Biomarkers in premalignant conditions of the gastrointestinal tract: Studies on Barrett's esophagus and primary sclerosing cholangitis

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CHAPTER 7

Genetic and epigenetic abnormalities in primary sclerosing cholangitis-associated cholangiocarcinoma


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Primary sclerosing cholangitis (PSC) is a cholestatic liver disease of unknown etiology, characterized by chronic inflammation of the biliary tree with subsequent fibrosis and cirrhosis of the liver. Patients with PSC are at increased risk for the development of cholangiocarcinoma (CCA), a highly malignant epithelial tumor arising from the intra- and extrahepatic bile ducts. Currently, orthotopic liver transplantation is the only curative treatment. The lack of efficient diagnostic methods for early detection and the limited therapeutic options for CCA are major problems and are associated with poor survival. The pathogenesis of PSC-associated CCA is complex and poorly understood. It seems that pro-inflammatory cytokines play an important role in genetic and epigenetic changes that contribute to the carcinogenic process. The mapping of genetic alterations may elucidate molecular targets that may be applied as biomarkers to facilitate early diagnosis of malignant degeneration in order to improve patient outcome. In the last decade the introduction of several novel molecular techniques available for genome-wide screening has advanced our knowledge on many of the genetic abnormalities that are prevalent in CCA and PSC-associated CCA. This review summarizes genetic and epigenetic abnormalities, which have important potential for clinical application.
INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic cholestatic disease of the intra- and extrahepatic bile ducts. It is characterized by diffuse inflammation of the biliary tree and periductal fibrosis, which causes obliteration of the bile ducts. These processes eventually lead to liver cirrhosis for which liver transplantation is the only known curative treatment. Approximately 60-80% of PSC patients are also affected by inflammatory bowel disease (IBD), mostly ulcerative colitis. The exact prevalence of PSC in the population is not known, but it ranges from 0 – 16.2 per 100,000 inhabitants with a trend that seems to increase. True population based studies are lacking but the highest numbers are found in Northern Europe and North America.

Patients with PSC are at increased risk for the development of cholangiocarcinoma (CCA), a highly malignant epithelial tumor arising from the intra- and extrahepatic bile ducts with a dismal prognosis. The development of CCA in PSC can occur at any stage of the disease, but approximately 30% of the CCAs are detected within the first year after the diagnosis of PSC. It can be argued that the development of symptomatic CCA can lead to the diagnosis of unrecognized PSC. The life-time risk of PSC patients for developing CCA ranges from 9-20%. Early diagnosis of CCA in PSC is a challenge as CCA can mimic benign fibrotic strictures on imaging modalities and tumor markers such as CEA and CA 19-9 have poor clinical utility as early diagnostic tool. Because the distinction between malignant and benign strictures may be blurred by the inflammatory effects associated with PSC, the interpretation of routine brush cytology, obtained by ERCP, is difficult. Also the tumor often presents as a ductal infiltrating desmoplastic lesion. Brush cytology obtained by endoscopic retrograde cholangiopancreatography (ERCP) has a very high specificity of 97-100%. In the majority of the studies the sensitivity is poor, ranging from 20 to 62.5%, although results up to 90% have been reported. At present, clinical biomarkers to detect the early neoplastic changes of CCA are lacking. As a consequence, the majority of CCA patients presents with advanced disease, which is associated with poor survival.

The etiology and pathogenesis of PSC is likely to be multifactorial in nature. The strong clinical association with IBD, its link to HLA haplotypes and the high prevalence of autoantibodies suggest that autoimmunity plays a role. Furthermore, evidence about familial occurrence of PSC indicates that genetic predisposition is also a contributing factor to the development of PSC. Similar to other malignancies, the neoplastic transformation of cholangiocytes to CCA may be initiated by inactivation or loss of tumor suppressor genes followed by activation of oncogenes. In PSC, the strong association between biliary dysplasia and malignancy suggests that the development of PSC-associated CCA is a multi-step process following the inflammation-dysplasia-carcinoma sequence. In addition to the potential toxic effect of bile acids on susceptible cholangiocytes in PSC, the release of inflammatory cytokines seem to play an important role in genetic alterations and epigenetic changes that contribute to the process of carcinogenesis.
Improving our knowledge on genetic abnormalities that contribute to the development of CCA is not only essential to understand this tumor’s biological behavior, but may also be utilized as diagnostic biomarkers. Several techniques are now available that allow detection of genetic abnormalities by genome wide screening using only limited amounts of tumor DNA. By applying comparative genome hybridization (CGH) overall losses and gains of chromosomal arms, small genetic abnormalities like losses and gains of specific gene loci can be detected.

