



# Detecting cholangiocarcinoma in patients with primary sclerosing cholangitis – The promise of DNA methylation and molecular biomarkers

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## Summary

**Cholangiocarcinoma (CCA) is a highly fatal malignancy of the bile ducts that arises in up to 20% of patients with primary sclerosing cholangitis (PSC). Current detection methods for CCA display suboptimal sensitivity and/or specificity, and there is no evidence-based screening strategy for CCA in patients with PSC. Consequently, CCA is often detected too late for surgical resection, contributing to the high mortality associated with this malignancy. Recently, biomarkers have emerged with potential to complement current detection methods, and/or be used for cancer surveillance in high-risk patient groups, including patients with PSC. Aberrant DNA methylation patterns represent promising biomarkers with great potential for CCA detection. Such aberrations are frequent in CCA, often occur early, and can be detected in liquid biopsies, including blood, bile and urine. This review summarises and highlights the most promising DNA methylation biomarkers identified for CCA detection so far, focusing on patients with PSC. Other promising molecular biomarkers for detection of PSC-associated CCA in liquid biopsies will also be briefly covered.**

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## Introduction

Cholangiocarcinoma (CCA) is a heterogeneous group of malignancies arising from the biliary duct epithelium. CCA is most commonly classified as perihilar (~50%), distal (~40%), or intrahepatic (~10%) based on anatomical origin.<sup>1</sup> With an incidence of 1–2/100,000 people worldwide, CCA is considered a rare malignancy, but several epidemiologic studies have demonstrated an increase in the incidence of CCA over the last decades.<sup>2</sup>

The majority of CCAs occur sporadically, but a well-established predisposing factor for CCA development in the Western world is primary sclerosing cholangitis (PSC).<sup>3</sup> The development of CCA prior to liver transplantation is a dreaded complication of PSC that is responsible for more than 30% of PSC-associated deaths.<sup>4</sup> The lifetime risk of developing CCA in PSC may be up to 20%, with an annual incidence of 1.5%.<sup>5–8</sup> Generally, patients with PSC are much younger at the time of diagnosis of CCA than the general population (fourth vs. seventh decade of life).<sup>5–7</sup>

The inflammatory biliary strictures commonly observed in PSC mimic early malignant changes, thus detecting CCA at an early stage in PSC is extremely challenging and current diagnostic approaches are restricted by low accuracy.<sup>9</sup> Multiple modalities used in combination are most often needed to diagnose CCA, including different imaging techniques (*i.e.*, CT, MRI, and/or endoscopic

retrograde cholangiopancreatography [ERCP]), cytological evaluation and/or fluorescent *in situ* hybridization (FISH) for polysomy using biliary brush material, and assessments of serum tumour markers (*i.e.*, carbohydrate antigen 19-9 [CA19-9] and carcinoembryonic antigen).<sup>10</sup> Consequently, up to 40% of CCAs in PSC are not diagnosed until intended liver transplantation or at autopsy.<sup>6</sup>

The only curative treatment option for patients with CCA is surgery, either resection or transplantation (with or without neoadjuvant or adjuvant therapy), which is primarily performed in highly selected patients with early stage perihilar tumours.<sup>2,11</sup> However, most patients are diagnosed at an advanced stage of malignancy, presenting with unresectable tumours. Consequently, the prognosis for CCA is poor, with an overall survival of less than 12 months in the absence of surgical resection.<sup>6</sup> Systemic chemotherapy may improve quality of life in the palliative setting, but it provides only a modest improvement in overall survival.<sup>12</sup> As the success rate for surgical resection and liver transplantation for locally advanced CCA has improved,<sup>13,14</sup> it is clear that novel biomarkers that enable earlier cancer detection could significantly increase survival in this setting.

In this review, we focus on the detection of PSC-associated CCA. We first provide a brief summary of current detection methods for CCA, and further elaborate on emerging biomarkers with potential

**Keywords:** Bile; Bile duct cancer; Biomarker; Blood; Cholangiocarcinoma; DNA methylation; Early detection; Liquid biopsy; Primary sclerosing cholangitis; Urine

Received 1 April 2020; received in revised form 25 June 2020; accepted 30 June 2020; available online XXX

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to complement and improve current detection methods. The focus will be on DNA methylation biomarkers, but other promising molecular biomarkers in liquid biopsies will also be briefly covered.

### The challenge of diagnosing cholangiocarcinoma – current methods

Although there is little evidence to support specific screening strategies for CCA detection in PSC, experts in the field recommend annual testing with CA19-9 and MRI or ultrasound, followed by ERCP with bile duct brushings for cytology and FISH if CA19-9 or imaging raise the suspicion of CCA, or if otherwise clinically indicated.<sup>9,15</sup> However, the problem is that none of these tests has satisfying accuracy for CCA detection, either suffering from low sensitivity and/or low specificity.

#### Serum CA19-9

CA19-9 is the most widely used serum biomarker for CCA in PSC but has several limitations. Numerous different cut-off values are used to define a positive CA19-9 test in the context of CCA, giving rise to highly variable sensitivities and specificities. Using a cut-off of 129 U/ml, Levy *et al.* reported a sensitivity, specificity and positive predictive value of 78.6%, 98.5%, and 56.6% for CCA, respectively.<sup>16</sup> Applying the same cut-off, Charatchroenwitthaya *et al.* obtained a similar specificity (100%), but a considerably lower sensitivity of 13% in patients with PSC<sup>15</sup> – using a cut-off of 20 U/ml, they obtained a sensitivity of 78% and a specificity of 67%, meaning that >30% of the those with a positive test were cancer free.<sup>15</sup> Importantly, both studies reported that high CA19-9 levels were associated with advanced, inoperable CCA, with CA19-9 levels tending to be normal or only marginally elevated in early malignancy.<sup>15,16</sup> Another recent study<sup>17</sup> analysed CA19-9 levels in a set of 89 pre-diagnosis serum samples obtained from 55 cases who developed biliary tract cancer and 91 matched cancer-free controls. Using a cut-off of 37 U/ml, CA19-9 provided 17% sensitivity at a specificity of 93%.<sup>17</sup> Other studies have used CA19-9 levels from 100 U/ml to 200 U/ml as cut-offs, with sensitivities varying from 67–89%, and specificities of 80–98%.<sup>18–20</sup> In addition, the ability to express CA19-9 depends on the activity of FUT3. Individuals who lack this activity (Lewis antigen negative, ~7% of the population), are unable to express CA19-9 irrespective of disease status.<sup>21</sup> In conclusion, the low sensitivity of CA19-9 makes it a suboptimal marker for the early diagnosis of CCA.

