

Other than in the blood, miRNA has also been detected in the tissues and bile. A study quantified the miRNA present in the extracellular vesicle of bile to develop a diagnostic panel for CCA. Its results showed that 54 miRNA species had amplifications beyond the specified cutoff value. Applying different mathematical approaches to assess predictive values provided reliable results. The sensitivity and specificity were 67% and 96%, respectively.⁸² In another study that assessed miRNA dysregulation in CCA patients, the CCA patient was shown to have 7.7-fold higher expression of miRNA compared with normal tissues. With regard to diagnosis performance, miR-21 accurately diagnosed 22 of 23 cancers and 17 of 17 normal tissues. Its sensitivity and specificity were 95% and 100%, respectively.⁸³

Human aspartyl beta-hydroxylase (AspH) is an enzyme in humans encoded by the AspH gene.¹¹⁰ An elevated level of HAAH has been correlated with several types of human

carcinomas, and as such it has been investigated for use as biomarkers for those carcinomas.^{111,112} Homeobox constitutes a large family of genes that usually regulates the formation of cell and organ structures during embryonic development.¹¹³ However, there are some homeobox genes that function as tumor suppressors.¹¹⁴ Therefore, changes in the activities of such genes have been associated with cancer formations. In a study to establish and validate a semiquantitative reverse transcriptase-polymerase chain reaction assay for detection of HAAH and HoxB7 mRNA, the detection limits of these proteins were 10^{-8} ng/mL and 10^{-9} ng/mL, respectively. The intra-assay variability was between 0.6% and 10.3% and the inter-assay variability was between 3.3% and 11.8%. The sensitivity and specificity were 73% and 80%, respectively.⁴⁶

Besides being the most commonly investigated biomarkers in the serum, CA and CEA were also found in several tissues. The diagnostic utility of markers such as CA19-9 and CEA along with other markers such as tumor protein p53 (p53), antigen Ki-67 (Ki-67), anticytokeratin CAM5.2 (CAM5.2), and intracytoplasmic lumina (ICL) improved the sensitivity of histologic evaluations without compromising the specificity. Histologic evaluation alone provided a sensitivity and specificity of 53% and 100%, respectively. Combining the histologic evaluation with the markers significantly increased the sensitivity. The highest improvement in diagnostic sensitivity was found in the combination of histologic evaluation with CEA (from 53% to 83%), and the lowest improvement was in the combination with CA19-9 (from 53% to 60%). The other markers (p53, Ki-67, CAM5.2, and ICL) improved the sensitivity to 75%, 73%, 60%, and 73%, respectively.⁴⁹

Mitogen-activated protein kinase 13 (MAPK13) or stress-activated protein kinase 4 (SAPK4) is a member of the p38 subfamily of the MAP kinases. It is an enzyme that, in humans, is encoded by the MAPK13 gene.¹¹⁵ p38 δ MAPK is believed to be activated by an inflammatory cytokine that is associated with biliary tract inflammation named interleukin-6.¹¹⁶ Thus, p38 δ MAPK can be detected in tumors of the biliary tract.¹¹⁷ In a study aiming to identify p38 δ MAPK as a novel marker for distinguishing CCA from hepatocellular carcinoma (HCC), p38 δ MAPK positive staining was detected in 92.6% of CCA cases but with only 9.3% of HCC cases ($P < 0.00001$). The sensitivity and specificity were 89.1% and 90.7%, respectively.⁷⁴

Biomarkers in both blood and tissue/bile. There are relatively limited articles related to the investigation of the biomarkers

that are present in both the blood (plasma/serum) and the tissue/bile samples. The markers studied included CA, CEA, and sIgG4.

Carbohydrate antigen 19-9 and CEA are two of the most commonly studied markers. In a study to evaluate the diagnostic accuracy of serum and bile levels of CA19-9 and CEA in differentiating PSC patients with or without CCA, serum CA19-9 levels in 33.3% of PSC patients with CCA were found to be greater than the upper reference value (200 ng/mL). In PSC patients without CCA, only 15.6% patients had values greater than the same reference value. The CEA level on the other hand, was similar in both the groups. In the bile samples, however, the individual distribution values of both CA19-9 and CEA were heterogeneous and as such no clear estimations could be made. It was concluded that there was no relationship between serum and bile levels of CA19-9 and CEA. The sensitivity and specificity of CA19-9 were 33% and 81%, respectively, at the cutoff value of 200 ng/mL.⁴² A retrospective study evaluated the diagnostic utility of serum CA19-9 when used in combination with other cytological techniques (ultrasonography, computed tomography, or magnetic resonance imaging) in detecting CCA in patients with PSC. Individually, CA19-9 was found to exhibit low accuracy in detecting the cancer (78% sensitivity and 67% specificity) at a cutoff value of 20 U/mL. The sensitivity significantly improved when CA19-9 was combined with the cytologic techniques. Carbohydrate antigen 19-9 combined with either ultrasonography, computed tomography, or magnetic resonance imaging increased the sensitivity to 91%, 100%, and 96%, respectively however, the specificity decreased to 62%, 38%, and 37%, respectively.⁴⁴ In another retrospective study, the efficiencies of brush cytology, CA19-9, and CEA for the diagnosis of CCA were evaluated. The brush cytology was shown to accurately diagnose 46.4% of CCA.⁶¹ This was further supported by significantly higher mean serum levels of CA19-9 and CEA in the CCA patients compared with non-CCA patients (68.4 versus 3.5 ng/mL and 5,994.1 versus 66.7 U/mL respectively; $P < 0.01$). Combination of brush cytology and serum CA19-9 provided the most accurate diagnosis (95.6%), whereas the most sensitive strategy for detecting CCA was the combination of CA19-9 with CEA (100%). The overall sensitivity and specificity were 66% and 97.7%, respectively.⁷³

