Novel Biomarkers for Cholangiocarcinoma

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Contents

Abstract

The development of cholangiocarcinoma is an uncommon event apart from countries where liver fluke is prevalent. It most commonly occurs as a consequence of chronic inflammation and, therefore, markers of the onset of malignant change need to distinguish between the process of chronic inflammation and neoplastic transformation. Access to samples of tumour is difficult because of its small size but biomarkers have been recognised in plasma, bile and brushings of strictures. The most available biomarkers are derived from the mucus produced by biliary epithelium, where although carbohydrate antigen 19-9 (CA 19-9) and carcinoembryonic antigen (CEA) are frequently relied on for advanced cases where their sensitivity and specificity is about 90 % and 98 %, the diagnostic accuracy is much poorer in early disease. Other mucoproteins have similar results but these markers do not distinguish between other forms of GI cancer. Markers of genetic alterations associated with neoplasia, such as aneuploidy and mutations of *P53*, have been shown to improve the cytological assessment of brushing samples from biliary strictures. Future understanding of the neoplastic mechanism through gene sequencing promises to give a more accurate picture. Proteomic analysis of serum has demonstrated the presence of some interesting proteins with m/z of 4462 and 11535, which add to the diagnostic value of CA19-9 and CEA to diagnose cholangiocarcinoma from patients with other benign diseases and from healthy volunteers. Leucin- rich alpha-2-glycoprotein, LRG1 is an interesting protein identified by the MALDI technique which has been shown to be concentrated in cholangiocarcinoma tissue and in the serum of these patients. When a serum protein panel combines this novel biomarker with CA19-9 and with the inflammatory marker IL-6 the ROC AUC was 0.98. A multiplexmeasure of biomarkers will be required to bring these novel findings into clinical practice.

"Novel" indicates a new kind of nature: strange; previously unknown.

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In some tumors, the understanding of abnormal biochemical pathways and genetic alterations allows for the discovery of interesting new markers to establish the diagnosis and to monitor treatment. Furthermore, these may lead to new and specific therapies; for example, the presence of c-Kit staining in gastrointestinal (GI) stromal tumors indicates that Glivec® should be an efficacious treatment. This chapter reviews some of the current knowledge about our progress with cholangiocarcinoma, an uncommon cancer but of increasing incidence in the Western world. However, in Southeast Asia, there are regions of high incidence due to the prevalence of chronic biliary inflammatory conditions.

Cholangiocarcinoma is generally thought to arise on a background of prolonged inflammatory events in the biliary tree. This inflammation may result from the presence of gallstones, choledochal cyst/s [1], a background of sclerosing cholangitis [2], or following radiotherapy. In the process of malignant transformation, hyperplasia of the biliary mucosa progresses to dysplasia and early carcinoma lesions [3], with subsequent changes in mucus production [4]. The changes are considered to be sufficiently widespread to have an influence on the tissue proteome (the entire complement of proteins expressed by the genome). The frequency of such lesions in patients without underlying pathology is less than 0.5 % of cholangiocarcinomas [4].

Only cholangiocytes, the epithelial cells lining the biliary tree, are considered to have the ability to differentiate into cholangiocarcinoma; although under severe injury or toxicity, they may develop a morphology suggestive of intestinal, pancreatic acinar, hepatocyte, or ductal cell origin. If the process continues to the development of cancer, the malignant cells continue to have a phenotype related to their metaplastic origin. Small hepatocellular carcinomas may imitate cholangiocarcinoma and produce similar mucins [5]. This great heterogeneity in the characteristics of cholangiocarcinoma and gallbladder carcinoma is often observed in the histology of gallbladders at the time of cholecystectomy where metaplasia similar to intestinal, gastric, or pancreatic epithelium is seen in association with dysplasia. It is therefore reasonable to expect that no one biomarker will exist which can distinguish cholangiocarcinoma from other chronic non-cancerous conditions of gastrointestinal ductal epithelium [6].

Knowledge about the biological processes involved in the initiation and progress of cancer has expanded with the ability to undertake cancer gene sequencing and to examine related epigenetic influences [7]. Srirakasa et al. [8] have reviewed hypermethylation of genes involving multiple important pathways related to tumor suppression, apoptosis, cell adhesion, and DNA repair, processes common to many malignant conditions. Interestingly, further investigation seeking to determine the influence of the epigenetic effect of microRNA in the control of protein expression is underway [9, 10].

Table 1 Aims of biomarkers

1. Screening, diagnosis, and prognosis
(a) To discover candidate biomarkers
(b) To quantify sensitivity and specificity of biomarkers
(c) To monitor outcome of treatment
2. Therapy efficacy
(a) To evaluate biomarkers in clinical trials
(b) To determine the dose effect of a treatment
(c) To identify new therapeutic possibilities
3. Prediction of therapy response
(a) To identify novel targets and/or pathways
(b) To identify agents which predict clinical efficacy
(c) To develop markers which predict response to specific therapy

A number of benign strictures may be confused with cholangiocarcinoma, including primary sclerosing cholangitis, follicular cholangitis, and sclerosing cholangitis with granulocytic epithelial lesion [11]. An important new differential diagnosis is autoimmune IgG4-related sclerosing cholangitis, which is typified by histopathology demonstrating a characteristic lymphoplasmacytic infiltrate of CD4- or CD8-positive lymphocytes and IgG4-positive plasma cells, and exhibits interstitial fibrosis and acinar cell atrophy in later stages. Accurate diagnosis is significant because autoimmune IgG4-related sclerosing cholangitis can be treated with steroids, thus avoiding surgical intervention. It usually develops with diverse manifestations such as autoimmune pancreatitis, retroperitoneal fibrosis, and tubulointerstitial nephritis, and although there may be elevated blood levels of IgG4, this feature is not always present. Therefore, it is most important to make a definitive diagnosis which may require a biopsy including analysis for specific biomarkers.

Biomarkers can assume many functions (Table 1). The ideal of having a single blood test which could establish a specific diagnosis of chloangiocarcinoma is very difficult to achieve. This is particularly so in a rare condition because only a small false-negative rate would make the test impractical for screening purposes. In subgroups of patients at high risk of cholangiocarcinoma, such as primary sclerosing cholangitis (PSC) where there is a dominant stricture, the pretest probability of a malignant cause may be as high as 15 % (http://www.gi.org/patients/gihealth/sclerosing.asp). In these cases, a hypothetical test with a sensitivity and specificity of 90 % would result in a posttest probability of 65 %, i.e., a result of clinical value. Preliminary results using proteomic techniques are now approaching these values. Similar tests can be undertaken on bile, but

difficulties with the preparation of the sample make reliable results hard to achieve.

Biomarkers on tissue specimens (incision or excision biopsies for histology, fine needle aspiration biopsies, and brushings from bile duct strictures) may also be useful for improving the accuracy of diagnosis and prognostication. Identification of these biomarkers requires an understanding of the complex biology of cholangiocarcinoma.