In this review, we aimed at summarizing the results of published studies looking at findings of genetic abnormalities in cholangiocarcinogenesis in general, and more specifically in the setting of PSC. We searched Medline and EMBASE from inception to August 2012, using the following search terms in varying combinations in order to identify published articles in English language: “primary sclerosing cholangitis”, “cholangiocarcinoma”, “biliary duct neoplasm”, “genetic alterations”, “comparative genomic hybridization”, “methylation”, and “fluorescence in situ hybridization”. Individual biomarkers were also included in the searches. The reference lists of identified articles were hand-searched for further relevant papers. A summary of genetic alterations found in PSC-associated CCA is shown in table 1.

**Chromosomal Instability**

**Changes in DNA content**

Aneuploidy, a change of DNA content per cell, reflects a heterogeneous category of major DNA abnormalities and is associated with more aggressive malignancies. In patients with malignant biliary strictures, it is reported that the survival of patients with a diploid tumor was significantly longer than those with aneuploid tumors. In a study by Bergquist et al., up to 80% of PSC-associated biliary malignancies displayed aneuploidy compared to 12% of PSC patients with benign strictures. The detection of aneuploidy assessed by digital image analysis (DIA) on brush cytology, showed comparable sensitivity to routine cytology at 43% and 41%, respectively for the diagnosis of PSC-associated CCA when positive and suspicious cytology results were considered positive. However, aneuploidy by DIA had lower specificity than routine cytology at 87% and 97%, respectively. Recently, Halme and colleagues conducted the first prospective study of 102 consecutive PSC patients referred for ERCP with a mean follow-up time of 5 years. There were 42 patients who reached an endpoint of either liver transplantation or diagnosis of biliary malignancy or dysplasia. DNA aneuploidy detected by flow cytometry yielded an overall sensitivity and specificity of 48% and 94%, respectively. Aneuploidy in combination with cytological suspicion improved the sensitivity up to 72%, with a decline in specificity to 82%. In addition, aneuploidy was found in patients who turned out to have low-grade dysplasia. These patients were initially classified as having no dysplasia by routine cytology, suggesting that aneuploidy may have a predictive value as an early diagnostic marker, independent of the cytological diagnosis.
Table 1. Genetic alterations described in PSC-associated CCA