Serum IgG4 elevation is characteristic of immunoglobulin G4-associated cholangitis (IAC). Immunoglobulin G4-associated cholangitis has many symptoms similar to CCA.¹¹⁸ Therefore, use of biomarkers to differentiate the two diseases could be of clinical benefit. Identification of the key factors that would assist in differential diagnosis of CCA from IAC was reported in a retrospective study using serum IgG4 (sIgG4), CA19-9, CA242, and CEA as markers. The average level of sIgG4 was as high as 16,028 mg/L in about 16.1% of the CCA patients, whereas the level was 896 mg/L in 100% of the IAC patients. The sIgG4 provided a sensitivity and specificity of 100% and 87.1%, respectively.⁴⁵

Biomarkers in urine. Availability of biomarkers in the urine samples would be of great advantage in the clinical settings as it will reduce the use of invasive methods that need to be applied to obtain other biomarkers. Urine contains proteins and peptides that originate as the ultrafiltrate of plasma. The urinary proteomes are highly sensitive toward changes that are of renal origin and also other nonrenal disorders. Urine proteomic analysis (UPA) of a urine peptide marker model was

shown to be able to differentiate CCA from other biliary diseases with an area under curve of 0.87 (95% CI: 0.80–0.92); $P = 0.0001$. The sensitivity and specificity were 83% and 79%, respectively.⁵⁹

DISCUSSION

Summary of evidence. Considering its malignancy and silent clinical characteristics, detecting CCA at the early stages is of particular clinical interest. Early detection will not only benefit the patients in receiving broader treatment methods, but will finally lessen the financial burden on the public health system that occurs with more intensive treatments at metastatic stages. The availability of biomarkers specific to the early stages of CCA appears to be one of the most promising approaches for early diagnosis.

Various potential biomarkers in serum as well as tissue and other body fluid samples have been reported for the diagnosis and disease differentiation for CCA. Because of the use of different equipment at different places and also personnel variations, results from different studies are conflicting even with the same biomarkers. Because there is such a large variation in the diagnostic accuracy of different biomarkers obtained by different studies, systematic analysis of the relevant studies to point out the most accurate biomarkers that can be used in routine clinical laboratories is important. This study reviewed all relevant articles that were related to the biomarkers for diagnosis of CCA. Several potential biomarkers that were useful in the diagnosis of CCA were reported. The biological fluids under analysis of the biomarkers included blood (plasma/serum), tissue from the bile duct, bile, and urine samples (Supplemental Table 1). Carbohydrate antigen 19-9 and CEA were the most commonly studied serum biomarkers. These two biomarkers, either alone or in combination provided satisfactory results with regard to specificity (89.7–100%), although with questionable sensitivity (47.2–98.2%). These results were in agreement with those reported in a previous review.¹¹⁹ The main tissue biomarkers studied were gene methylations and DNA related. Both were found to provide favorable specificity (98–100%) but inconsistent sensitivity (58–87%) in diagnosing CCA. The efficiency of combination of the markers that are found in the serum and in the tissue samples has also been reported. Although the combined biomarkers improved the accuracy of diagnosing CCA, none was considered adequate for clinical application.

Because all of the previously mentioned sample collection procedures for the biomarker analysis are invasive methods, other samples including urine have been investigated. This UPA method provided a number of advantages over other methods; it is noninvasive, urine can be obtained in large quantities, and it is more stable and is sensitive to the renal-related changes as well as other non-renal disorders. However, the accuracy of the UPA is questionable with sensitivity and specificity of 83% and 79%, respectively. It is noted however that, despite the availability of several candidate biomarkers for CCA, yet at present, no single biomarker can be used for clinical settings. One limitation of the current review is that articles in languages other than English were not included in the analysis. This might have left out some important articles that contain information that could lead to alterations in the conclusions. The serum CA19-9 has been

proposed as the gold standard biomarker for the diagnosis of CCA.¹²⁰ However, its wide variation of serum levels and diagnostic sensitivity limit its clinical application. In addition, CA19-9 cannot be used in patients who are negative for the Lewis antigen.¹²¹ Keeping in mind that histopathological examination which is the current universally accepted gold standard method for diagnosis of CCA is an invasive method involving a lot of risks to the patient, application of biomarkers in routine laboratory tests would provide more of benefit than harm.

Although no single biomarker can be used to ensure accurate diagnosis of CCA, combining these potential biomarkers with other laboratory tests would be expected to greatly improve the accuracy of diagnosis. This is useful, in particular as a screening tool for those people who are at high risk of *O. viverrini* infections in endemic areas.