1 Biological Considerations for Understanding of Biomarker Concepts

Cholangiocytes are arranged in a single layer and have important and diverse functions which affect bile flow and prevent the absorption of toxic substances in bile. They are also closely associated with dendritic cells as a protection from bacteria and other antigens. Cholangiocytes are strongly connected by cytokeratins, and they secrete bicarbonate and a number of specialized mucins to provide protection from the bile [12]. One area of importance when searching for new biomarkers is the rich mucin pool derived from cholangiocytes. The established serum biomarkers, carcino-embryonic antigen (CEA), and cancer antigen (CA) 19-9 are glycoproteins that are useful for monitoring the progress of treatment, but their sensitivity and specificity (60–80 %) make them poor diagnostic biomarkers, particularly because they are elevated in chronic inflammatory conditions which lead to the induction of cholangiocarcinoma. As they are also frequently elevated in other malignant conditions of the GI tract, they are poor discriminators between cancers of the GI tract.

Along with the production of mucin, cholangiocytes produce trefoil factor family (TFF) peptides which also protect cholangiocytes and act as receptors, inducing hyperplasia or apoptosis. These proteins have intense cross-linking with sulfur bridges. The synthesis and release of TFFs are regulated by a number of environmental and local agents, estrogens, and pro-inflammatory and anti-inflammatory cytokines [13]. It is worth noting that the majority of cholangiocarcinoma specimens demonstrate an increased production of spliced TFF2 and that when this occurs, it confers a better survival [14].

The significance of MUC mucins in the developing and the adult liver, various hepatobiliary diseases, and intrahepatic cholangiocarcinoma has recently been reviewed [15].

1.1 Importance of Glycoproteins for Cholangiocytes

When chronic inflammation induces metaplasia, this may take on an intestinal, gastric, or pancreatic appearance. Inflammatory biliary conditions and tumors of the biliary tree are associated with altered expression of mucins. It is interesting that alteration of mucin production begins as early as the process of metaplasia leading to dysplasia and that this early switch is carried on through the malignant progression of cholangiocarcinomas [16]. Histological assessment of tissues may gain important diagnostic and prognostic information from the immunohistochemical study of the many mucins related to cholangiocarcinoma. When the metaplasia is of gastric cell type, it is likely to be associated with the production of *MUC1*, while metaplasia of intestinal cell arrangement is associated with *MUC2* over-production, implying slightly different malignant potential.

Hughes et al. [17] found that most cases of dysplastic biliary epithelium and cholangiocarcinoma display a Brunner or pyloric gland cell phenotype and a gastric foveolar cell phenotype. However, while aggressive invasive cholangiocarcinoma frequently is associated with *MUC1* over-expression, altered *MUC1* gene expression also occurs in inflammatory diseases and carcinomas of the GI tract and breast [18, 19], making *MUC1* a poor discriminator between tumors.

Cholangiocarcinomas with a better prognosis, particularly those of intraduct papillary type, produce large quantities of gelatinous mucin which is predominantly *MUC2*. Notably, there is a similar progression from preinvasive lesions in the pancreas with mucin production having a dichotomy in the dysplasia-CIS-invasive carcinoma sequence. In a study of 268 pancreatic tumors, 54 % of the intraductal papillary mucinous neoplasms expressed *MUC2*, whereas none of the pancreatic intraepithelial neoplasms (PanINs) did. In contrast, PanINs, especially higher-grade lesions, were often positive for *MUC1* (61 % of PanIN-3), whereas the expression of this glycoprotein was infrequent in intraductal papillary mucinous neoplasms (20 %). This dichotomy was further accentuated in the invasive carcinoma group [20]. The *MUC2* expression in the intrahepatic biliary system, including intestinal metaplasia, intraductal papillary tumors, and mucinous carcinoma, is dependent on the *CDX2* homeobox gene, which induces intestinal differentiation [21, 22].

Over-expression of mucins *MUC4* and *MUC5AC* has also been observed in the early phase of the development of hyperplasia and dysplasia in cholangiocarcinoma [15]. *MUC4* is a novel intramembrane ligand for receptor tyrosine kinase ErbB2 (HER-2) [23], which has been shown to be associated with a poorer prognosis in patients with mass-forming intrahepatic cholangiocarcinoma [24]. The expression of *MUC5AC* was associated with the dysplasia–carcinoma sequence.

In summary, tumors which predominantly express the gelatinous mucins *MUC2*, *MUC5AC*, *MUC5B*, and *MUC6*

are more likely to have a good prognosis, while those associated with the transmembrane mucins *MUC1*, *MUC3*, *MUC4*, *MUC12*, and *MUC17* have a poorer prognosis.

In a study of four cases of oncocytic biliary intraductal papillary neoplasms (IPNs), the IPNs were composed of distinctive oncocytic cells. The invasive carcinomas accompanying two of the cases were also composed of oncocytes. None of the cases showed aberrant expression of the Wnt/β-catenin pathway proteins which frequently have a central role regulating cell fate decisions in neoplasia by integrating signals from many other pathways, including retinoic acid, FGF, TGF-β, and BMP. Despite this, cyclin D1 was markedly over-expressed in all four cases. Three of four cases had positive staining for *MUC3*, *MUC4*, *MUC5AC*, *MUC5B*, and *MUC6*. Thus, the Wnt pathway proteins (especially beta-catenin and E-cadherin) are expressed normally in oncocytic variants of IPNs of the biliary tree, and the mucin profile is similar to their counterparts in the pancreas [25].

Diagnosis of cholangiocarcinoma is further complicated by the presence of intrahepatic peribiliary glands, which, particularly when dysplastic, add to the complexity of the microscopic appearance of the biliary tree. These glands are present in the large intrahepatic bile ducts [26–28]. The lobules of branched tubuloalveolar seromucous glands communicate with bile ducts via conduits [29] with serous, mucous, and endocrine cells which stain positively for somatostatin, serotonin, and pancreatic polypeptide [30, 31], which adds to the variety of cell types which may become malignant. These glands have been shown to secrete a seromucin rich in amylase and lipase [32]. As well, the bile duct wall intramural glands have sparsely branching tubular mucus glands with tall columnar cells. These glands could be confused with invasive carcinoma.

At the ampulla of Vater, the distinction between tumors arising from biliary, intestinal, or pancreatic tissue may be helped by a study of the mucus subtypes. Ampullary tumors can be classified histologically as either intestinal type or pancreaticobiliary type and display different features according to tumor location, association with adenoma, and *MUC2* expression. Furthermore, KRAS mutation is supposed to be associated with tumors arising in the area from the ampulloduodenum to the ampullopancreatic duct, with metaplastic mucus occurring in both intestinal and pancreaticobiliary types [33].

2 CA19-9 and CEA: Current Markers for Cholangiocarcinoma

CA19-9 and CEA are the established tumor markers with clinical utility in the management of cholangiocarcinoma and gallbladder carcinoma. Numerous studies show that the mean values for these markers are elevated in patients with these carcinomas above those of patients presenting similarly who are found to have benign pathology [34]. However, there are numerous reasons for the limited value of these markers. Firstly, they can be extremely high [35, 36] in some patients with benign conditions, but this may be partly adjusted for by dividing the CA19-9 value by the serum CRP concentration, because the benign cases are frequently associated with inflammation [37]. Even with adjustment, however, the sensitivity, specificity, and positive predictive values remain low at 76.5, 68.6, and 70.9 %, respectively. Secondly, both CA19-9 and CEA are elevated in patients with other forms of gastrointestinal cancer and indeed cancers of the genitourinary system. Lastly, CA19-9 cannot be demonstrated in about 10 % of the population who have Lewis negative blood factors [38]. The use of these tumor markers for diagnosis of cholangiocarcinoma in patients with PSC is unfortunately not as valuable as previously reported [39]. The serum levels of CA19-9 frequently rise temporarily in association with a "biochemical relapse" of PSC (shown by increased values of serum alkaline phosphatase). However, although the marker product of CA19-9 and CEA has a low sensitivity, it has a relatively high specificity for the detection of cholangiocarcinoma in PSC patients [40]. Therefore, assessment of patients with elevated values needs to be made with the knowledge of these variations. These markers are of most value when used in conjunction with other tests, such as radiological findings.