<table>
<thead>
<tr>
<th>Genetic Abnormalities</th>
<th>Author</th>
<th>Year</th>
<th>Cohort</th>
<th>Study Size (N)</th>
<th>PSC (N)</th>
<th>Method</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA content abnormalities</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aneuploidy</td>
<td>Bergquist</td>
<td>2000</td>
<td>PSC-CCA (n=10), PSC without CCA (n=15)</td>
<td>28</td>
<td>28</td>
<td>Flowcytometry</td>
<td>80% in PSC-CCA, 12% in PSC without CCA (p=0.0007).</td>
</tr>
<tr>
<td></td>
<td>Moreno Luna</td>
<td>2006</td>
<td>PSC, 17 developed CCA</td>
<td>86</td>
<td>86</td>
<td>DIA</td>
<td>Sensitivity 43%, specificity 87%.</td>
</tr>
<tr>
<td></td>
<td>Lindberg</td>
<td>2006</td>
<td>PBS including PSC-CCA (n=10) and PSC without CCA (n=25)</td>
<td>159</td>
<td>35</td>
<td>Flowcytometry</td>
<td>Sensitivity 43%, specificity 96%.</td>
</tr>
<tr>
<td></td>
<td>Halme92</td>
<td>2011</td>
<td>PSC</td>
<td>102</td>
<td>102</td>
<td></td>
<td>Sensitivity 48%, specificity 94%.</td>
</tr>
<tr>
<td>Tumor suppressor loci</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p16</td>
<td>Ahrendt49</td>
<td>1999</td>
<td>PSC-associated CCA</td>
<td>10</td>
<td>10</td>
<td>MSA</td>
<td>Allelic loss in 90%.</td>
</tr>
<tr>
<td></td>
<td>Taniai89</td>
<td>2002</td>
<td>PSC (n=10), PSC-CCA (n=10), PBC (n=10), controls (n=10)</td>
<td>40</td>
<td>20</td>
<td>PCR</td>
<td>Mutations in 80% of PSC-CCA, in 50% of PSC without CCA versus 0% in PBC and controls.</td>
</tr>
<tr>
<td>Oncogenes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K-ras</td>
<td>Boberg90</td>
<td>2000</td>
<td>PSC-CCA</td>
<td>33</td>
<td>33</td>
<td>PCR</td>
<td>K-ras mutations in 33% (11/33).</td>
</tr>
<tr>
<td></td>
<td>Ahrendt48</td>
<td>2000</td>
<td>PSC-CCA</td>
<td>12</td>
<td>12</td>
<td>DNA sequencing and a mutation ligation assay</td>
<td>K-ras mutations in 33% (4/12).</td>
</tr>
<tr>
<td>Other</td>
<td>Melum80</td>
<td>2008</td>
<td>PSC (n=365) and healthy controls (n=368), 49 developed PSC-CCA</td>
<td>733</td>
<td>365</td>
<td>Genotyping with SNPs</td>
<td>2 NKG2D SNPs associated with increased risk of CCA; rs1105378: OR, 2.08; 95% CI, 1.31-3.29; rs2617167: OR, 2.32; 95% CI, 1.47-3.66.</td>
</tr>
</tbody>
</table>

Only studies that reported results specified for PSC-associated CCA were recorded in the table. CCA, cholangiocarcinoma; PBS, pancreaticobiliary strictures; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; PSC-CCA, PSA associated CCA; DIA, digital image analysis; PCR, polymerase chain reaction; MSA, microsatellite analysis; NKG2D, natural killer cell receptor G2D; OR, odds ratio.
In spite of their advantages, flow cytometry and DIA offer no additional information about detailed chromosomal aberrations. Other techniques such as fluorescence in situ hybridization (FISH) or CGH are able to evaluate chromosomal abnormalities more in detail. These include more specific detection of losses or gains of chromosomal arms and gene loci.

**Polysomy**

FISH is a technique in which small fluorescently labelled DNA probes are used for the detection of chromosomal and specific gene aberrations. DNA FISH applied on cytology specimen can facilitate the diagnosis of indeterminate biliary strictures, associated with CCA.\textsuperscript{13,33,34} Several studies have shown that chromosome specific probes (chromosome 3, 7 and 17) to detect polysomy, serve as an indicator of chromosomal instability. The sensitivity of conventional cytology varied from 4-21% and increased to 35%-60% when combined with assessment for polysomy with FISH while preserving the specificity of conventional cytology. Similarly, FISH can potentially detect malignant biliary strictures in patients with PSC.\textsuperscript{14,35,36} With FISH, the sensitivity of cytological examination of brush specimens from PSC patients was increased from 50% to 86% in the presence of polysomy. However, the specificity declined from 97% to 83%.\textsuperscript{14} In prospective studies, polysomy also revealed to have prognostic value. PSC patients with serial polysomy were significantly more likely to develop CCA and polysomy may be detected by FISH a year or more before radiological or pathological evidence of carcinoma is observed.\textsuperscript{36} To date, only a few genetic abnormalities using DNA FISH-markers have been evaluated. These include the loss of the 9p21 (p16) gene locus and aberrations for chromosomes 3, 7, and 17 in order to detect polysomy. A summary of the diagnostic performance of FISH-based studies, using brush cytology are outlined in Table 2.