#### Non-invasive imaging modalities

Non-invasive imaging modalities, including ultrasound, CT and combined MRI/magnetic resonance cholangiopancreatography, have the advantage of being both non-invasive and relatively cheap to perform, representing valuable tools for CCA detection. Abnormalities observed using imaging, including mass forming lesions or thickening of the bile duct wall, might be indicative of CCA, but can also represent benign changes in PSC.<sup>9</sup> Considering only definite findings as positive for CCA, the sensitivity for ultrasound, CT and MRI was reported to be 10%, 25% and 32%, respectively, with 100% specificity.<sup>15</sup> If definite, probable and possible findings were considered positive for CCA, the sensitivities increased to 57% for ultrasound, 75% for CT and 63% for MRI, but specificities dropped to 94%, 80% and 79%, respectively, indicative of a high false positive detection rate.<sup>15</sup>

#### Key points

- Detecting CCA at an early and curable stage in patients with PSC is challenging, with current diagnostic tools restricted by low accuracy.
- Earlier CCA detection in patients with PSC could significantly improve survival.
- The unmet clinical need for improved CCA detection may be met by molecular biomarkers.
- Several DNA methylation biomarkers, which improve the diagnostic accuracy of CCA, have been identified.
- DNA methylation markers in liquid biopsies, such as bile, plasma or serum, may prove to be affordable, feasible and accurate for early CCA detection.

#### Cholangiography-based techniques

##### ERCP

ERCP combined with biliary brush cytology and FISH has been recommended as the method of choice to confirm a suspected CCA diagnosis.<sup>9</sup> A sensitivity of 91% and specificity of 66% were reported when using ERCP alone, with all definite, probable and possible findings considered positive for CCA. If only considering definite findings as positive for malignancy, the specificity increased to 100%, but the sensitivity dropped to only 13%.<sup>15</sup>

##### Biliary brushing

###### Conventional brush cytology

Conventional brush cytology obtained via ERCP has high specificity (95–100%) for the diagnosis of CCA in PSC, but unfortunately the sensitivity is limited, ranging from 8–46%.<sup>15,20,22,23</sup> In a meta-analysis, the pooled sensitivity and specificity was 47% and 97%, respectively.<sup>24</sup> The low sensitivity can be explained by the inflammation associated with PSC, which complicates cytological assessments.<sup>25</sup> In addition, the tumour may be located in areas not accessible by biliary brushing, furthermore, it can be challenging to obtain adequate cellular material because of fibrotic connective tissue surrounding the tumour.<sup>22</sup>

##### FISH

Specimens obtained by ERCP may be analysed for chromosomal alterations by FISH. In one study, FISH polysomy had a sensitivity of 47% and a specificity of 100% for the diagnosis of CCA in PSC.<sup>23</sup> A meta-analysis reported that FISH polysomy had a pooled sensitivity of 51% and specificity of 93% for diagnosing CCA in patients with PSC.<sup>26</sup> The disadvantage of FISH is that the technique is expensive, labour intensive, prone to subjective interpretation errors and requires significant technical expertise.<sup>27</sup>

#### Combination of diagnostic tests

As each of the current diagnostic modalities for CCA in PSC is restricted by low diagnostic accuracy, multiple modalities – including CA19-9, imaging, biliary cytology and FISH – are usually required to arrive at a definitive diagnosis of CCA.<sup>15</sup>

#### Novel biomarkers – potential for improved detection of cholangiocarcinoma

There is a broad consensus that biomarkers for improved CCA detection are an unmet clinical need. Biomarkers that enable earlier cancer detection would be of high clinical value as they could significantly improve survival, especially for patients with high-risk PSC.

### Search strategy

A literature search was performed in PubMed and Web of science. Keywords were cholangiocarcinoma, bile duct cancer, DNA methylation. Poster abstracts and non-English articles were excluded from further review, along with articles not including patient samples. Articles listing number of methylated samples (sensitivity) or AUC values were included in this review. The list was supplemented by any references appearing in the reference list of included articles, not detected by the initial search but fulfilling the criteria above. The search was completed in February 2020.

### DNA methylation biomarkers for detection of CCA

DNA hypermethylation of gene promoters is a stable and early event in carcinogenesis, which has been identified in precancerous lesions and early stage cancers. In the setting of CCA, aberrant DNA methylation has been reported in both intraductal papillary neoplasm of the liver/bile ducts (IPNL/B) and biliary intraepithelial neoplasia (BillIN), the 2 main precursor lesions of CCA.<sup>28,29</sup> For instance, methylation of *CDKN2A* (p16) was reported in 55% of the 44 IPNL foci analysed by Ishikawa *et al.*<sup>28</sup> In another study, Kim *et al.* found that 4 loci specifically methylated in CCA samples (*TMEFF2*, *HOXA1*, *NEUROG1*, *RUNX3*) had significantly higher methylation levels in BillIN compared to normal samples, suggesting that hypermethylation contributes to the development of BillIN or the progression of BillIN to CCA.<sup>29</sup> In addition to being an early event in carcinogenesis, DNA methylation can easily be detected by PCR-based technologies, suggesting that such aberrations may have clinical value for early cancer detection, and/or for cancer surveillance among high-risk individuals. Interestingly, in addition to being methylated in IPNL foci, *CDKN2A* (p16) has been reported to be methylated in 46% of bile samples from patients with PSC (n = 11).<sup>30</sup> This could indicate early malignant transformation in predisposed individuals. However, none of the patients with PSC showed any signs of malignancy in the relevant study,<sup>30</sup> suggesting that *CDKN2A* (p16) is not a good marker for early cancer detection among high-risk patients.