An important drawback of CA19-9 as a tumor marker is that it does not detect early disease. A study of 208 patients with PSC who were followed longitudinally for 5 years with a cutoff of change in CA19-9 concentration of 63.2 U/ml gave 90 % sensitivity and 98 % specificity. However, only two of the 14 patients identified with cholangiocarcinoma were candidates for curative resection. Further, in a study of 866 patients with a presentation of general biliary symptoms, CA19-9 was investigated as a screening test for early pancreatic or biliary cancer. Of 117 subjects with an elevated level above the normal range, 115 did not develop a biliary or pancreatic malignancy after 2-years follow-up and therefore had a false-positive result [41]. Thus, a test with such a low specificity as CA19-9 is quite unacceptable as a screening test.

3 Use of CA15-3 and CA27.29 for Screening, Diagnosis, and Staging

Assays of the markers CA15-3 and CA27.29 are well characterized for the detection of circulating *MUC1* antigen in peripheral blood. This circulating marker has prognostic relevance in early-stage breast cancer [42]. The production

of *MUC1* in breast cancer is very limited compared to that in cholangiocytes, and yet, this topic has been more extensively studied in relation to breast cancer. Given the importance of mucin production by cholangiocytes, it is perhaps surprising that there is a dearth of publications studying the usefulness of such measures for the management of gallbladder carcinoma and cholangiocarcinoma. Two general types of assay measuring *MUC1* gene-derived glycoprotein are used: The assays for CA15-3 are sandwich assays, while those for CA27.29 are competitive assays. These types of assay measure slightly different parts of this tandem-repeat molecule. As long as the tests are calibrated carefully, CA15-3 and CA27.29 measurement of *MUC1* gives comparable results [43]. While it is likely that serum tumor markers CA15-3 and CA27.29 have prognostic value, their role in the management of early-stage breast cancer is unclear [44], and although they have value in detecting recurrence [45], there is no prospective randomized clinical trial to demonstrate survival benefit and so their role remains uncertain [46]. CA15-3 or CA27.29 can be used in conjunction with diagnostic imaging, history, and physical examination for the monitoring of patients with metastatic disease during active therapy, but they should not be used in isolation.

An interesting cross-sectional study evaluating two GI markers (CA19-9 and CEA) and four breast cancer markers (CA27.29, CA15-3, MCA and CEA) in 213 patients demonstrated sensitivity of 90 %, but specificity was 40.3 % for CEA and 32.3 % for CA19-9 when GI tumors were compared to benign GI disease. This was not as good as the result for breast cancer where a sensitivity of 90 % and specificity of 70 % was obtained for CA27.29, 67.5 % for CA15-3, 52.5 % for MCA, and 40 % for CEA. Comparison of breast cancer and GI malignancies with other malignancies leads to a marked shift of the receiver operating characteristic (ROC) curve to the right and loss of specificity. High serum antigen levels were found in late-stage tumors. Further, the presence of liver metastases in breast cancer was associated with abnormal levels of CA27.29 ($P = 0.028$). Pancreatic adenocarcinomas had a higher CA19-9 antigen level ($P < 0.001$) than other GI malignancies. None of the above markers retains its specificity for pancreaticobiliary cancer when compared with a control group consisting of other malignancies [47].

4 Markers of Proliferation

Markers of cellular proliferation can be obtained from tissue samples. For many tumors, such markers can be used as predictors of a poorer prognosis. In general, markers of elevated proliferative rate correlate with a worse prognosis in untreated patients and may predict benefit from chemotherapy [48]. The implementation of DNA flow cytometry to measure proliferative rate is complicated by variation in methods of tissue preparation, differences in instrumentation, and methods for converting information on the histograms to the estimate of the cell cycle S-phase. In addition, interpretation of individual studies is difficult because many are too small to have statistical power, cutoffs have not been prospectively defined, and study populations have not been controlled for adjuvant systemic treatments.

A small number of studies have examined the value of measuring cellular proliferation in managing cholangiocarcinoma. The utility of identifying aneuploidy has been demonstrated in samples taken from paraffin blocks, indicating that this may also be a clinically useful approach in managing cholangiocarcinoma [49]. DNA flow cytometry determination of S-phase is one of the several markers of proliferative rate in tumor specimens, which is applicable to cytology specimens from the biopsy of masses or brush cytology at the time of endoscopic retrograde cholangiopancreatography (ERCP). In pancreatic cancer, aneuploidy has been shown to be predictive of a poorer outcome. Aneuploidy was associated with higher-than-normal levels of other biological markers of prognosis such as HER-2 [50]. Despite these findings, measures of proliferation rate in cholangiocarcinoma are not routinely used in clinical practice.

DNA analysis has been shown to add to the accuracy of CA19-9 and CEA for the diagnosis of cholangiocarcinoma in bile duct strictures. In 57 patients with a diagnosis of PSC undergoing ERCP, brush samples were taken from strictures for cytology and DNA analysis by flow cytometry to obtain measures of proliferation. The tumor markers CA19-9 and CEA were determined both in serum and bile fluid. Thirty-nine patients were found to have malignant strictures (seven with PSC), and a diagnostic sensitivity of 100 % and specificity of 85 % were reached when the results of brush cytology, DNA analysis, serum CA19-9, and serum CEA were combined. Analyses of CA19-9 and CEA in bile fluid yielded no diagnostic significance. The authors concluded that the combination of positive brush cytology at ERCP plus aneuploidy improves the results of serum CA19-9 and CEA. The results were valuable for distinguishing between malignant and benign biliary strictures, especially in PSC patients [51]. A recent review supports the use of fluorescent in situ hybridization (FISH) to identify cells with chromosomal abnormalities to improve sensitivity from that of routine cytology and digital image analysis to identify aneuploidy, but the sensitivity remains low at 40 % [52]. Examination of specific genetic changes in the biliary epithelium may give insights into these important mechanisms and improve our diagnostic ability.