**Comparative genome hybridization (CGH)**

With CGH, it is now possible to screen entire genomes for DNA sequence copy number changes using only small amounts of tumor DNA.\textsuperscript{37} These molecular cytogenetic techniques can potentially assess genetic changes in the development of CCA. To date there are no CGH studies, exploring genetic changes in cholangiocarcinogenesis in the setting of PSC. Compared to other types of solid tumors such as hepatocellular carcinoma, only few cytogenetic studies on CCA in general have been performed. Gains of the chromosomal arms 1q, 7p, 7q, 8q, 17q, 20q and losses of 3p, 4q, 6q, 8p, 9p, 17p and 18q were frequently found in CCA and described in at least two separate studies.\textsuperscript{38–43} Frequently amplified regions included 1q21-q25, 7p21-p22, 15q21-q25, 20q11-q13. In particular, gain of 20q11-q13 is often described which suggests that this region may harbor candidate oncogenes.\textsuperscript{38,40,41,43} Gain of 20q is also a common finding in other solid tumors such as pancreatic and colorectal cancer (CRC).\textsuperscript{38} Recently, TPX2 (20q11) and AURKA (20q13.2) were identified in CRC as two genes that promote 20q amplicon-driven progression from colorectal adenoma to CRC.\textsuperscript{44} On the other hand, frequently deleted regions included 3p13-p21.
Table 2. Review of literature; diagnostic value of FISH on brush cytology in pancreaticobiliary strictures and PSC-associated CCA

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Cohort</th>
<th>Patients (N)</th>
<th>PSC patients (N)</th>
<th>Cut-off values for FISH-positive status</th>
<th>Cytology (malignancy)</th>
<th>FISH (malignancy)</th>
<th>FISH (PSC-CCA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kipp *</td>
<td>2004</td>
<td>PBS</td>
<td>131</td>
<td>71</td>
<td>Polysomy</td>
<td>15%</td>
<td>98%</td>
<td>34%</td>
</tr>
<tr>
<td>Moreno Luna*</td>
<td>2006</td>
<td>PBS</td>
<td>233</td>
<td>86</td>
<td>Polysomy/trisomy 3 or 7</td>
<td>16%</td>
<td>100%</td>
<td>60%</td>
</tr>
<tr>
<td>Barr Fritcher**</td>
<td>2007</td>
<td>PBS</td>
<td>284</td>
<td>107</td>
<td>Polysomy</td>
<td>15%</td>
<td>100%</td>
<td>44%</td>
</tr>
<tr>
<td>Charatcharoen-witthaya**</td>
<td>2008</td>
<td>PSC</td>
<td>230</td>
<td>230</td>
<td>Polysomy/trisomy 3 or 7</td>
<td>8%</td>
<td>100%</td>
<td>NA</td>
</tr>
<tr>
<td>Levy**</td>
<td>2008</td>
<td>PBS</td>
<td>86</td>
<td>34</td>
<td>Trisomy 7</td>
<td>11%</td>
<td>97%</td>
<td>64%</td>
</tr>
<tr>
<td>Barr Fritcher**</td>
<td>2009</td>
<td>PBS</td>
<td>498</td>
<td>189</td>
<td>Polysomy, tri-/Tetrasomy</td>
<td>20%</td>
<td>100%</td>
<td>63%</td>
</tr>
<tr>
<td>Bangarulingam**</td>
<td>2010</td>
<td>PSC</td>
<td>235</td>
<td>235</td>
<td>Polysomy, tri-/Tetrasomy</td>
<td>59%</td>
<td>100%</td>
<td>NA</td>
</tr>
<tr>
<td>Kipp**</td>
<td>2010</td>
<td>PBS</td>
<td>132</td>
<td>40</td>
<td>Polysomy</td>
<td>26%</td>
<td>NR</td>
<td>50%</td>
</tr>
<tr>
<td>Barr Fritcher**</td>
<td>2011</td>
<td>PBS</td>
<td>85</td>
<td>26</td>
<td>Polysomy</td>
<td>24%</td>
<td>97%</td>
<td>62%</td>
</tr>
<tr>
<td>Barr Fritcher**</td>
<td>2011</td>
<td>PSC</td>
<td>30</td>
<td>30</td>
<td>Serial Polysomy</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Gonda**</td>
<td>2012</td>
<td>PBS</td>
<td>50</td>
<td>21</td>
<td>Polysomy p16 deletion</td>
<td>21%</td>
<td>100%</td>
<td>84%</td>
</tr>
<tr>
<td>Smoczynski**</td>
<td>2012</td>
<td>PBS</td>
<td>81</td>
<td>1</td>
<td>Polysomy/trisomy 3 or 7</td>
<td>35%</td>
<td>100%</td>
<td>52%</td>
</tr>
</tbody>
</table>