### Tissue

DNA methylation of *CDKN2A* (p16) has been extensively studied in CCA, with reported sensitivities and specificities between 14–83% and 64–100%, respectively, but only non-PSC controls have been included in these studies (Table 1). Several other genes have been reported to be aberrantly methylated in tissue, biliary brushes, bile and plasma among patients with CCA (Table 1). In tissue, *SEMA3B* hypermethylation could differentiate between CCA and normal tissue with 100% sensitivity and specificity.<sup>31</sup> However, only a limited number of samples (15 CCA and 15 adjacent normal tissue) were analysed. Also *HOXA1*,<sup>32</sup> *OPCML*,<sup>33,34</sup> *SFRP1*,<sup>33</sup> *RUNX3*,<sup>35</sup> *CDO1*,<sup>36,37</sup> and *HPP1*<sup>32</sup> have shown promise as CCA biomarkers in tissue, all with >70% sensitivity and >90% specificity.

### Biliary brushes

DNA can be obtained from biliary brush material, and further analysed for DNA methylation. Using biliary brushes, Parsi *et al.* showed that methylation of *TFPI2*, *NPTX2* and *CCND2* could differentiate biliary tract cancers (n = 10) from patients with benign liver conditions with a sensitivity of 80% and a specificity of 86%.<sup>38</sup> We have also shown the suitability of analysing DNA methylation markers in biliary brushes. In an initial study

analysing tissue specimens, a 4-gene biomarker panel (*CDO1*, *DCLK1*, *SFRP1* and *ZSCAN18*) was shown to have high sensitivity (87%) and specificity (100%) for detection of CCA.<sup>36</sup> These genes, in addition to several other markers, were further validated in biliary brush samples, including 49 CCAs and 60 benign PSC controls, divided into a test and a validation series. Across both series, a 4 marker panel (*CDO1*, *CNRIP1*, *SEPT9*, and *VIM*) could differentiate between CCA and PSC controls with high sensitivity (85%) and specificity (98%), and an AUC of 0.944.<sup>39</sup> Importantly, this panel had better diagnostic accuracy for CCA than standard brush cytology (samples from both benign PSC controls and PSC complicated with CCA were included) and could thus improve the detection of PSC-associated CCA. Finally, methylation of *HOXA1*, first identified in tissue samples, has been validated in biliary brushes, showing 89% sensitivity and 100% specificity. In the same study, *NEUROG1* and *CDKN2A* (p16) methylation were associated with sensitivities of 67% and 100%, respectively, both with 100% specificity. However, only 9 CCA samples were included.<sup>40</sup>

### Bile and blood

Similar to biliary brushes, bile is obtained during ERCP. Since cells and DNA from the entire bile duct is shed into the bile, the main advantage of analysing DNA from bile is its potential to capture the true heterogeneity of CCAs. Despite the promising potential of using bile as a source for biomarker discovery and CCA detection, very few DNA methylation studies have been performed. In 2003, Klump and colleagues analysed *CDKN2A* (p16) and *CDKN2A* (p14) in 21 and 23 CCA samples, respectively, and in 32 samples from patients with benign biliary disorders, in addition to the 11 PSC samples previously mentioned (section 2.1). Excluding the PSC samples, *CDKN2A* (p16) and *CDKN2A* (p14) methylation had a sensitivity of 52% and 48%, and a specificity of 94% and 97%, respectively.<sup>30</sup> In another study, the DNA methylation levels of 19 markers were analysed in bile, showing sensitivities ranging between 3–93% and specificities between 14–100%.<sup>41</sup> *CDKN2A* (p16) was one of the markers included, displaying 74% sensitivity and 64% specificity.<sup>41</sup> Finally, analysing 77 CCA samples and 48 samples from patients with benign biliary disorder, Shin *et al.* reported that methylation of the marker panel *CCND2*, *CDH13*, *GRIN2B*, *RUNX3*, and *TWIST1* in bile had a 76% sensitivity and 100% specificity for CCA. The samples were divided into a training, validation and test set; all reached ≥70% sensitivity, underscoring the robustness of the results and the promise of these markers for CCA detection.<sup>42</sup> However, apart from the study by Klump *et al.*, none of the aforementioned studies in bile included PSC controls.

An alternative and minimally invasive source for biomarker discovery is blood. However, like bile, few studies have been performed on DNA methylation markers. The combination of *SHOX2* and *SEPT9* promoter hypermethylation in plasma from 20 patients with CCA and 100 controls had a 45% sensitivity and 99% specificity for CCA.<sup>43</sup> In tissue samples, the same markers achieved a sensitivity and specificity of 74% and 100%, respectively, underscoring the potential drop in accuracy when using blood instead of tissue samples.<sup>43</sup> *OPCML* methylation was originally identified in tissue samples,<sup>34</sup> and was also recently found in serum from 80% of CCA samples (n = 40) and in only 10% of samples from people with benign biliary disorders (90% specificity).<sup>44</sup> If methylation of *OPCML* was combined with methylation of *HOXD9* the specificity increased to 100%, but the

Table 1. DNA methylation biomarkers for detection of CCA.