CONCLUSION

Cholangiocarcinoma is becoming a burdensome public health issue in many countries, especially in the Southeast Asian countries. Current management strategies provide inconsistent results mainly because of the silent features of the disorder. Most of the cases are diagnosed only at the metastatic stages that are late for any invasive treatments. Early diagnosis might be possible if biomarkers that are specific to the early stages are identified and standardized. After reviewing the articles that investigated the biomarkers for diagnosis of CCA, we found out that there are many biomarkers which could diagnose and differentiate CCA against other similar disorders and healthy controls with many of them showing significant results. However, there is no single marker that can be used alone for diagnosis of CCA with satisfactory accuracy. Nevertheless, certain markers could be used as a screening tool for those people in risky areas.

Received November 12, 2017. Accepted for publication February 26, 2018.

Published online April 9, 2018.

Note: Supplemental tables appear at www.ajtmh.org.

Acknowledgments: The study was supported by Chulabhorn International College of Medicine (CICM) of Thammasat University, Center of Excellence in Pharmacology and Molecular Biology of Malaria and Cholangiocarcinoma of Thammasat University, National Research Council of Thailand (NRCT) and National Research University Project of Thailand (NRU), Office of Higher Education Commission of Thailand.

Authors' addresses: Gyem Tshering, Palden Wangyel Dorji, and Wanna Chaijaroenkul, Chulabhorn International College of Medicine, Thammasat University, Rangsit Center, Pathum Thani, Thailand, E-mails: wangten_gyem@hotmail.com, paldenwangyal@yahoo.com, and wn_ap39@yahoo.com. Kesara Na-Bangchang, Center of Excellence in Pharmacology and Molecular Biology of Malaria and Cholangiocarcinoma, Chulabhorn International College of Medicine, Thammasat University, Rangsit Center, Pathum Thani, Thailand, E-mail: kesaratmu@yahoo.com.

REFERENCES

1. Elkins DB, Mairiang E, Sithithaworn P, Mairiang P, Chaiyakum J, Chamadol N, Loapaiboon V, Haswell-Elkins MR, 1996. Cross-sectional patterns of hepatobiliary abnormalities and possible precursor conditions of cholangiocarcinoma associated with *Opisthorchis viverrini* infection in humans. *Am J Trop Med Hyg* 55: 295–301.

2. Pinlaor S, Yongvanit P, Hiraku Y, Ma N, Semba R, Oikawa S, Murata M, Sripa B, Sithithaworn P, Kawanishi S, 2003. 8-nitroguanine formation in the liver of hamsters infected with *Opisthorchis viverrini*. *Biochem Biophys Res Commun* 309: 567–571.
3. Thamavit W, Bhamarapavati N, Sahaphong S, Vajrasthira S, Angsubhakorn S, 1978. Effects of dimethylnitrosamine on induction of cholangiocarcinoma in *Opisthorchis viverrini*-infected Syrian golden hamsters. *Cancer Res* 38: 4634–4639.
4. Vatanasapt V, Sriamporn S, Vatanasapt P, 2002. Cancer control in Thailand. *Jpn J Clin Oncol* 32 (Suppl): S82–S91.
5. Kluhaphrema TSP, Attasara P, Sriplung H, Wiangnon S, Sumitsawan Y, 2010. *Cancer in Thailand*. Bangkok, Thailand: National Cancer Institute.
6. Chainuvati T, Paosawadhi A, Sripranoth M, Manasatith S, Viranuvatti V, 1976. Carcinoma of the cystic duct associated with opisthorchiasis. *Southeast Asian J Trop Med Public Health* 7: 482–486.
7. Haswell-Elkins MR, Sithithaworn P, Elkins D, 1992. *Opisthorchis viverrini* and cholangiocarcinoma in northeast Thailand. *Parasitol Today* 8: 86–89.
8. Prayong P, Mairiang E, Pairojkul C, Chamgramol Y, Mairiang P, Bhudisawasdi V, Sripa B, 2014. An interleukin-6 receptor polymorphism is associated with opisthorchiasis-linked cholangiocarcinoma risk in Thailand. *Asian Pac J Cancer Prev* 15: 5443–5447.
9. Yothaisong S, Thanee M, Namwat N, Yongvanit P, Boonmars T, Puapairoj A, Loilome W, 2014. *Opisthorchis viverrini* infection activates the PI3K/AKT/PTEN and Wnt/β-catenin signaling pathways in a cholangiocarcinogenesis model. *Asian Pac J Cancer Prev* 15: 10463–10468.
10. Montomoli J, Erichsen R, Norgaard M, Hoyer M, Hansen JB, Jacobsen JB, 2011. Survival of patients with primary liver cancer in central and northern Denmark, 1998–2009. *Clin Epidemiol* 3 (Suppl 1): 3–10.
11. Patel T, 2001. Increasing incidence and mortality of primary intrahepatic cholangiocarcinoma in the United States. *Hepatology* 33: 1353–1357.
12. Khan SA, Emadossady S, Ladep NG, Thomas HC, Elliott P, Taylor-Robinson SD, Toledano MB, 2012. Rising trends in cholangiocarcinoma: is the ICD classification system misleading us? *J Hepatol* 56: 848–854.
13. Anderson CD, Pinson CW, Berlin J, Chari RS, 2004. Diagnosis and treatment of cholangiocarcinoma. *Oncologist* 9: 43–57.
14. Meyer CG, Penn I, James L, 2000. Liver transplantation for cholangiocarcinoma: results in 207 patients. *Transplantation* 69: 1633–1637.
15. Sudan D, DeRoover A, Chinnakotla S, Fox I, Shaw B Jr., McCashland T, Sorrell M, Tempero M, Langnas A, 2002. Radiochemotherapy and transplantation allow long-term survival for nonresectable hilar cholangiocarcinoma. *Am J Transplant* 2: 774–779.
16. Takada T, Amano H, Yasuda H, Nimura Y, Matsushiro T, Kato H, Nagakawa T, Nakayama T, 2002. Is postoperative adjuvant chemotherapy useful for gallbladder carcinoma? A phase III multicenter prospective randomized controlled trial in patients with resected pancreaticobiliary carcinoma. *Cancer* 95: 1685–1695.
17. Valle J et al., 2010. Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer. *N Engl J Med* 362: 1273–1281.
18. Bengala C et al., 2010. Sorafenib in patients with advanced biliary tract carcinoma: a phase II trial. *Br J Cancer* 102: 68–72.
19. Todoroki T, Ohara K, Kawamoto T, Koike N, Yoshida S, Kashiwagi H, Otsuka M, Fukao K, 2000. Benefits of adjuvant radiotherapy after radical resection of locally advanced main hepatic duct carcinoma. *Int J Radiat Oncol Biol Phys* 46: 581–587.
20. Todoroki T, Kawamoto T, Otsuka M, Koike N, Yoshida S, Takada Y, Adachi S, Kashiwagi H, Fukao K, Ohara K, 1999. Benefits of combining radiotherapy with aggressive resection for stage IV gallbladder cancer. *Hepatogastroenterology* 46: 1585–1591.
21. Nakagohri T, Kinoshita T, Konishi M, Takahashi S, Gotohda N, 2008. Surgical outcome and prognostic factors in intrahepatic cholangiocarcinoma. *World J Surg* 32: 2675–2680.
22. Loilome W, Wechagama P, Namwat N, Jusakul A, Sripa B, Miwa M, Kuver R, Yongvanit P, 2012. Expression of oxysterol binding protein isoforms in opisthorchiasis-associated cholangiocarcinoma:

- a potential molecular marker for tumor metastasis. *Parasitol Int* 61: 136–139.
23. Adisakwattana P, Suwandittakul N, Petmitr S, Wongkham S, Sangvanich P, Reamtong O, 2015. ALCAM is a novel cytoplasmic membrane protein in TNF- α stimulated invasive cholangiocarcinoma cells. *Asian Pac J Cancer Prev* 16: 3849–3856.
 24. Subrungruanga I, Thawornkunob C, Chawalitchewinkoon-Petmitr P, Pairojkul C, Wongkham S, Petmitr S, 2013. Gene expression profiling of intrahepatic cholangiocarcinoma. *Asian Pac J Cancer Prev* 14: 557–563.
 25. Faca VM, Hanash SM, 2009. In-depth proteomics to define the cell surface and secretome of ovarian cancer cells and processes of protein shedding. *Cancer Res* 69: 728–730.
 26. Briggs CD, Neal CP, Mann CD, Steward WP, Manson MM, Berry DP, 2009. Prognostic molecular markers in cholangiocarcinoma: a systematic review. *Eur J Cancer* 45: 33–47.
 27. Mon NN, Kokuryo T, Hamaguchi M, 2009. Inflammation and tumor progression: a lesson from TNF-alpha-dependent FAK signaling in cholangiocarcinoma. *Methods Mol Biol* 512: 279–293.
 28. Pinlaor S, Prakobwong S, Hiraku Y, Pinlaor P, Laothong U, Yongvanit P, 2010. Reduction of periductal fibrosis in liver fluke-infected hamsters after long-term curcumin treatment. *Eur J Pharmacol* 638: 134–141.
 29. Tanimura Y et al., 2005. Tumor necrosis factor alpha promotes invasiveness of cholangiocarcinoma cells via its receptor, TNFR2. *Cancer Lett* 219: 205–213.
 30. Levy C, Lymp J, Angulo P, Gores GJ, Larusso N, Lindor KD, 2005. The value of serum CA 19-9 in predicting cholangiocarcinomas in patients with primary sclerosing cholangitis. *Dig Dis Sci* 50: 1734–1740.
 31. Pungpak S, Akai PS, Longenecker BM, Ho M, Befus AD, Bunnag D, 1991. Tumor markers in the detection of opisthorchiasis-associated cholangiocarcinoma. *Trans R Soc Trop Med Hyg* 85: 277–279.
 32. Patel AH, Harnois DM, Klee GG, LaRusso NF, Gores GJ, 2000. The utility of CA 19-9 in the diagnoses of cholangiocarcinoma in patients without primary sclerosing cholangitis. *Am J Gastroenterol* 95: 204–207.
 33. Qin XL, Wang ZR, Shi JS, Lu M, Wang L, He QR, 2004. Utility of serum CA19-9 in diagnosis of cholangiocarcinoma: in comparison with CEA. *World J Gastroenterol* 10: 427–432.
 34. Loilome W, Yongvanit P, Wongkham C, Tepsiri N, Sripa B, Sithithaworn P, Hanai S, Miwa M, 2006. Altered gene expression in *Opisthorchis viverrini*-associated cholangiocarcinoma in hamster model. *Mol Carcinog* 45: 279–287.
 35. Srisomsap C, Sawangareetrakul P, Subhasitanont P, Panichakul T, Keeratichamroen S, Lirdprapamongkol K, Chokchaichamnankit D, Sirisinha S, Svasti J, 2004. Proteomic analysis of cholangiocarcinoma cell line. *Proteomics* 4: 1135–1144.
 36. Srisomsap C, Sawangareetrakul P, Subhasitanont P, Chokchaichamnankit D, Chiablaem K, Bhudhisawasdi V, Wongkham S, Svasti J, 2010. Proteomic studies of cholangiocarcinoma and hepatocellular carcinoma cell secretomes. *J Biomed Biotechnol* 2010: 437143.
 37. Tit-Oon P, Chokchaichamnankit D, Khongmanee A, Sawangareetrakul P, Svasti J, Srisomsap C, 2014. Comparative secretome analysis of cholangiocarcinoma cell line in three-dimensional culture. *Int J Oncol* 45: 2108–2116.
 38. Desmetz C, Mange A, Maudelonde T, Solassol J, 2011. Auto-antibody signatures: progress and perspectives for early cancer detection. *J Cell Mol Med* 15: 2013–2024.
 39. Andresen K et al., 2012. Novel target genes and a valid biomarker panel identified for cholangiocarcinoma. *Epigenetics* 7: 1249–1257.
 40. Andresen K et al., 2015. Four DNA methylation biomarkers in biliary brush samples accurately identify the presence of cholangiocarcinoma. *Hepatology* 61: 1651–1659.
 41. Bertram S et al., 2016. Novel immunohistochemical markers differentiate intrahepatic cholangiocarcinoma from benign bile duct lesions. *J Clin Pathol* 69: 619–626.
 42. Bjornsson E, Kilander A, Olsson R, 1999. CA 19-9 and CEA are unreliable markers for cholangiocarcinoma in patients with primary sclerosing cholangitis. *Liver* 19: 501–508.
 43. Brychtova V, Zampachova V, Hrstka R, Fabian P, Novak J, Hermanova M, Vojtesek B, 2014. Differential expression of anterior gradient protein 3 in intrahepatic cholangiocarcinoma and hepatocellular carcinoma. *Exp Mol Pathol* 96: 375–381.