Table 2 Studies of *P53* in cholangiocarcinoma

Reference	Number of cases	*P53* protein expression	
		Percent (%)	Effect on survival
Ahrendt et al. [95]	12	50	Reduced survival
Bergan et al. [96]	60 ductal type	25	Reduced survival: 0.76 vs. 1.4 years
	22 intestinal	50	
Cong et al. [97]	22	37	Reduced survival
Havlik et al. [98]	29		Reduced survival
Isa et al. [99]	23	21	No effect
Jarnagin et al. [65]	128	27	None, but effect of p27 and Mdm2 seen
Kim et al. [100]	25	37	No effect
Liu et al. [58]	36	51	Reduced survival
Kuroda et al. [66]	55	32	Reduced survival
Tannapfel et al. [101]	41	32	Reduced survival
Washington and Gottfried [102]	41	58	No effect
Shin et al. [59]	36	61	Reduced survival
Wang [107]	294		Meta-analysis reduced survival but not definitive

5 *P53* as a Marker for Cholangiocarcinoma

Inactivation of the tumor suppressor gene *p53*, either by mutation or by methylation, is the most common genetic abnormality in human cancer and has been implicated as a late event in the genesis of cholangiocarcinoma [53] and in gallbladder carcinogenesis [54]. Germline abnormalities appear to have a poor association with the onset of cholangiocarcinoma [55]. It is therefore assumed that the onset is caused by the exposure of cholangiocytes to toxic substances excreted in bile. *P53* (protein) may be measured in paraffin-fixed tissue by immunohistochemistry (IHC) and *p53* genetic changes by gene sequencing. *P53* is accumulated in the nucleus in up to 50 % of cholangiocarcinoma cases, reflecting a minor abnormality of the protein and an inhibition of its natural degradation. Notably, about 90 different mutations of *p53* have been recognized and there is little difference in the nature of these along the course of the biliary tree. The structure and function of *p53* and its role in linking cancer to specific carcinogens by way of mutational signatures have been reviewed [56], and recently, the ratio of two different isoforms, 133p53/Tap53, was shown to be a potential prognostic biomarker. In a study of 36 patients with cholangiocarcinoma [57], clinical outcome was compared for abnormalities of sequencing of *p53* gene in the region of exon 5–8 and for *P53* protein accumulation to find which measure is the better predictor of outcome. *p53* gene mutations were found in 22 of 36 (61.1 %) patients, and for *P53* protein, expression was positive in 19 of 36 (52.8 %) patients. There were significant differences in the extent of differentiation and invasion between tumors with positive and negative expression of *P53* protein. However, there were no significant differences in pathologic parameters between the mutated and non-mutated tumors. The authors concluded that the identification of alterations of the *p53* gene evaluated by DNA sequence analysis is relatively accurate, but despite this, the over-expression of *P53* protein could not act as an independent index to estimate the prognosis of cholangiocarcinoma [58]. Fluke-associated cholangiocarcinoma appears more likely to over-express *p53* than sporadic cholangiocarcinoma. This may be because of the greater likelihood of an intestinal goblet cell phenotype which over-expresses *p53* arising in fluke-associated cholangiocarcinoma as with gallbladder cancer [54]. Differences in the aetiopathology of the cancers may reflect different pathways to the development of cholangiocarcinoma [17].

Several studies of patients with cholangiocarcinoma suggest that high tissue *P53* protein levels measured by IHC or mutations or deletions in the *p53* gene measured by single-strand conformational gel electrophoresis, manual sequencing, or allele-specific polymerase chain reaction (PCR) appear to predict poor outcome (Table 2). Results in studies showing no effect of *P53* accumulation on survival may have been affected by small study numbers. These studies indicate that about 36 % of cases accumulate *P53* in the nucleus and that in these cases, there is a poorer survival outcome. However, it seems unlikely that for IHC, *P53* will provide sufficient accurate results to be clinically useful, given that it detects both mutated *p53* and stabilized wild-type *p53*, and conversely will miss *p53* deletions. This is confirmed by a study where the patients with wild-type *P53* exhibited longer overall survival than those with defective *P53* [57]. Methods

to define genetic abnormalities in *p53* more precisely and conveniently might determine specific mutations of *p53* which strongly correlate with clinical outcomes and may be a predictor of benefit from systemic therapies. However, at present, methodologies to do so are cumbersome, expensive, and not widely available as routine clinical assays, limiting the utility of this marker in clinical practice. Furthermore, no prospective studies assessing clinical benefits using these new techniques have been published.

6 Markers of Epigenetic Influences on Gene Function

This is a rapidly progressing field with the advent of gene sequencing and the knowledge of the importance of epigenetic regulators of RNA function for many neoplasms. Recent development of epigenetic evaluation of cancer has demonstrated systematic aberrations where methylation silences specific genes. In particular, cholangiocarcinoma has consistent changes in *CDO1*, *DCLK1*, *SFRP1*, and *ZSCAN18* [7]. These genetic abnormalities were seen to occur in cell lines and fresh frozen samples of cholangiocarcinoma and were confirmed in paraffin blocks. When these potential biomarkers were combined as a panel of four, the sensitivity and specificity were 100 % in fresh frozen samples, but the sensitivity fell back to 87 % when tested in validation paraffin samples in this relatively small cohort. The advantage of this technology is that the DNA is stable in bile and so can be measured in bile collected at ERCP or other forms of biliary drainage. Unfortunately, the effect of dilution in bile collections reduces the sensitivity of the method. In many cases, brush cytology is available and this material has been a useful means of obtaining samples for epigenetic studies. Shin and colleagues used a five-marker panel of *CCND2*, *CDH13*, *GRIN2B*, *RUNX3*, *and TWIST1,* which improved the sensitivity of cytology from 43 to 83 % [59].

Another study reported *OPCML* methylation in 72 % of cholangiocarcinoma specimens which was not found in the uninvolved adjacent tissue. Previous studies have demonstrated that *OPCML* methylation in cholangiocarcinoma is associated with poorer differentiation and as such it should be a marker of poor outcome [60].

7 Urokinase Plasminogen Activator (uPA), Its Receptor uPAR, and Plasminogen Activator Inhibitor 2 (PAI-2) as Markers of Invasiveness in Cholangiocarcinoma

The uPA system has been shown to increase invasiveness, and increased expression of these factors has been associated with poor outcome in some cancers. This system involves a cell surface receptor, uPAR, which becomes active when the uPA protein binds to it. Activation of the uPA/uPAR mechanism may be inhibited by the small proteins PAI-1 and PAI-2. Studies of pancreatico-biliary cancers indicate that poor outcome is predicted by increased expression of uPA and uPAR and further that PAI-2 is an independent predictor of improved outcome by suppression of the uPAR mechanism. Several assay formats for these markers have been evaluated, including IHC, quantitative real-time reverse transcriptase polymerase chain reaction (qRT)-PCR, and enzyme-linked immunosorbent assays (ELISA) [61]. Both qRT-PCR and IHC have been shown to be predictive of survival [62] and to indicate the presence of lymph node metastasis in cholangiocarcinoma [63].

8 Expression of Cathepsin and Cyclin Proteins as Markers of Tumor Progression in Cholangiocarcinoma

Present data are insufficient to recommend use of cathepsin measurements for management of patients with cholangiocarcinoma although studies indicate that different cathepsins are involved in the mechanism of metastasis [64].

Similarly, the cyclin proteins which are expressed in the late G1 phase and promote the transition to the S-phase of the cell cycle are abnormally expressed in some cases of cholangiocarcinoma [25, 65, 66]. They can be measured by IHC in formalin-fixed paraffin-embedded (FFPE) tissue, and mRNA for cyclin E has been quantitated by RT-PCR in fresh frozen specimens [67]. Low molecular weight (LMW) forms of cyclin E have been measured by Western blot analysis of proteins in fresh frozen tissue [68]. Discordance between IHC and Western blot analysis in assessment of the prognostic value of cyclin E may be related to the antibodies used for each assay, given that the reagents that detect intact cyclin E may not react with the LMW fragments. Further work is required to demonstrate the role of these markers in the management of hepatobiliary tumors.