Gene name	Cancer samples	Controls	Sensitivity	Specificity	AUC	Material	PSC controls	PSC-CCA	Ref.
14-3-3 $\sigma$	79 CCA	15 (BBD)	60%	100%	–	Tissue	–	–	53
ALX4	5 BDC	5 (AN)	80%	80%	–	Tissue	–	–	54
APC	72 CCA	10 (AN)	46%	90%	–	Tissue	–	–	55
APC	79 CCA	15 (BBD)	27%	100%	–	Tissue	–	–	53
APC	111 CCA	38 (BBD)	26%	100%	–	Tissue	No	–	32
BCL2	111 CCA	38 (BBD)	23%	97%	–	Tissue	No	–	32
BLU	15 CCA	15 (AN)	20%	100%	–	Tissue	–	–	31
CCD2	10 BTC	27 (PSC) 39 (BBD)	10%	93% (PSC) 100% (BBD)	–	Biliary brushes	Yes	–	38
CCD2, NPTX2, TFPI1	10 BTC	27 (PSC) 39 (BBD)	80%	85% (PSC) 87% (BBD)	–	Biliary brushes	Yes	–	38
CCND2, CDH13, GRIN2B, RUNX3, and TWIST1	77 CCA 20 (Training) 33 (Validation) 24 (Test)	48 (BBD) 20 (Training) 12 (Validation) 24 (Test)	76% 70% (Training) 74% (Validation) 83% (Test)	100%	–	Bile	–	–	42
CDH1	65 BDC	–	60%	–	–	Tissue	–	–	56
CDH1	72 CCA	10 (AN)	43%	100%	–	Tissue	–	–	55
CDH1	23 BDC	2 (NM controls)	35%	100%	–	Tissue	–	–	35
CDH1	111 CCA	38 (BBD)	30%	95%	–	Tissue	No	–	32
CDH1	79 CCA	15 (BBD)	22%	100%	–	Tissue	–	–	53
CDKN2A (p14)	21 CCA	32 (BBD) 11 (PSC)	48%	97% (BBD) 40% (PSC)	–	Bile	Yes	Yes	30
CDKN2A (p14)	92 CCA	–	40%	–	–	Tissue	–	–	57
CDKN2A (p14)	72 CCA	10 (AN)	38%	100%	–	Tissue	–	–	55
CDKN2A (p14)	52 CCA	–	25%	n.a.	–	Tissue	–	–	58
CDKN2A (p14)	36 CCA	36 (AN)	24%	–	–	Tissue	–	–	59
CDKN2A (p14)	111 CCA	38 (BBD)	11%	100%	–	Tissue	No	–	32
CDKN2A (p14)	79 CCA	15 (BBD)	9%	100%	–	Tissue	–	–	53
CDKN2A (p15)	72 CCA	10 (AN)	51%	90%	–	Tissue	–	–	55
CDKN2A (p15)	92 CCA	–	49%	–	–	Tissue	–	–	57
CDKN2A (p16)	41 CCA	–	83%	n.a.	n.a.	Tissue	–	–	60
CDKN2A (p16)	90 CCA	–	77%	–	–	Tissue	–	–	61
CDKN2A (p16)	51 CCA	–	76%	n.a.	–	Tissue	–	–	58
CDKN2A (p16)	23 CCA	36 (BBD)	74%	64%	–	Bile	No	–	41
CDKN2A (p16)	9 CCA	9 (GBEC)	67%	100%	0.88	Biliary brushes	–	–	40
CDKN2A (p16)	23 CCA	32 (BBD) 11 (PSC)	52%	94% (BBD) 54% (PSC)	–	Bile	Yes	Yes	30
CDKN2A (p16)	72 CCA	10 (AN)	50%	90%	–	Tissue	–	–	55
CDKN2A (p16)	21 CCA	–	43%	n.a.	–	Tissue	–	–	62
CDKN2A (p16)	92 CCA	–	28%	–	–	Tissue	–	–	57
CDKN2A (p16)	23 BDC	2 (NM controls)	26%	100%	–	Tissue	–	–	35
CDKN2A (p16)	79 CCA	15 (BBD)	18%	100%	–	Tissue	–	–	53
CDKN2A (p16)	65 BDC	–	17%	–	–	Tissue	–	–	56
CDKN2A (p16)	111 CCA	38 (BBD)	14%	100%	–	Tissue	No	–	32
CDO1	39 CCA	54 (30 PSC +14 BBD)	77%	100%	0.907	Tissue	Yes	Yes	36
CDO1	49 CCA	54 (30 PSC +14 BBD)	77%	98%	0.933	Biliary brushes	Yes	Yes	39
CDO1	108 BTC (81 CCA)	101 (AN)	76%	92%	0.89	Tissue	–	–	37
CDO1, CNRIP1, SEPT9, VIM	49 CCA	54 (30 PSC +14 BBD)	85%	98%	0.944	Biliary brushes	Yes	Yes	39
CDO1, DCLK1, SFRP1, ZSCAN18	39 CCA	54 (30 PSC +14 BBD)	87%	100%	0.924	Tissue	Yes	Yes	36
CHFR	23 BDC	2 (NM controls)	17%	100%	–	Tissue	–	–	35
CNRIP1	49 CCA	54 (30 PSC +14 BBD)	70%	100%	0.901	Biliary brushes	Yes	Yes	39
COX2	79 CCA	15 (BBD)	5%	100%	–	Tissue	–	–	53
DAPK	65 BDC	–	54%	–	–	Tissue	–	–	56
DAPK	23 BDC	2 (NM controls)	17%	100%	–	Tissue	–	–	35
DAPK	79 CCA	15 (BBD)	8%	100%	–	Tissue	–	–	53
DAPK	72 CCA	10 (AN)	3%	100%	–	Tissue	–	–	55
DAPK1	36 CCA	36 (AN)	31%	94%	–	Tissue	–	–	59,63
DAPK1, CDKN2A (p14), ASC	36 CCA	36 (AN)	41%	86%	–	Tissue	–	–	59
DCLK1	39 CCA	54 (30 PSC +14 BBD)	44%	100%	0.795	Tissue	Yes	Yes	36
DcR1	102 CCA	29 (AN)	28%	97%	–	Tissue	–	–	34
DKK2	23 CCA	36 (BBD)	48%	75%	–	Bile	No	–	41
DKK3	23 CCA	36 (BBD)	70%	61%	–	Bile	No	–	41

(continued on next page)

Table 1 (continued)