All studies used a probe set with FISH probes that target the centromeric regions of chromosomes 3, 7, and 17 and the 9p21 band (p16). FISH scoring criteria: Patients are classified into different strata according to the number of copies of the FISH markers per cell: (i) polysomy (≥5 cells showed gains of ≥2 of the 4 probes), (ii) trisomy (if ≥10 cells showed 3 copies of chromosome 7 or 3 and ≤2 copies of the other probes) (iii) tetrasomy (if ≥10 cells showed 4 copies of all probes). * Studies originating from the Mayo Clinic Cohort.

These studies used a higher cut-off for trisomy 7 or trisomy 3 (if ≥10 cells showed 3 copies of chromosome 7 or 3 and 2 or fewer copies of the other 3 probes because signal splitting could lead to false-positive trisomic signals. When polysomy was considered positive for malignancy the sensitivity and specificity were 44% en 100%, respectively, for malignancy and 47% and 100%, respectively, for PSC-related CCA.

When polysomy and trisomy 7 FISH results were considered positive, the sensitivity and specificity were 68% and 81%, respectively.

Most patients had more than one FISH test during repeated ERCP. Patients with any FISH-positive test were considered as positive, even though some of them on subsequent testing had a negative result.

Patients with a polysomy/FISH result without definitive imaging or pathological evidence of malignancy at the time of the first polysomy, were included in the analysis.

Both homozygous and heterozygous deletion of p16 (9p21) were considered positive. Homozygous deletion was considered when both copies were absent in at least 10 cells as per scoring criteria suggested by the manufacturer, or if there was heterozygous deletion in at least 6% of the total cells analyzed.

Abbreviations: PSC, primary sclerosing cholangitis; CCA, cholangiocarcinoma; PBS, pancreaticobiliary strictures; NR, not reported; NA, not applicable.
Although there are common features, the patterns of chromosomal alterations found by CGH differ significantly between studies. This may be partially caused by tumor heterogeneity and differences in etiological background of CCA. Two large studies were performed on CCA case series from Korea, where parasitic infections such as liver flukes, including Opisthorchis viverrini, are the most important risk factors for CCA unlike in Europe where PSC is the most common associated risk factor. DNA micro-array analysis identified a total of 49 genes that were significantly differentially expressed in Opisthorchiasis-associated CCAs and non-Opisthorchiasis-associated CCAs, suggesting that different genetic pathways might play a role in these groups. A summary of the published CGH studies in CCA is presented in Table 3.

**Tumor Suppressor Genes**

**P53 in CCA**
The protein encoded by the p53 tumor suppressor gene is located on the short arm of chromosome 17 (17p13), which is crucial in the regulation of cell cycle progression, DNA repair, and apoptosis. Abnormalities of this gene represent one of the most frequent occurring genetic changes in human cancer. Mutations of this gene result in the synthesis of a P53 protein with a longer half-life leading to protein accumulation, making it detectable by immunohistochemistry. Recently, Wang et al reported in a meta-analysis that patients with an extrahepatic CCA with overexpression of P53 had more advanced disease and worse prognosis. Their study suggested that p53 appeared to be a useful prognostic marker. Mutation of the p53 gene has been found in 30% to 60% of CCAs. In terms of clinical outcome, several studies on P53 protein-overexpression have conflicting results. Only one study has demonstrated that P53 overexpression is an independent prognostic marker in CCA.