It is considered that the location of a cholangiocarcinoma may be related to the etiology of that tumor which may influence the pathways in the dysplasia–carcinoma sequence. In a study of cell cycle proteins, tissue arrays from tumors at different sites in the biliary tree have been examined by IHC. p27, Cyclin D1, and Bcl2 were more frequently over-expressed in proximal tumors, while *p53* and Mdm2 were more frequently over-expressed in distal tumors. While cholangiocarcinomas differentially express cell cycle regulatory proteins based on tumor location and morphology, these differences were not sufficiently distinct to be of diagnostic importance. Vascular invasion, lymph node metastases, absence of p27 expression, and Mdm2 over-expression independently predicted poor outcome on

Table 3 Proteomic techniques in use

Method	Description	Advantage
2-D gel electrophoresis (2-DE)	Uses isoelectric properties and SDS-PAGE gel electrophoresis to separate protein spots. Discovered proteins are biased toward abundant proteins [76]. 2-DE does not identify proteins which are small, very basic, very acidic, or hydrophobic. 2-DE is a slow process	MS can subsequently identify the proteins of interest, now aided by new software for analysis of protein spots [74]
MALDI-TOF	Matrix solution + sample are dried on glass slide. A laser directed at surface ionizes the complex. The ionized complex is accelerated through an electric potential along a flight tube to a detector. The time of flight is related to the mass-to-charge ratio (m/z) of the compound	Measures proteins up to 30 kDa. Can be helpful in sequencing of proteins and oligonucleids
SELDI-TOF MS	Similar principles to MALDI-TOF, but the glass chips have specific surfaces to select a subset of proteins. This is then covered by a matrix. The m/z of the proteins in the sample is measured by time-of-flight technique. The identification of unknown proteins requires further separation of the sample	Can identify patterns of proteins in low concentration. Not easy to collect individual proteins for identification
Multiplex ELISA	Multiple antibodies placed in different wells, measured by luminescence. Each antibody may require different test conditions	A multiplex phosopoarray study demonstrated cholangiocarcinoma survival was associated with increased tissue pAKT and pMTOR and reduced pTEN [103]
Phage display	Screens for protein–protein and protein–DNA interactions using genetic sequences from a DNA library of interactions. Many proteins can be tested at the same time by integrating their sequence into a suitable phage	Suitable for testing large sample sizes
SILAC	Stands for "stable isotope labeling by amino acids in cell culture." Measures in vivo incorporation of specific amino acids into mammalian proteins [75]	Has identified proteins which are up-regulated in cholangiocarcinoma tissue [104]
Protein microarray	Different proteins are affixed in ordered fashion to a glass slide. Substrates, e.g., protein kinase, or biologically active small molecules, are identified when they bind, by luminescence or similar technique	Has been recently reviewed as an emerging technique to improve cancer diagnosis and prognosis [105]
Aptamer microarray	An aptamer is a nucleic acid (DNA or RNA) or a peptide macromolecule that binds tightly to a specific molecular target. They can be attached to nanoparticles and therefore help target diagnostic or therapeutic agents	Binds 1,000-fold more tightly than many factors. This technology is progressing rapidly and has been recently reviewed [106]

multivariate analysis, and there may be prognostic roles for the proteins Mdm2 and p27. However, these measures did not provide a strong guide for prognosis [65].

9 Proteomic Analysis of Biliary Carcinoma

New technology is revealing a complex array of proteins and peptides in tissue and blood samples and that the pattern of these is distinct for different conditions. Various mixtures of truncated peptide fragments, or of modifications of proteins or peptides, such as glycosylation, cysteinylation, lipidation, and glutathionylation, require careful evaluation to determine their biological role and the value of this new knowledge for improved diagnosis and therapeutic possibilities. It is expected that these differences, either in tissue, in the circulation, or in secreted fluids, will be sufficiently specific to evaluate many different clinical questions. For proteomic pattern analysis, computer-based algorithms have been developed to distinguish bile duct cancer from benign diseases [34]. More work is required on larger numbers of samples from patients to answer specific questions such as identifying the proteins which distinguish patients with PSC from those with cholangiocarcinoma.

Protein expression in tumors reflects the activation of biological pathways, and the degree of activation of these pathways is predictive of patient outcome [69]. Furthermore, tissue may be available for proteomic assessment from samples taken at surgery and through needle biopsy and from FNA or ERCP with cytology. Although cancer mechanisms are best studied in the cancer cells taken with laser dissection, many of the samples acquired include stroma. However, stroma may also hold important messages about cancer biology because the migration of tumor cells relies on an interaction with the stroma and the immune system through dendritic cells. Therefore, many opportunities exist for the discovery of new markers in the holistic cancer biology mechanism. These may be of necessity in low concentrations.