Gene name	Cancer samples	Controls	Sensitivity	Specificity	AUC	Material	PSC controls	PSC-CCA	Ref.
DLEC1	172 CCA	10 (BBD)	23%	100%	-	Tissue	No	-	64
FHIT	19 CCA	-	42%	-	-	Tissue	-	-	65
GSTP	72 CCA	10 (AN)	18%	100%	-	Tissue	-	-	55
GSTP1	79 CCA	15 (BBD)	6%	100%	-	Tissue	-	-	53
HIC1	102 CCA	29 (AN)	38%	100%	-	Tissue	-	-	34
HOXA1	111 CCA	38 (BBD)	90%	95%	-	Tissue	No	-	32
HOXA1	9 CCA	9 (GBEC)	89%	100%	0.94	Biliary brushes	-	-	40
HOXA9	102 CCA	22 (AN)	86%	-	-	Tissue	-	-	66
HOXAD9	102 CCA	24 (AN)	89%	-	-	Tissue	-	-	66
HOXD9	40 CCA	40 (BBD)	68%	90%	0.788	Serum	-	-	44
HPP1	111 CCA	38 (BBD)	73%	92%	-	Tissue	No	-	32
IGF2	111 CCA	38 (BBD)	23%	89%	-	Tissue	No	-	32
ITGA4	29 CCA	34 (19 AN +15 NM controls)	55%	91%	-	Tissue	-	-	67
MGMT	72 CCA	10 (AN)	33%	100%	-	Tissue	-	-	55
MGMT	79 CCA	15 (BBD)	11%	100%	-	Tissue	-	-	53
MHL1	52 CCA	10 (AN)	24%	100%	-	Tissue	-	-	55
MINT1	79 CCA	15 (BBD)	41%	100%	-	Tissue	-	-	53
MINT1	111 CCA	38 (BBD)	38%	100%	-	Tissue	No	-	32
MINT12	79 CCA	15 (BBD)	51%	100%	-	Tissue	-	-	53
MINT2	111 CCA	38 (BBD)	7%	100%	-	Tissue	No	-	32
MINT2	79 CCA	15 (BBD)	0%	100%	-	Tissue	-	-	53
MINT25	79 CCA	15 (BBD)	15%	100%	-	Tissue	-	-	53
MINT31	111 CCA	38 (BBD)	15%	100%	-	Tissue	No	-	32
MINT31	79 CCA	15 (BBD)	1%	100%	-	Tissue	-	-	53
MINT32	79 CCA	15 (BBD)	35%	100%	-	Tissue	-	-	53
miR-373	48 CCA	48 (AN)	79%	87%	-	Tissue	-	-	68,69
MLH1	65 CCA	-	45%	100%	-	Tissue	-	-	70
MLH1	65 BDC	-	43%	-	-	Tissue	-	-	56
MLH1	23 BDC	2 (NM controls)	13%	100%	-	Tissue	-	-	35
NEUROG1	9 CCA	9 (GBEC)	100%	100%	1.00	Biliary brushes	-	-	40
NEUROG1	111 CCA	38 (BBD)	53%	89%	-	Tissue	No	-	32
NPTX2	23 CCA	36 (BBD)	57%	72%	-	Bile	No	-	41
NPTX2	10 BTC	27 (PSC) 39 (BBD)	40%	89% (PSC) 95% (BBD)	-	Biliary brushes	Yes	-	38
OPCML	73 CCA	10 (AN)	89%	100%	0.932	Tissue	-	-	33
OPCML	40 CCA	40 (BBD)	80%	90%	0.85	Serum	-	-	44
OPCML	102 CCA	29 (AN)	73%	100%	-	Tissue	-	-	34
OPCML, HOXD9	40 CCA	40 (BBD)	63%	100%	0.812	Serum	-	-	44
p73	72 CCA	10 (AN)	36%	100%	-	Tissue	-	-	55
PCDH8	8 CCA	50 (NM controls)	88%	78%	-	Tissue	-	-	71
ppENK	23 CCA	36 (BBD)	57%	72%	-	Bile	No	-	41
PTEN	102 CCA	29 (AN)	35%	93%	-	Tissue	-	-	34
PTEN	79 CCA	15 (BBD)	0%	100%	-	Tissue	-	-	53
RAR-b	72 CCA	10 (AN)	18%	100%	-	Tissue	-	-	55
RARB2	111 CCA	38 (BBD)	14%	100%	-	Tissue	No	-	32
RASSF1	65 BDC	-	46%	-	-	Tissue	-	-	56
RASSF1A	13 CCA	-	69%	-	-	Tissue	No	-	72
RASSF1A	19 CCA	-	68%	-	-	Tissue	-	-	65
RASSF1A	15 CCA	15 (AN)	67%	100%	-	Tissue	-	-	31
RASSF1A	72 CCA	10 (AN)	65%	80%	-	Tissue	-	-	55
RASSF1A	48 CCA	12 (AN)	58%	83%	-	Tissue	-	-	73
RASSF1A	23 BDC	2 (NM controls)	30%	50%	-	Tissue	-	-	35
RASSF1A	111 CCA	38 (BBD)	28%	100%	-	Tissue	No	-	32
RASSF1A	9 CCA	9 (GBEC)	56%	100%	0.78	Biliary brushes	-	-	40
RBP1	111 CCA	38 (BBD)	14%	100%	-	Tissue	No	-	32
RIZ1	81 CCA	69 (AN)	38%	93%	-	Tissue	-	-	74
RUNX3	23 BDC	2 (NM controls)	78%	100%	-	Tissue	-	-	35
RUNX3	53 CCA	-	49%	-	-	Tissue	-	-	75
RUNX3	111 CCA	38 (BBD)	33%	100%	-	Tissue	No	-	32
SEMA3B	15 CCA	15 (AN)	100%	100%	-	Tissue	-	-	31
SEPT9	43 CCA (Tissue)	41 (AN)	19%	100%	0.541	Tissue	-	-	43
SEPT9	20 CCA (Plasma)	100 (NM controls)	25%	99%	0.699	Plasma	-	-	43
SEPT9	49 CCA	54 (30 PSC +14 BBD)	57%	100%	0.795	Biliary brushes	Yes	Yes	39

(continued on next page)

Table 1 (continued)