**P53 in PSC-associated CCA**
Komori et al. studied the association between chronic inflammation and carcinogenesis in 20 liver tissue specimens from patients with PSC who underwent liver transplantation. They found an overexpression of the inflammatory cytokine activated induced cytidine deaminase (AID) in 93% of resection specimens from patients with CCA. AID was also found in 16/20 explanted livers of PSC patients. Aberrant expression of AID resulted in somatic mutations in tumor-related genes including p53, c-myc and p16. These results might link bile duct inflammation to cancer development by inducing genetic alterations in the biliary epithelium. Rizzi et al. evaluated the expression of P53 with immunohistochemistry in PSC-associated CCA and observed abnormalities of P53 in 78.5% at a late stage in the development of the malignant process. Overexpression of P53 is confirmed in several studies concerning PSC-associated CCA in up to 93%.

**P16 / 9p21**
The tumor suppressor gene p16 is located on human chromosome 9 in the region 9p21 and is frequently silenced in biliary cancer. The diagnosis of adenocarcinoma in indeterminate
Table 3. Comparative genomic hybridization studies reporting regions of copy number alterations

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Cohort</th>
<th>Patients (N)</th>
<th>PSC patients (N)</th>
<th>Method</th>
<th>Loci of interest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rijken(^{97})</td>
<td>1999</td>
<td>CCA</td>
<td>14</td>
<td>NR</td>
<td>CGH</td>
<td>Gain of 8q, 20q, 12p, 17q, Xp, 2q, 6p, 7p, 11q, 13q, 19q. Loss of 18q, 6q, 10p, 8p, 12q, 17p, 7q, 12p, 22q.</td>
</tr>
<tr>
<td>Koo(^{98})</td>
<td>2001</td>
<td>CCA</td>
<td>11</td>
<td>NR</td>
<td>CGH</td>
<td>Gain of 5p, 7p, 8q, 13q, 20q. Loss of 16q, 17p, 18q.</td>
</tr>
<tr>
<td>Shiraishi(^{38})</td>
<td>2001</td>
<td>Biliary tract cancer</td>
<td>42</td>
<td>NR</td>
<td>CGH</td>
<td>Gain of 1q, 7p, 8q, 12p, 17q21, 20q. Loss of 3p, 5q, 6q, 7p, 8p, 9p, 18q.</td>
</tr>
<tr>
<td>Wong(^{39})</td>
<td>2002</td>
<td>CCA</td>
<td>13</td>
<td>0</td>
<td>CGH</td>
<td>Gain of 1q, 3q, 8q, 15q, 17q. Loss of 3p, 4q, 6q, 9p, 17p, 18q.</td>
</tr>
<tr>
<td>Lee(^{41})</td>
<td>2004</td>
<td>CCA</td>
<td>33</td>
<td>NR</td>
<td>DOP--PCR CGH</td>
<td>Gain of 20q, 17, 11q11-13, 8p12-qter, 18p, 15q22-qter, 16p, 6p21, 3q25-qter, 1q41-qter, 5p14-q11.2. Loss of 1p32-pter, 4q.</td>
</tr>
<tr>
<td>Homayounfar(^{42})</td>
<td>2009</td>
<td>CCA</td>
<td>22</td>
<td>NR</td>
<td>CGH</td>
<td>Gain of 1q, 7q, 7p, 8q. Loss of -6q, 3p, 9p, 14q, 13q.</td>
</tr>
<tr>
<td>Miller(^{40})</td>
<td>2009</td>
<td>Biliary tract cancer</td>
<td>34</td>
<td>NR</td>
<td>CGH</td>
<td>Gain of 1q, 3q, 5p, 7p, 7q, 8q, and 20q. Loss of 11p, 3p, 6q, 8p, 9p, and 14q.</td>
</tr>
</tbody>
</table>