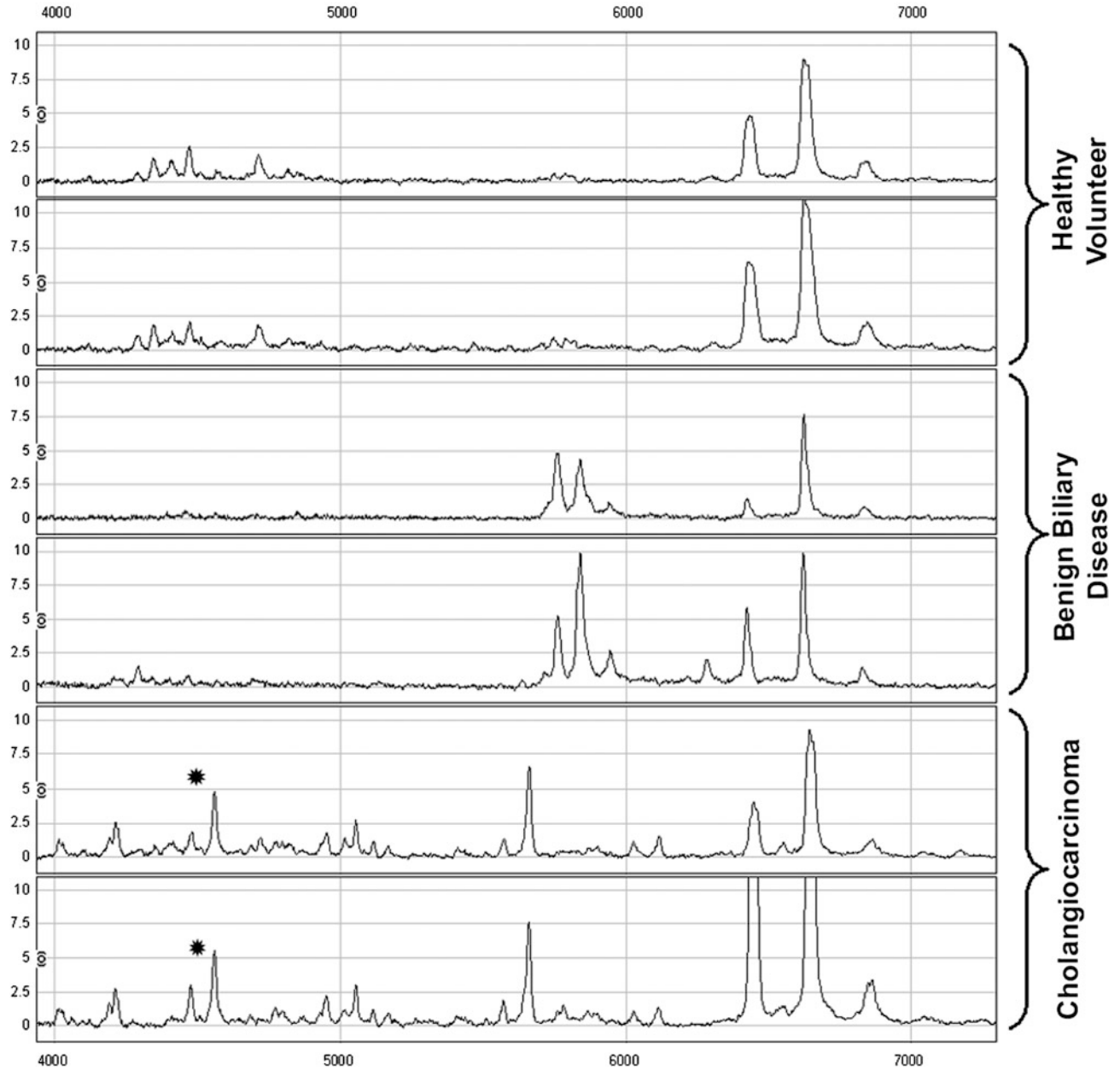

Fig. 1 The printout of the protein mass profile for a small segment of the SELDI MS curve. Results are from the spectrum of two subjects from each of the groups: healthy controls, benign biliary disease, and cholangiocarcinoma. The *asterisk marks* the m/z 4462 peak. Modified from Scarlett et al. [34]

Many technological advances allow the assessment of numerous proteins at very low concentrations, which is useful in analysis of serum. The majority of serum proteins which differentiate patients with cancer from those without are actually not derived from the neoplastic cells, but are host-specific proteins originating in tissues such stroma, liver, or immunological material [70]. New methods that allow isolation of low abundance serum proteins which are more likely to represent tumor markers are in development [71, 72]. Once a number of candidate proteins have been identified and a limited panel is shown to be discriminatory for the tumor of interest, they may be measured by IHC or serum-based immunoassays. Markers can then be validated individually or in combination as a profile or signature. The development of a clinically valuable panel will require validation on a large independent sample set. Although many teams are working to this end, many large collaborative studies are still needed to validate these results.

9.1 Proteomic Pattern Analysis

Analysis of multiple proteins or peptide fragments simultaneously can be approached in several ways, and each has its positive and negative features [73]. Some of these methods include multiplex ELISA, phage display, and aptamer arrays and are summarized in Table 3 [74–76]. However, the most widely studied methods involve identification of proteomic profiles as peaks on mass spectrometric (MS) analysis with precise charge-to-mass ratios. In some cases, proteins have been designated by their apparent molecular weight and isoelectric point within two-dimensional (2-D) gel analysis [76]. Specific peptides can be identified from their amino acid sequence identity or homology to known proteins or their fragments. Some studies have used whole tumor specimens that include both epithelial cells and stroma, whereas others have used microdissected epithelial cells. If isolation of epithelial cells is not required, a fine-needle aspirate can provide adequate material [76]. Before mass spectroscopic analysis, preliminary separation of proteins can be performed with 2-D gel analysis or by binding of proteins to chips or specific surfaces to attract subsets of proteins, called surface-enhanced laser desorption and ionization (SELDI) [77] and matrix-associated laser desorption and ionization (MALDI) [78, 79], respectively. After desorption and ionization, the pattern of charged peptides generally has been analyzed by time-of-flight (TOF) mass spectroscopy. While these methods are excellent for measuring many proteins of low abundance, it may not be necessary to identify the protein in each peak, which may be very difficult when they are of low abundance [80]. Demonstrating a pattern of proteins associated with a cancer type may be sufficient to help make a diagnosis. The multiplex ELISA method can also be used to detect several different proteins simultaneously [81]. In addition, multiple peptides can be measured by phage displays or aptamers [82, 83]. Indeed, screening protein arrays with sera from patients with cancer would facilitate the identification of autoantibody signatures that can be used for diagnosis and/or prognosis of patients. The usefulness of multiplexed measurements lies not only in the ability to screen many individual marker candidates but also in evaluating the use of multiple markers in combination. The advantage of protein and serum screening of peptides and cDNA repertoires displayed on phages as well as the fabrication of protein microarrays for probing immune responses in patients has been recently reviewed [82].

9.2 Proteomic Pattern Analysis

Proteomic pattern analysis was a new field in 2007, when there were 362 articles listed in PubMed containing the key words "proteomic analysis" and "neoplasms." By 2012, these had increased to 2,069. SELDI-TOF was originally used to profile proteins in serum and tissue from cholangiocarcinoma subjects because it was able to screen samples from a large cohort of patients in a short time. A study demonstrated the potential of SELDI to authenticate serum biomarkers which differentiated cholangiocarcinoma from benign disease and/or healthy individuals [34].

In this preliminary study, SELDI-TOF MS proteomic profiling differentiated tissue and sera of cholangiocarcinoma from non-malignant subjects. Previous studies involving different cancer types [84–86] showed similar findings, but the pattern of biomarkers varied between the cancer types. The most interesting discovery of the study of the cholangiocarcinoma patients was the finding of a SELDI-derived peak (*m/z* 4462) which is demonstrated in Fig. 1. This peak was as effective as the tumor markers CEA or CA19-9 at discriminating between sera from cancer patients and disease controls. The relevant ROC curves are demonstrated in Fig. 2a, b. Diagnostic accuracy was improved when these three serum markers were combined in a panel. The diagnosis could be further enhanced using data generated from a panel of other proteins, suggesting that analysis of proteomic profiles, rather than individual proteins, may yield improved diagnostic ability. The value of this technology is in its capacity to analyze large numbers of proteins rapidly to determine which may become potential biomarkers. The LMW portion of the proteome, previously undetectable by the limited resolution of 2-D gel electrophoresis, appears to carry an abundance of tumor-specific information with the potential to improve diagnosis and the understanding of tumor pathogenesis.

A remarkable finding in that paper was that 14 peaks were common to both the tissue and serum of cancer patients. Of these, one peak was significantly up-regulated in both cancer subgroups: *m/z* 11664 ($P = 0.001$ for tissue, $P < 0.001$ for serum). Interestingly, the tenfold cross-validation/multivariate logistic regression models did not select either of these proteins for any of the putative biomarker panels used above. Nonetheless, these peaks are of significant interest for future investigation.

Alterations in the *serum* protein profile would also seem likely as a result of both the malignant process itself and as secondary to the inflammatory response, and would include release of cytokines and acute-phase proteins from the liver. It was therefore crucial to have a control group of patients who did not have cancer but who had a variety of biliary inflammatory processes with matched liver dysfunction measures.