 44. Charatcharoenwithaya P, Enders FB, Halling KC, Lindor KD, 2008. Utility of serum tumor markers, imaging, and biliary cytology for detecting cholangiocarcinoma in primary sclerosing cholangitis. *Hepatology* 48: 1106–1117.
 45. Du S et al., 2016. Differential diagnosis of immunoglobulin G4-associated cholangitis from cholangiocarcinoma. *J Clin Gastroenterol* 50: 501–505.
 46. Feldmann G et al., 2006. Detection of human aspartyl (asparaginyl) beta-hydroxylase and homeobox B7 mRNA in brush cytology specimens from patients with bile duct cancer. *Endoscopy* 38: 604–609.
 47. Correa-Gallego C et al., 2016. Circulating plasma levels of microRNA-21 and microRNA-221 are potential diagnostic markers for primary intrahepatic cholangiocarcinoma. *PLoS One* 11: e0163699.
 48. Hooper JE, Morgan TK, Grompe M, Sheppard BC, Troxell ML, Corless CL, Streeter PR, 2012. The novel monoclonal antibody HPC2 and N-cadherin distinguish pancreatic ductal adenocarcinoma from cholangiocarcinoma. *Hum Pathol* 43: 1583–1589.
 49. Jahng AW, Chung D, Pham B, Reicher S, Yee B, Abramyan L, Venegas R, French S, Eysselein VE, 2009. Staining for intracytoplasmic lumina and CAM5.2 increases the detection rate for bile duct cancers. *Endoscopy* 41: 965–970.
 50. Khoontawad J et al., 2014. Increase of exostosin 1 in plasma as a potential biomarker for opisthorchiasis-associated cholangiocarcinoma. *Tumour Biol* 35: 1029–1039.
 51. Kisiel JB et al., 2013. Methylated bone morphogenetic protein 3 (BMP3) gene: evaluation of tumor suppressor function and biomarker potential in biliary cancer. *J Mol Biomark Diagn* 4: 1000145.
 52. Kraiklang R, Pairojkul C, Khuntikeo N, Imtawil K, Wongkham S, Wongkham C, 2014. A novel predictive equation for potential diagnosis of cholangiocarcinoma. *PLoS One* 9: e89337.
 53. Li YG, Zhang N, 2009. Clinical significance of serum tumor M2-PK and CA19-9 detection in the diagnosis of cholangiocarcinoma. *Dig Liver Dis* 41: 605–608.
 54. Li Y, Li DJ, Chen J, Liu W, Li JW, Jiang P, Zhao X, Guo F, Li XW, Wang SG, 2015. Application of joint detection of AFP, CA19-9, CA125 and CEA in identification and diagnosis of cholangiocarcinoma. *Asian Pac J Cancer Prev* 16: 3451–3455.
 55. Liu L, Wang J, Liu B, Dai S, Wang X, Chen J, Huang L, Xiao X, He D, 2008. Serum levels of transthyretin down-regulation in cholangiocarcinoma. *J Cell Biochem* 104: 745–755.
 56. Lumachi F, Lo Re G, Tozzoli R, D'Aurizio F, Facomer F, Chiara GB, Basso SM, 2014. Measurement of serum carcinoembryonic antigen, carbohydrate antigen 19-9, cytokeratin-19 fragment and matrix metalloproteinase-7 for detecting cholangiocarcinoma: a preliminary case-control study. *Anticancer Res* 34: 6663–6667.
 57. Matsuda A et al., 2010. *Wisteria floribunda* agglutinin-positive mucin 1 is a sensitive biliary marker for human cholangiocarcinoma. *Hepatology* 52: 174–182.
 58. Matsuda A et al., 2013. Glycoproteomics-based cancer marker discovery adopting dual enrichment with *Wisteria floribunda* agglutinin for high specific glyco-diagnosis of cholangiocarcinoma. *J Proteomics* 85: 1–11.
 59. Metzger J et al., 2013. Urine proteomic analysis differentiates cholangiocarcinoma from primary sclerosing cholangitis and other benign biliary disorders. *Gut* 62: 122–130.
 60. Monsouvanh A, Proungvitaya T, Limpaboon T, Wongkham C, Wongkham S, Luvira V, Proungvitaya S, 2014. Serum cathepsin B to cystatin C ratio as a potential marker for the diagnosis of cholangiocarcinoma. *Asian Pac J Cancer Prev* 15: 9511–9515.
 61. Navaneethan U, Lourdasamy V, Poptic E, Hammel JP, Sanaka MR, Parsi MA, 2015. Comparative effectiveness of pyruvate kinase M2 in bile, serum carbohydrate antigen 19-9, and biliary brushings in diagnosing malignant biliary strictures. *Dig Dis Sci* 60: 903–909.
 62. Nischalke HD, Schmitz V, Luda C, Aldenhoff K, Berger C, Feldmann G, Sauerbruch T, Spengler U, Nattermann J, 2012. Detection of IGF2BP3, HOXB7, and NEK2 mRNA expression in brush cytology specimens as a new diagnostic tool in patients with biliary strictures. *PLoS One* 7: e42141.