Only studies with at least 10 patients with CCA were included in this table.
Abbreviations: PSC, primary sclerosing cholangitis; CCA, cholangiocarcinoma; CGH, comparative genomic hybridization; DOP-PCR CGH, degenerate oligonucleotide primed-comparative genomic hybridization PCR; NR, not reported.
biliary strictures can improve from 58% to 89% with the detection of p16 deletion by FISH in addition to polysomy, as compared to 21% with conventional cytology.\textsuperscript{33} Both promoter hypermethylation (76-83%) and genetic mutation or deletion have been described (20-80%) in biliary cancers.\textsuperscript{33-35} Using methylation specific PCR, bile specimen of 71 patients with biliary disease were evaluated (including 11 PSC patients). About 52% of bile duct specimen of patients with CCA revealed p16 promoter methylation. A similar rate of methylation (46%) was detected in PSC patients without evidence of CCA.\textsuperscript{60} Ahrendt et al. studied paraffin-embedded sections from PSC patients who developed CCA and determined an allelic loss of chromosome 9p21 in 90% of the samples by using microsatellite analysis.\textsuperscript{18} Homozygous deletion of p16 was also frequently detectable in PSC-associated biliary dysplasia. These results suggest that loss of p16 may be useful to detect PSC patients at increased risk for developing CCA.\textsuperscript{59}

\textbf{RASSF1A}

The locus 3p21 has been established as a susceptibility locus for PSC.\textsuperscript{20} RASSF1A, a tumor suppressor gene located at 3p21.3, is implicated in the activation of many human cancers. Inactivation of RASSF1A is mainly caused by aberrant methylation or loss of heterozygosity (LOH). RASSF1A regulates several functional pathways including cell cycle control by inhibition of cyclin D1 expression and modulation of multiple apoptotic pathways. It is suggested that P53 protein specifically binds to the RASSF1A promoter and inhibits its expression.\textsuperscript{61} Concomitant loss of RASSF1A and P53 seemed to further undermine genomic stability, accelerate tumorigenesis, and progression to aneuploidy.\textsuperscript{62} Candidate gene analysis in CCA has revealed frequent deletion of RASSF1A in 40%-60% with concurrent findings of hypermethylation of RASSF1A in simultaneous expression analysis.\textsuperscript{38,39} Inactivation of RASSF1A is associated with an advanced tumor stage and poor prognosis in several human cancers.\textsuperscript{63} In summary, RASSF1A may be considered as a potential biomarker for CCA in PSC patients.

\textbf{ONCOGENES}

\textbf{KRAS}

Several studies have evaluated detection of mutations in KRAS in bile duct specimen. The ras-gene product links growth promoting signals from the cell surface to the nucleus and has a key role in controlling cell growth and differentiation by GTPase activity.\textsuperscript{64} KRAS alterations have been detected in a wide range of 21% to 100% of CCAs in surgically resected tissue samples.\textsuperscript{16,65,66} KRAS has not been proven to be superior to conventional cytology.\textsuperscript{16,34,65,67} Although most patients with KRAS mutation may remain tumor-free even with long follow-up, the presence of KRAS mutations in bile fluid of PSC patients has been associated with malignant progression.\textsuperscript{68} Anderson et al. performed comprehensive molecular (n=104) and genomic (n=69) characterization of surgically resected CCAs. Patients were classified into survival subgroups. Although KRAS mutations were only found in 24.6% of the specimen, the
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Presence of a KRAS mutation was associated with poor overall survival and early recurrence. Furthermore, the subgroup of patients with poor prognosis was characterized by multiple aberrantly regulated oncogenic pathways, including activation of Her2/neu and epidermal growth factor receptor (EGFR) signaling.