Discrimination between patients with PSC and those with the added complication of cholangiocarcinoma is perhaps one of the most difficult clinical challenges, because transplantation for malignancy can lead to early recurrence. In a

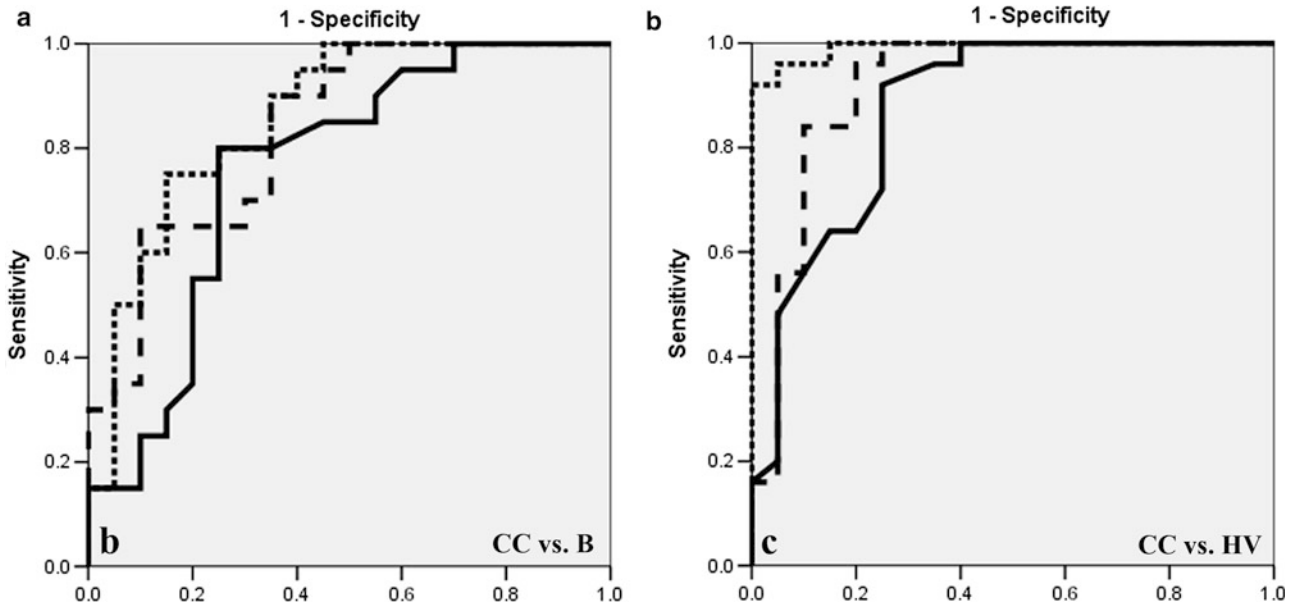

Fig. 2 ROC curves for the serum results from the following: **a** cholangiocarcinoma versus benign disease. *Solid line* marker m/z 4462, *dashed line* 2-marker panel, and *dotted line* CEA added to the panel and **b** cholangiocarcinoma versus healthy volunteers. *Solid line* marker m/z 11535, *dashed line* 3-marker panel, *dotted line* CEA, and CA19-9 added to the panel. Modified from Scarlett et al. [34]

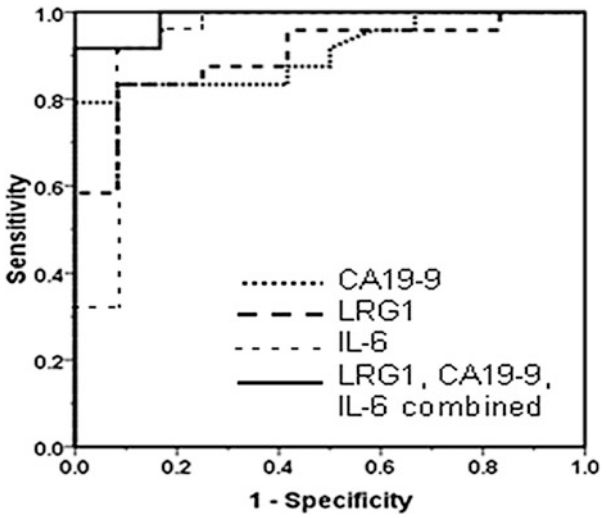

Fig. 3 ROC curves for CA19-9, LRG1, and IL-6 and for CA19-9, LRG1, and IL-6 combined (AUC 0.98) in discriminating cholangiocarcinoma from benign biliary disease

prospective study [87] involving 84 subjects, the novel tumor markers trypsinogen-1, trypsinogen-2, tumor-associated trypsin inhibitor, human chorionic gonadotropin-beta, and trypsin-2-alpha-antitrypsin were evaluated. 46 subjects had undergone transplantation for PSC, and three of these were found to have an unsuspected cholangiocarcinoma. Five of the patients with cholangiocarcinoma had PSC. These markers were measured by the immunofluorescence technique. Serum trypsinogen-2 showed the highest accuracy in differentiating between cholangiocarcinoma and PSC, with an area under the curve (AUC) of 0.804, while for CA19-9, this was 0.613. For patients with simultaneous cholangiocarcinoma, serum trypsinogen-2 also showed the highest accuracy for differentiation between PSC and cholangiocarcinoma, with an AUC of 0.759. This finding needs to be considered within a multimarker platform using a method such as advanced protein microarray.

Studies of the discriminatory power of low abundance proteins can be greatly enhanced using immunoaffinity depletion, whereby large proteins are removed from the sample. It is then concentrated to increase the amplitude of the peaks measuring the smaller proteins of interest. This was undertaken in pooled samples of serum from patients with bile duct cancer. The results were compared with controls from patients with benign strictures and from healthy people. The remaining proteins were compared on a 2-dimensional difference gel electrophoresis (2D-DIGE), which demonstrated an over-expressed protein in the cholangiocarcinoma sample. This protein was able to be identified by nanoflow liquid chromatography electrospray ionization tandem mass spectroscopy and found to be leucine-rich alpha-2-glycoprotein 1 (LRG1). Subsequently, serum LRG1 was shown to have predictive diagnostic ability, both independently and combined in a panel with CA19-9 and IL-6 [88] (Fig. 3). The AUC of 0.98 for the panel indicates the value of utilizing a panel rather than a single marker.

Although this was an early study requiring confirmation, it indicates different influences on the development of cholangiocarcinoma. Importantly, LRG1 was found to be in