Gene name	Cancer samples	Controls	Sensitivity	Specificity	AUC	Material	PSC controls	PSC-CCA	Ref.
SEPT9, SHOX2	43 CCA (Tissue)	41 (AN-tissue)	74% (Tissue)	100% (Tissue)	0.932	Tissue	-	-	43
	20 CCA (Plasma)	100 (NM controls-plasma)	45% (Plasma)	99% (Plasma)	0.752 (Plasma)	Plasma	-	-	
SFRP1	73 CCA	10 (AN)	84%	100%	0.951	Tissue	-	-	33
SFRP1	39 CCA	54 (30 PSC +14 BBD)	59%	100%	0.769	Tissue	Yes	Yes	36
SFRP1	102 CCA	29 (AN)	34%	97%	-	Tissue	-	-	34
SFRP2	23 CCA	36 (BBD)	74%	78%	-	Bile	No	-	41
SHOX2	43 CCA (Tissue)	41 (AN-tissue)	72% (Tissue)	100% (Tissue)	0.921	Tissue	-	-	43
	20 CCA (Plasma)	100 (NM controls-plasma)	35% (Plasma)	99% (Plasma)	0.691 (Plasma)	Plasma	-	-	
SOCS3	16 CCA	16 (AN)	44%	100%	-	Tissue	-	-	76
TFPI1	10 BTC	27 (PSC)	40%	96% (PSC)	-	Biliary brushes	Yes	-	38
		39 (BBD)		92% (BBD)					
THBS1	79 CCA	15 (BBD)	11%	100%	-	Tissue	-	-	53
TIG1	111 CCA	38 (BBD)	22%	97%	-	Tissue	No	-	32
TIMP3	79 CCA	15 (BBD)	9%	100%	-	Tissue	-	-	53
TMS1/ASC	36 CCA	36 (AN)	36%	92%	-	Tissue	-	-	59,77
VIM	49 CCA	54 (30 PSC +14 BBD)	45%	100%	0.797	Biliary brushes	Yes	Yes	39
ZNF331	39 CCA	54 (30 PSC +14 BBD)	26%	100%	-	Tissue	Yes	Yes	78
ZSCAN18	39 CCA	54 (30 PSC +14 BBD)	54%	100%	0.752	Tissue	Yes	Yes	36

AN, adjacent normal; BBD, benign biliary disorders (non-PSC); BDC, bile duct cancer; BTC, biliary tract cancer; GBEC, gallbladder epithelial cells; HC, healthy controls; NM, non-malignant.

sensitivity decreased to 63%.<sup>44</sup> No blood samples from PSC controls were analysed in either of these studies.<sup>43,44</sup>

### Molecular biomarkers for detection of CCA in patients with PSC

In addition to DNA methylation markers, several other biomarkers, including microRNAs (miRNAs) and peptide/protein markers have been suggested as potential candidates for improved detection of CCA among patients with PSC (Table 2). In the current review, only markers detected in liquid biopsies (blood, bile and urine) or biliary brushes have been included.

#### Molecular biomarkers in biliary brushes, blood, bile and urine

Singhi *et al.* recently developed a next-generation sequencing assay (BiliSeq) applied on DNA from ERCP-obtained biliary brushes that could improve the detection and management of patients with malignant bile duct strictures.<sup>27</sup> BiliSeq achieved a sensitivity of 73% and a specificity of 100%, when used to analyse 150 samples from patients with biliary tract cancers or high-grade dysplasia, and 70 samples from patients with benign biliary disorders. Importantly, when including only patients with PSC, a sensitivity of 83% (10/12) and a specificity of 100% (25/25) was obtained.<sup>27</sup> Although including few patients, these results are interesting and warrant further testing.

Several studies have investigated proteins or protein profiles for CCA detection. In 2011, Lankisch *et al.* identified a bile proteomic profile that could differentiate CCA from PSC with 84% sensitivity, 78% specificity, and an AUC of 0.87. Of the 10 samples from patients with PSC-associated CCA, 8 were correctly identified (80% sensitivity).<sup>45</sup> A proteomic profile for CCA detection in bile has also been identified by Voigtländer *et al.* Interestingly, combining the proteomic profile from bile and urine increased the specificity from 80% to 96%, although the sensitivity decreased from 83% to 72%.<sup>46</sup> Using only urine, a distribution profile including 42 peptides was positive in 83% of the 42

analysed CCA samples, and in 22% of the PSC samples (78% specificity). Of further interest, the panel was able to identify all of the PSC-associated CCAs (n = 10). Higher specificities were obtained when including control samples from patients with only non-PSC benign biliary disorders (81%) or healthy individuals (86%).<sup>47</sup>

miRNAs have also been analysed in both bile and serum, with the aim of differentiating between CCA and PSC, with varying sensitivities and specificities. In serum, 5 miRNAs could distinguish between CCA (n = 31) and PSC (n = 40) with sensitivities, specificities and AUCs ranging from 32–68%, 88–93%, and 0.67–0.85, respectively.<sup>48</sup> Analysing a limited number of samples in bile, Voigtländer and colleagues further showed that 4 other miRNAs (miR-640, miR-412, miR-1537 and miR-3189) could differentiate between patients with PSC (n = 52) and those with PSC complicated by CCA (n = 12), with sensitivities between 50–67% and specificities between 89–92%.<sup>48</sup> One of the identified markers in serum, miR-122, had a sensitivity of 32% for CCA detection with a specificity and AUC of 90% and 0.65, respectively. Recently, the same miRNA was found to have a much higher accuracy (AUC = 0.895) than initially reported for differentiating CCA (n = 90) and PSC (n = 10).<sup>49</sup> In the same study, several other miRNAs were also included, showing a trend of decreased accuracy when including PSC controls compared to non-PSC controls.<sup>49</sup> Analysing miRNAs in extracellular vesicles isolated from bile has been suggested as a better way of developing miRNAs for disease detection compared to using whole bile.<sup>50</sup> By extracting RNA from extracellular vesicles isolated from bile, a miRNA-panel with 67% sensitivity and 96% specificity for CCA detection was obtained, including only patients with PSC. The marker panel could correctly identify 3/4 patients with CCA (75% sensitivity) and 12/13 controls with PSC (92% specificity).<sup>50</sup> Serum extracellular vesicles have also been reported to contain protein biomarkers for PSC and CCA. Arbelaiz *et al.* identified several proteins in serum extracellular vesicles that could