63. Nishino R et al., 2008. Identification of novel candidate tumor marker genes for intrahepatic cholangiocarcinoma. *J Hepatol* 49: 207–216.
64. Ono Y, Hiratsuka Y, Murata M, Takasawa A, Fukuda R, Nojima M, Tanaka S, Osanai M, Hirata K, Sawada N, 2016. Claudins-4 and -7 might be valuable markers to distinguish hepatocellular carcinoma from cholangiocarcinoma. *Virchows Arch* 469: 417–426.
65. Patsenker E, Wilkens L, Banz V, Osterreicher CH, Weimann R, Eisele S, Keogh A, Stroka D, Zimmermann A, Stickel F, 2010. The alphavbeta6 integrin is a highly specific immunohistochemical marker for cholangiocarcinoma. *J Hepatol* 52: 362–369.
66. Pattanapairoj S, Silsirivanit A, Muisuk K, Seubwai W, Cha'on U, Vaeteewoottacharn K, Sawanyawisuth K, Chetchotsak D, Wongkham S, 2015. Improve discrimination power of serum markers for diagnosis of cholangiocarcinoma using data mining-based approach. *Clin Biochem* 48: 668–673.
67. Ramage JK, Donaghy A, Farrant JM, Iorns R, Williams R, 1995. Serum tumor markers for the diagnosis of cholangiocarcinoma in primary sclerosing cholangitis. *Gastroenterology* 108: 865–869.
68. Rucksaken R, Pairojkul C, Pinlaor P, Khuntikeo N, Roytrakul S, Selmi C, Pinlaor S, 2014. Plasma autoantibodies against heat shock protein 70, enolase 1 and ribonuclease/angiogenin inhibitor 1 as potential biomarkers for cholangiocarcinoma. *PLoS One* 9: e103259.
69. Sato Y, Harada K, Sasaki M, Yasaka T, Nakanuma Y, 2012. Heat shock proteins 27 and 70 are potential biliary markers for the detection of cholangiocarcinoma. *Am J Pathol* 180: 123–130.
70. Shi Y, Deng X, Zhan Q, Shen B, Jin X, Zhu Z, Chen H, Li H, Peng C, 2013. A prospective proteomic-based study for identifying potential biomarkers for the diagnosis of cholangiocarcinoma. *J Gastrointest Surg* 17: 1584–1591.
71. Shin SH, Lee K, Kim BH, Cho NY, Jang JY, Kim YT, Kim D, Jang JJ, Kang GH, 2012. Bile-based detection of extrahepatic cholangiocarcinoma with quantitative DNA methylation markers and its high sensitivity. *J Mol Diagn* 14: 256–263.
72. Silsirivanit A et al., 2013. CA-S27: a novel Lewis a associated carbohydrate epitope is diagnostic and prognostic for cholangiocarcinoma. *Cancer Sci* 104: 1278–1284.
73. Siqueira E, Schoen RE, Silverman W, Martin J, Rabinovitz M, Weissfeld JL, Abu-Elmagd K, Madariaga JR, Slivka A, 2002. Detecting cholangiocarcinoma in patients with primary sclerosing cholangitis. *Gastrointest Endosc* 56: 40–47.
74. Tan FL et al., 2010. p38delta/MAPK13 as a diagnostic marker for cholangiocarcinoma and its involvement in cell motility and invasion. *Int J Cancer* 126: 2353–2361.
75. Tao LY, Cai L, He XD, Liu W, Qu Q, 2010. Comparison of serum tumor markers for intrahepatic cholangiocarcinoma and hepatocellular carcinoma. *Am Surg* 76: 1210–1213.
76. Tolek A, Wongkham C, Proungvitaya S, Silsirivanit A, Roytrakul S, Khuntikeo N, Wongkham S, 2012. Serum alpha1beta-glycoprotein and afamin ratio as potential diagnostic and prognostic markers in cholangiocarcinoma. *Exp Biol Med (Maywood)* 237: 1142–1149.
77. Voigtlander T, David S, Thamm K, Schlue J, Metzger J, Manns MP, Lankisch TO, 2014. Angiopoietin-2 and biliary diseases: elevated serum, but not bile levels are associated with cholangiocarcinoma. *PLoS One* 9: e97046.
78. Yang Z, 2015. The utility of villin and mammaglobin in the differential diagnosis between intrahepatic cholangiocarcinoma and breast cancer. *Appl Immunohistochem Mol Morphol* 23: 19–25.
79. Zen Y, Britton D, Mitra V, Pike I, Sarker D, Itoh T, Heaton N, Quaglia A, 2014. Tubulin β -III: a novel immunohistochemical marker for intrahepatic peripheral cholangiocarcinoma. *Histopathology* 65: 784–792.
80. Bernuzzi F et al., 2016. Serum microRNAs as novel biomarkers for primary sclerosing cholangitis and cholangiocarcinoma. *Clin Exp Immunol* 185: 61–71.
81. Wang LJ, Zhang KL, Zhang N, Ma XW, Yan SW, Cao DH, Shi SJ, 2015. Serum miR-26a as a diagnostic and prognostic biomarker in cholangiocarcinoma. *Oncotarget* 6: 18631–18640.