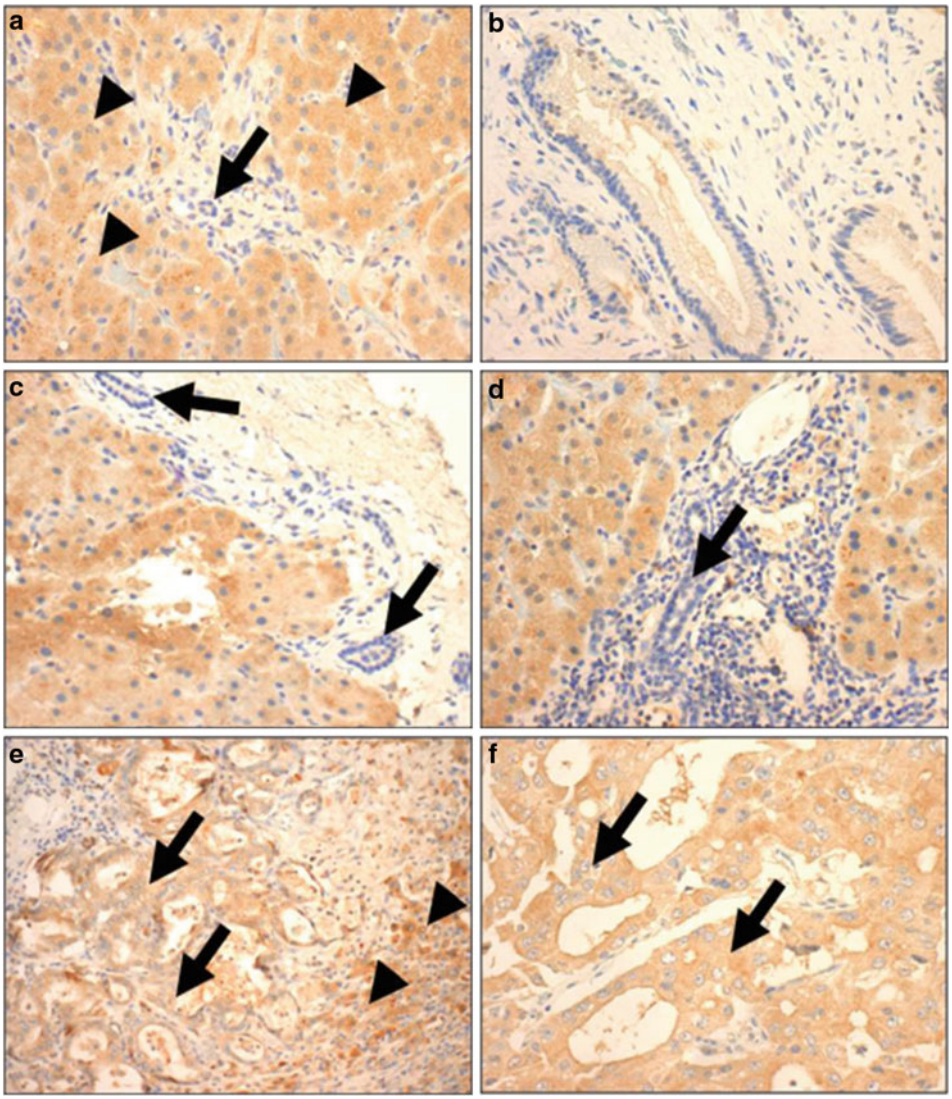

Fig. 4 LRG1 immunohistochemistry analysis demonstrating **a** moderate expression of LRG1 in normal liver (*arrowheads*) with absent expression in biliary epithelium (*arrow*) (original magnification 400×); **b** absent expression of LRG1 in normal biliary epithelium from gallbladder (original magnification 400×); **c** PSC showing positive staining of hepatocytes with absent staining of the biliary epithelium (*arrows*) (original magnification 400×); **d** PBC showing positive staining of hepatocytes with absent staining of the biliary epithelium (*arrow*) (original magnification 400×), **e** cholangiocarcinoma showing positive expression in malignant cells (*arrows*) and in adjacent non-neoplastic hepatocytes of the liver (*arrowheads*) (original magnification 200×); and **f** cholangiocarcinoma showing positive expression of LRG (*arrows*) (original magnification 400×). Adapted from Sandanayake et al. [88]

high concentrations in cholangiocarcinoma cells and less strongly in hepatocytes, but was not expressed in normal biliary epithelium (Fig. 4). The finding of elevated LRG1 in patients' serum can indicate the expression of this protein in the cancer cells' cytoplasm. This signifies a change in cellular metabolism from that of normal cholangiocytes. CA19-9 may be a response to biliary obstruction, and the altered expression of LRG1 in the biliary epithelium indicates a significant change in the biliary mucosa, while IL-6 implies the influence of inflammation on the etiology of cholangiocarcinoma.

10 Proteomic Analysis of Bile

Bile is a rich source of proteins, but the complexity of bile with its ample array of mucins and lipids, its high pH, concentrated inorganic ions, and active bile salts creates problems with analysis. Bile is freely accessible through ERCP, and it is clear that there will be important biomarkers present if some of these difficulties can be overcome. Although it is early in the discovery of the complex map of proteins in bile, recent papers demonstrate that current methods are reproducible and that specific proteins can be recognized [89, 90]. Delipidation, desalination, and nucleic acid removal are necessary before the bile proteome can be examined by the widely accepted 2-DE technique or by tryptic digestion [91]. A 2-DE methodological study undertook a variety of sample preparation options to remove bile contaminants. A large number of protein spots were separated in 2-D maps from the experimental and control groups, with means of 250 and 216 spots on pH 3–10 IPG strips, and 182 and 176 spots on pH 4–7 strips, respectively. When the authors compared bile from a patient with malignancy with bile from a patient with benign disease, approximately 16 and 23 spots,

respectively, were differentially expressed. This study established a reliable sample preparation process suitable for 2-DE examination of bile fluid. The differentially displayed proteomes in the 2-D biliary maps from the experimental and control groups indicated the potential application for bile fluid analysis to identify disease-associated biomarkers, especially for biliary tract tumors [89]. A further paper has identified 97 proteins which are differentially expressed, of which 38 were up-regulated [92]. The authors found that phosphoglycerate mutase 1 (PGAM-1), protein disulfide isomerase family A, member 3 (PDIA3), heat shock 60-kDa protein 1 (chaperonin) (HSPD1) and SSP411 protein were confirmed to be up-regulated by Western blot analysis. Further, SSP411 displayed value as a potential serum diagnostic biomarker with a sensitivity of 90 % and specificity of 83 % at a cutoff value of 0.63.

One novel marker, Mac-2BP, found in bile using tandem mass spectrometry, was demonstrated to be as frequently elevated as CA19-9 in cholangiocarcinoma patients. Further analysis with ELISA indicated that Mac-2BP could discriminate cholangiocarcinoma specimens from patients with PSC, with a ROC AUC of 0.70. When both bile markers were combined, the AUC of the ROC curve increased to 0.75 [90]. Further markers have been sought using cell culture techniques which suggest that CK7, CK19, U2/2, and galectin-3 may be useful markers to differentiate cholangiocarcinoma from hepatocellular carcinoma [93].

Thus, there is a rich pool of proteins to study, but the methodology needs to be developed before its clinical utility can be realized. The protein patterns of biomarkers of cholangiocarcinoma will become apparent as we become familiar with the biliary proteome. Such markers could then add diagnostic value to bile cytology.

11 Multiparameter Markers

This chapter describes a number of emerging technologies which hold promise for the future, despite the heterogeneous nature of the neoplastic process. Within the development of each technology, there has already been an expansion of the number of potential novel biomarkers identified. The new technologies are exploring measures of DNA, microRNA, proteins, aptomers, and epigenetic factors, among others. They have found biomarkers, all correlating with neoplasia, some specifically with cholangiocarcinoma. A diagnosis, prognosis, and even potential therapies can be derived from these. Any one individual discovery, however, does not appear to have sufficient specificity and sensitivity to make it an ideal biomarker. Therefore, a process which allows the measures of independent markers to be brought together in a multiparameter panel will improve diagnostic accuracy to a level which has widespread utility. The biomarkers discussed here will not be used in isolation in the clinical setting, but integrated with the results of clinical, standard blood pathology, and cytology tests, along with FISH and DNA morphology and in conjunction with different radiological findings to increase diagnostic accuracy [94].

Furthermore, correlation of protein levels with altered pathways within the cancer cells should give new insights into the mechanisms underlying the differences in proteins associated with cholangiocarcinoma. Biomarkers will provide an improved understanding of cholangiocarcinoma and the host response to it.

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