**Table 2. Molecular biomarkers for detection of CCA among patients with PSC.**

Marker	Cancer samples	Controls	Sensitivity	Specificity	AUC	Material	PSC controls	PSC-CCA	Ref
A1AG1	43 CCA	30 (PSC)	77%	70%	0.794	Serum (EV)	Yes	No	10
Angpt-2	49 CCA	48 (34 PSC +14 BDS)	74%	94%	0.85	Serum	Yes	Yes	79
BiliSeq (28 gene NGS panel)	12 CCA	25 (PSC)	83%	100%	0.86	Biliary brushes, bile	Yes	Yes	27
CDKN2A (p16) (loss)	12 CCA	29 (PSC)	67%	69%	0.68	Biliary brushes	Yes	Yes	80
CYFRA 21-1	66 BTC (62 CCA)	58 (19 PSC +39 BBD)	56%	88%	-	Serum, Plasma	Yes	Yes	81
CYFRA21-1	66 CCA	62 (PSC)	65%	76%	0.732	Serum	Yes	-	17
ELF score (HA, TIMP-1, PIIINP)	32 CCA	36 (PSC)	81%	60%	0.74	Serum	Yes	Yes	82
fc-Fetuin-A	39 CCA	39 (PSC)	62%	90%	0.812	Serum	Yes	-	83
FIBG	43 CCA	30 (PSC)	88%	63%	0.796	Serum (EV)	Yes	No	10
miR-122	90 CCA	40 (NM controls) 10 (PSC)	95%	100% (NM controls)	0.992 (NM controls) 0.895 (PSC)	Serum	Yes	-	49
miR-122	31 CCA	40 (PSC)	32%	90%	0.65	Serum	Yes	No	48
miR-126	31 CCA	40 (PSC)	68%	93%	0.87	Serum	Yes	No	48
miR-1281	31 CCA	40 (PSC)	55%	90%	0.83	Serum	Yes	No	48
miR-1537	12 CCA	52 (PSC)	67%	90%	0.78	Bile	Yes	Yes	48
miR-155	90 CCA	40 (NM controls) 10 (PSC)	61%	80% (NM controls)	0.664 (NM controls) 0.787 (PSC)	Serum	Yes	-	49
miR-192	90 CCA	40 (NM controls) 10 (PSC)	85%	95% (NM controls)	0.927 (NM controls) 0.857 (PSC)	Serum	Yes	-	49
miR-222	40 CCA	40 (PSC)	-	-	0.71	Serum	Yes	Yes	84
miR-222, miR-483-5p	40 CCA	40 (PSC)	-	-	0.77	Serum	Yes	Yes	84
miR-26a	31 CCA	40 (PSC)	52%	93%	0.78	Serum	Yes	No	48
miR-29b	90 CCA	40 (NM controls) 10 (PSC)	66%	100% (NM controls)	0.838 (NM controls) 0.831 (PSC)	Serum	Yes	-	49
miR-30b	31 CCA	40 (PSC)	52%	88%	0.78	Serum	Yes	No	48
miR-3189	12 CCA	52 (PSC)	67%	89%	0.8	Bile	Yes	Yes	48
miR-412	12 CCA	52 (PSC)	50%	89%	0.81	Bile	Yes	Yes	48
miR-483-5p	40 CCA	40 (PSC)	-	-	0.7	Serum	Yes	Yes	84
miR-640	12 CCA	52 (PSC)	50%	92%	0.81	Bile	Yes	Yes	48
miR-panel	46 CCA	50 (13 PSC +37 BBD)	67%	96%	-	Bile (EV)	Yes	Yes	50
MUC5AC	66 CCA	62 (PSC)	61%	82%	0.72	Serum	Yes	-	17
MYC (gain)	12 CCA	29 (PSC)	58%	79%	0.69	Biliary brushes	Yes	Yes	80
ON-PC, S-PC	8 CCA	21 (6 PSC +15 BBD)	100%	83%	0.91	Bile	Yes	-	85
PC(34:3), histidine	20 CCA (Discovery) 15 CCA (Validation)	20 (PSC: discovery) 15 (PSC: validation)	100% (Discovery) 93% (Validation)	70% (Discovery) 93% (Validation)	0.99 (Discovery) 0.995 (Validation)	Serum	Yes	-	51
Peptide model	42 CCA	45 (PSC) 36 (BBD) 101 (HC)	83%	78% (PSC) 81% (BBD) 86% (HC)	0.87 (PSC+BBD)	Urine	Yes	Yes	47
Peptide model	25 CCA	18 (PSC)	84%	78%	0.87	Bile	Yes	Yes	45
PKM2	66 CCA	62 (PSC)	76%	83%	0.839	Serum	Yes	-	17
PKM2, CYFRA21-1, MUC5AC	66 CCA	62 (PSC)	76%	90%	0.899	Serum	Yes	-	17
Proteomic model	36 CCA	51 (33 PSC +18 BBD)	83%	80%	0.85	Bile	Yes	Yes	46
Proteomic model	36 CCA	51 (33 PSC +18 BBD)	72%	96%	-	Bile + urine	Yes	Yes	46
Proteomic model	36 CCA	51 (33 PSC +18 BBD)	89%	86%	0.93	Urine	Yes	Yes	46
RNU2-1f	12 CCA	11 (PSC)	67%	91%	0.856	Bile	Yes	No	86
S10A8	43 CCA	30 (PSC)	70%	67%	0.759	Serum (EV)	Yes	No	10
S10A9	43 CCA	30 (PSC)	74%	60%	0.746	Serum (EV)	Yes	No	10

(continued on next page)

Table 2 (continued)

Marker	Cancer samples	Controls	Sensitivity	Specificity	AUC	Material	PSC controls	PSC-CCA	Ref
SAMP	43 CCA	30 (PSC)	79%	58%	0.74	Serum (EV)	Yes	No	<sup>10</sup>
VOC	6 CCA	10 (PSC)	80%	100%	0.9	Urine	Yes	-	<sup>87</sup>
VOC	11 CCA	11 (PSC)	91%	73 %	0.89	Bile	Yes	Yes	<sup>88</sup>

BBD, benign biliary disorders (non-PSC); BTC, biliary tract cancer; EV, extracellular vesicles; HC, healthy controls; HGD, high-grade dysplasia; VOC, volatile organic compounds.

differentiate patients with CCA from those with PSC, with the best marker (FIBG) showing an AUC of 0.796 with 88% sensitivity and 63% specificity.<sup>10</sup>

Finally, it has been suggested that specific changes in serum concentrations of certain metabolites may be useful to differentiate CCA from PSC, and could help in the early diagnosis of CCA among patients with PSC. By combining 2 metabolites, PC(34:3) and histidine, Banales *et al.* reported accurate differentiation between patients with PSC (n = 35) and CCA (n = 35), both in a discovery cohort (AUC = 0.99) and a validation cohort (AUC = 0.995).<sup>51</sup> Although the specificity in the discovery cohort was suboptimal (70%), the results are interesting and deserve further validation.