82. Li L et al., 2014. Human bile contains microRNA-laden extracellular vesicles that can be used for cholangiocarcinoma diagnosis. *Hepatology* 60: 896–907.
83. Selaru FM et al., 2009. MicroRNA-21 is overexpressed in human cholangiocarcinoma and regulates programmed cell death 4 and tissue inhibitor of metalloproteinase 3. *Hepatology* 49: 1595–1601.
84. Voigtlander T, Gupta SK, Thum S, Fendrich J, Manns MP, Lankisch TO, Thum T, 2015. MicroRNAs in serum and bile of patients with primary sclerosing cholangitis and/or cholangiocarcinoma. *PLoS One* 10: e0139305.
85. Magnani JL, Steplewski Z, Koprowski H, Ginsburg V, 1983. Identification of the gastrointestinal and pancreatic cancer-associated antigen detected by monoclonal antibody 19-9 in the sera of patients as a mucin. *Cancer Res* 43: 5489–5492.
86. Malesci A, Tommasini MA, Bonato C, Bocchia P, Bersani M, Zerbi A, Beretta E, Di Carlo V, 1987. Determination of CA 19-9 antigen in serum and pancreatic juice for differential diagnosis of pancreatic adenocarcinoma from chronic pancreatitis. *Gastroenterology* 92: 60–67.
87. Flamini E et al., 2006. Free DNA and carcinoembryonic antigen serum levels: an important combination for diagnosis of colorectal cancer. *Clin Cancer Res* 12: 6985–6988.
88. Staab HJ, Anderer FA, Brummendorf T, Hornung A, Fischer R, 1982. Prognostic value of preoperative serum CEA level compared to clinical staging: II. Stomach cancer. *Br J Cancer* 45: 718–727.
89. Cummins JM, Velculescu VE, 2006. Implications of micro-RNA profiling for cancer diagnosis. *Oncogene* 25: 6220–6227.
90. Chen L et al., 2009. The role of microRNA expression pattern in human intrahepatic cholangiocarcinoma. *J Hepatol* 50: 358–369.
91. Meng F, Henson R, Lang M, Wehbe H, Maheshwari S, Mendell JT, Jiang J, Schmittgen TD, Patel T, 2006. Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. *Gastroenterology* 130: 2113–2129.
92. Wang C et al., 2015. A panel of five serum miRNAs as a potential diagnostic tool for early-stage renal cell carcinoma. *Sci Rep* 5: 7610.
93. Silakit R, Loilome W, Yongvanit P, Chusorn P, Techasen A, Boonmars T, Khuntikeo N, Chamadol N, Pairojkul C, Namwat N, 2014. Circulating miR-192 in liver fluke-associated cholangiocarcinoma patients: a prospective prognostic indicator. *J Hepatobiliary Pancreat Sci* 21: 864–872.
94. Wang LG, Gu J, 2012. Serum microRNA-29a is a promising novel marker for early detection of colorectal liver metastasis. *Cancer Epidemiol* 36: e61–e67.
95. Ng EK, Chong WW, Jin H, Lam EK, Shin VY, Yu J, Poon TC, Ng SS, Sung JJ, 2009. Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. *Gut* 58: 1375–1381.
96. Busse-Wicher M, Wicher KB, Kusche-Gullberg M, 2014. The exostosin family: proteins with many functions. *Matrix Biol* 35: 25–33.
97. Mach L, Stuwe K, Hagen A, Ballaun C, Glossl J, 1992. Proteolytic processing and glycosylation of cathepsin B. The role of the primary structure of the latent precursor and of the carbohydrate moiety for cell-type-specific molecular forms of the enzyme. *Biochem J* 282: 577–582.
98. Sloane BF, 1990. Cathepsin B and cystatins: evidence for a role in cancer progression. *Semin Cancer Biol* 1: 137–152.
99. Calkins CC, Sloane BF, 1995. Mammalian cysteine protease inhibitors: biochemical properties and possible roles in tumor progression. *Biol Chem Hoppe Seyler* 376: 71–80.
100. Nagai A, Terashima M, Harada T, Shimode K, Takeuchi H, Murakawa Y, Nagasaki M, Nakano A, Kobayashi S, 2003. Cathepsin B and H activities and cystatin C concentrations in cerebrospinal fluid from patients with leptomeningeal metastasis. *Clin Chim Acta* 329: 53–60.
101. Sabirzhanov B, Stoica BA, Hanscom M, Piao CS, Faden AI, 2012. Over-expression of HSP70 attenuates caspase-dependent and caspase-independent pathways and inhibits neuronal apoptosis. *J Neurochem* 123: 542–554.