**Growth Factors and Receptors**

**EGFR and Her2/neu**

EGFR and human epidermal growth factor receptor 2 (Her2/neu) antagonists have become important biomarkers and are often used in different types of adenocarcinomas. Her2/neu has become a successful target of therapy in advanced breast cancer. This raises the issue whether agents that specifically target these receptors could also be of potential benefit for CCA treatment. It seems that both aberrant EGFR and Her-2/neu expression are associated with carcinogenesis of CCA. However, results in the literature regarding the status of Her-2/neu and EGFR in CCA vary widely. Expression of Her-2/neu mostly assessed on the protein level is seen in up to 82% of CCA cases. To date, only few studies investigated Her-2/neu gene amplification in CCA mostly showing rates below 10%. A study by Yoko Ukita et al. detected amplification of Her-2/neu in all the 22 cases of CCA studied by DNA FISH. Comparable results are found for EGFR. EGFR overexpression is described in 10.7–81% of CCAs and EGFR mutation in 0–15%. These results indicate the need for further studies to assess the contribution of genetic abnormalities of Her-2/neu and EGFR and the overexpression of these receptors.

**NKG2D Polymorphisms and the MICA receptor-ligand**

The natural killer cell receptor G2D (NKG2D) is an activating receptor which is expressed on the surface of T-lymphocytes and natural killer (NK) cells. It plays a significant role in tumor surveillance by NK-cells as the ligands of NKG2D are induced by cellular stress and specifically by some tumor cells. Major histocompatibility complex class I chain related molecule A (MICA) is an important ligand for NKG2D. The 5.1 allele of the gene encoding MICA has been associated with PSC. Melum et al. investigated the influence of genetic variations in the NKG2D receptor by genotyping 365 PSC patients with seven single nucleotide polymorphisms (SNPs) covering the NKG2D-gene. Two of the NKG2D SNPs were significantly associated with an increased risk of CCA. Furthermore, carrier of the MICA 5.1 allele was associated with resistance against CCA.

**Methylation**

Epigenetics is defined as the study of changes in gene expression caused by other mechanisms than changes in DNA sequence. Epigenetic changes, including aberrant DNA methylation, have been described early in carcinogenesis and may serve as targets for therapy or as markers of early progression. Numerous genes are aberrantly methylated in CCA in general including but not limited to, RASSF1A (27-74%), p16 (76-83%), CDH1 (22-43%), TFPI-2 (40%), NPTX2 (40%) and APC (26-46%).
Recently, the first study was published that used an epigenome-wide approach to identify methylation biomarkers in CCA. Cancer lines from the gastrointestinal tract including tumor material from CCA’s with and without concomitant PSC were compared with gene expression profiles of primary tumors and benign controls. A DNA methylation based biomarker panel compromising CDO1, DCLK1, SFRP1 and ZACAN18 yielded a 87% sensitivity and 100% specificity.87

**Conclusion**

Although our understanding of the genetic and epigenetic abnormalities in PSC and its associated CCA has increased in the last decade, there are several gaps in knowledge that need to be explored. In summary, the release of inflammatory cytokines seems to influence the somatic mutation of several tumor-related genes. Aneuploidy and several genes, including p53, p16, EGFR and Her2/neu may also be contributory to the carcinogenic process. Polymorphisms of NKG2D have been associated with an increased risk of CCA in PSC patients. In addition, results of genome wide screening by CGH are yielding novel information about potential gene targets. Gains of 1q, 7p, 7q, 8q, 17q, 20q and losses of 3p, 4q, 6q, 8p, 9p, 17p and 18q are frequently described in CCA. More specifically, the region of RASSF1A and 20q11-q13 offer promising potential as biomarkers. Epigenetic changes are important due to their role in early carcinogenesis. These discoveries offer potential targets for early detection, risk stratification, and therapeutic surveillance in PSC-associated CCA. However, further investigations are clearly needed. The true challenge is finding biomarkers that can reliably and timely predict malignant degeneration, so that resection or liver transplantation is curative. Currently, when CCA is detected in a PSC patient, the prognosis is dismal, irrespective of major surgery.19,88 In most transplant centers, PSC patients with a known CCA are still rejected for liver transplantation. In order to find novel markers or establishing the role of the abovementioned biomarkers, given the rarity of the disease and the approximately 1% annual incidence of cholangiocarcinoma, multicenter initiatives with biobanking are much needed. Within the International PSC Study Group consortium, such an endeavor has now been initiated.

**References**


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Biomarkers in premalignant conditions of the gastrointestinal tract


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