### Perspectives and conclusions

Diagnosing CCA in patients with PSC is complicated, mainly owing to the challenge of differentiating benign from malignant strictures using imaging or by assessment of biliary brush specimens. Furthermore, the tumour might reside in areas not reached by biliary brushings, or the material obtained by biliary brushings might be too scarce to obtain a proper evaluation by cytology or FISH. Unfortunately, no evidence-based surveillance strategy for CCA development in patients with PSC exists, and current detection methods struggle with low sensitivity and/or specificity.

Several DNA methylation biomarkers have been identified that improve the diagnostic accuracy of CCA. Aberrant DNA methylation has also been reported frequently in precancerous lesions, underscoring the great potential of such biomarkers for early cancer detection. Furthermore, DNA methylation is a stable marker that can be detected with cost-efficient and clinically manageable methods. However, only a handful of studies so far include benign PSC controls and/or PSC complicated with CCA (Fig. 1 and Table 1). Considering that increased DNA methylation has been observed in patients with PSC compared to controls without PSC, the inclusion of controls with PSC is important to identify biomarkers for CCA surveillance in the setting of PSC. We have published one of the few DNA methylation biomarker studies including patients with PSC, where we identified a marker panel in tissue that was validated in biliary brush specimens with high accuracy.<sup>39</sup> Since biliary brush specimens are regularly obtained for standard cytological evaluation when CCA is suspected, DNA methylation analysis of such samples represents a good addition to standard detection methods. However, like regular brush cytology there is a chance of not accessing or obtaining sufficient material from the sites in the biliary tree where CCA develops and consequently missing the tumour cells. Further validation of these markers, preferentially in bile, will therefore be of great interest. Several sources of material might be used for biomarker analyses in addition to bile, including blood and urine. Although non- or minimally invasive

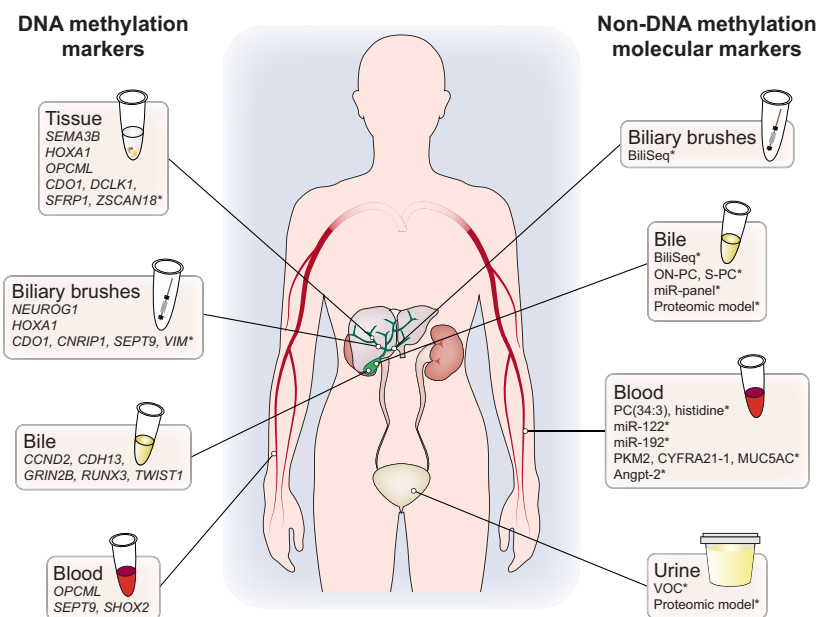


Fig. 1. Promising DNA methylation biomarkers (left) and non-DNA methylation molecular biomarkers for detection of CCA. \*Samples analysed include PSC controls. CCA, cholangiocarcinoma; PSC, primary sclerosing cholangitis.



procedures are preferential, it has been hypothesized that a tumour-derived miRNA profile is more likely to exist in bile than in serum, since CCA-affected biliary epithelium will be in direct contact with bile.<sup>50</sup> We further hypothesize that the same is true for DNA methylation markers. Since cells from the entire bile duct are shed into the bile, analysing DNA from bile may solve the problem of sampling bias using biliary brushes. Still, most DNA methylation studies included in Table 1 use tissue samples, with only a handful of the studies exploring the potential of bile, serum or plasma as sources of DNA methylation biomarkers for CCA detection.

In addition, other studies have focused on developing RNA expression-, miRNA-, or proteomic profiles in bile, urine or blood for more precise detection of CCA in patients with PSC. Some of these profiles have clearly shown the potential to facilitate more accurate CCA diagnosis in patients with PSC, like

PC(34:3) and histidine in serum,<sup>51</sup> or BiliSeq in bile/biliary brushes<sup>27</sup> (Fig. 1, Table 2). However, only a limited number of patients are included in the analyses. Studies focusing on molecular markers have generally included few patients with PSC or PSC complicated by CCA, and the methods used to detect these markers may unfortunately be too laborious and expensive for clinical practice. In this regard, it is important to both validate promising biomarker candidates for CCA in larger and independent cohorts, and to implement new methods for identifying these biomarkers so that they can be used in routine patient care.<sup>52</sup>

To conclude, novel early detection methods for CCA that could be implemented in surveillance algorithms for patients with PSC are warranted. In this context, DNA methylation markers in liquid biopsies may prove to be affordable, feasible and accurate for early CCA detection in PSC and thereby improve survival.

### Abbreviations

BillIN, biliary intraepithelial neoplasia; CA19-9, carbohydrate antigen 19-9; CCA, cholangiocarcinoma; ERCP, endoscopic retrograde cholangiopancreatography; FISH, fluorescent in situ hybridization; IPNL/B, intraductal papillary neoplasm of the liver/bile ducts; PSC, primary sclerosing cholangitis.

### Financial support

This research was funded by South-Eastern Norway Regional Health Authority, grant number 2019074.

### Conflict of interest

The authors declare no conflicts of interest that pertain to this work. Please refer to the accompanying ICMJE disclosure forms for further details.

### Authors' contributions

All authors contributed in the conception and design. HMV drafted the article and all authors contributed in reviewing and editing. All authors have approved the final version.

### Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhepr.2020.100143>.

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