102. Mayer MP, Bukau B, 2005. Hsp70 chaperones: cellular functions and molecular mechanism. *Cell Mol Life Sci* 62: 670–684.
103. Augustin HG, Koh GY, Thurston G, Alitalo K, 2009. Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. *Nat Rev Mol Cell Biol* 10: 165–177.
104. Tang D et al., 2006. Angiogenesis in cholangiocellular carcinoma: expression of vascular endothelial growth factor, angiopoietin-1/2, thrombospondin-1 and clinicopathological significance. *Oncol Rep* 15: 525–532.
105. Hanahan D, Weinberg RA, 2011. Hallmarks of cancer: the next generation. *Cell* 144: 646–674.
106. Hatziaepostolou M, Iliopoulos D, 2011. Epigenetic aberrations during oncogenesis. *Cell Mol Life Sci* 68: 1681–1702.
107. Brooks J, Cairns P, Zeleniuch-Jacquotte A, 2009. Promoter methylation and the detection of breast cancer. *Cancer Causes Control* 20: 1539–1550.
108. Belinsky SA, 2004. Gene-promoter hypermethylation as a biomarker in lung cancer. *Nat Rev Cancer* 4: 707–717.
109. Park SY, Kwon HJ, Lee HE, Ryu HS, Kim SW, Kim JH, Kim IA, Jung N, Cho NY, Kang GH, 2011. Promoter CpG island hypermethylation during breast cancer progression. *Virchows Arch* 458: 73–84.
110. Koriath F, Gieffers C, Frey J, 1994. Cloning and characterization of the human gene encoding aspartyl beta-hydroxylase. *Gene* 150: 395–399.
111. Ince N, de la Monte SM, Wands JR, 2000. Overexpression of human aspartyl (asparaginy) beta-hydroxylase is associated with malignant transformation. *Cancer Res* 60: 1261–1266.
112. Palumbo KS, Wands JR, Safran H, King T, Carlson RI, de la Monte SM, 2002. Human aspartyl (asparaginy) beta-hydroxylase monoclonal antibodies: potential biomarkers for pancreatic carcinoma. *Pancreas* 25: 39–44.
113. Boncinelli E, 1997. Homeobox genes and disease. *Curr Opin Genet Dev* 7: 331–337.
114. Cillo C, Cantile M, Faiella A, Boncinelli E, 2001. Homeobox genes in normal and malignant cells. *J Cell Physiol* 188: 161–169.
115. Ben-Menachem T, 2007. Risk factors for cholangiocarcinoma. *Eur J Gastroenterol Hepatol* 19: 615–617.
116. Park J, Tadlock L, Gores GJ, Patel T, 1999. Inhibition of interleukin 6-mediated mitogen-activated protein kinase activation attenuates growth of a cholangiocarcinoma cell line. *Hepatology* 30: 1128–1133.
117. Agrawal S, Kuvshinov BW, Khoury T, Yu J, Javle MM, LeVea C, Groth J, Coignet LJ, Gibbs JF, 2007. CD24 expression is an independent prognostic marker in cholangiocarcinoma. *J Gastrointest Surg* 11: 445–451.
118. Bjornsson E, Chari ST, Smyrk TC, Lindor K, 2007. Immunoglobulin G4 associated cholangitis: description of an emerging clinical entity based on review of the literature. *Hepatology* 45: 1547–1554.
119. Alvaro D, 2009. Serum and bile biomarkers for cholangiocarcinoma. *Curr Opin Gastroenterol* 25: 279–284.
120. Steinberg W, 1990. The clinical utility of the CA 19-9 tumor-associated antigen. *Am J Gastroenterol* 85: 350–355.
121. Goonetilleke KS, Siriwardena AK, 2007. Systematic review of carbohydrate antigen (CA 19-9) as a biochemical marker in the diagnosis of pancreatic cancer. *Eur J Surg Oncol* 33: 266–270.