# Prognostic molecular markers in cholangiocarcinoma: A systematic review.

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**Abstract**

The worldwide incidence of cholangiocarcinoma (CC) is steadily rising, the United Kingdom incidence now exceeding 1000 cases per year. It is an aggresive malignancy typified by unresponsiveness to existing chemotherapy and radiotherapy regimes in the vast majority of cases. Surgery offers the only hope of a cure, though postoperative disease recurrence is common, with 5-year survival rates following resection of less then 25%. Developments in molecular techniques and improved understanding of the basis of carcinogenesis in CC has led to examination of the role of biomarkers in predicting poor outcome. This systematic review examines published evidence relating to the prognostic significance of these molecular markers in CC. Of the molecular markers which have been investigated to date p53 mutation, cyclins, proliferation indices, mucins, CA19-9, CRP, and aneuploidy appear to hold significant potential as predictors of outcome in CC. These and other biomarkers may themselves represent novel therapeutic targets for CC.

**Keywords**

## 1. Introduction

Cholangiocarcinoma (CC) is a primary malignancy of the epithelial lining of the biliary tree which carries a poor prognosis despite combined therapeutic strategies. The current reported incidence is only 1-2 per 100,000 population per year in the United Kingdom, however it is an increasingly prevalent disease both here[1, 2] and worldwide[3]. Induction of biliary dysplasia and subsequent malignant transformation is thought to be partly due to local inflammation in the biliary tree which may be caused by a number disease states. Endemic factors, such as parasitic infestation, may account for the reported increasing incidence in south-east Asia[4]. In western populations the escalation in cases may be due to an increase in the incidence of hepatitis C virus and subsequent long-term biliary inflammation[3]. Other known associated conditions include primary sclerosing cholangitis (PSC), chronic intra-ductal gallstone disease, choledochal cysts, congenital liver malformations, human immunodeficiency virus infection, smoking and exposure to certain chemicals (possibly related to employment in the aircraft, rubber and wood finishing industries).

CC may arise anywhere in the biliary tree, from the small, peripheral hepatic ducts to the distal common bile duct. Commonly used classification systems utilise anatomical location to group tumours into three main categories: intrahepatic (20-25%), hilar (also known as Klatskin tumour – 50%) and extra-hepatic (20-25%)[5]. In published studies hilar tumours may be classified together with either intrahepatic or extra-hepatic type. Gallbladder carcinoma is also considered in some series to represent a sub-type of CC, but is not included here.

Despite advances in chemo-, immuno-, photodynamic- and radio-therapies, CC caries a poor overall survival rate. Surgery offers the only hope of cure and, with careful selection and increasingly radical surgical strategies, 5-year survival as high as 41% has been reported(6-9). However, existing pre-operative radiological, pathological and operative (laparoscopic) staging strategies do not allow accurate determination of long term prognosis in operable patients. The ability to predict tumour behaviour on the basis of molecular markers from either biopsy or serum samples would inform the patient and clinician during the decision-making process. With rapid advances in the understanding of tumour biology there is sufficient new evidence available to gain further insight into this disease. Furthermore, biomarkers associated with adverse outcome may themselves represent novel therapeutic targets.

The aim of this systematic review was to summarise the results of published studies looking at molecular biomarkers and their prognostic significance in CC. The PubMed and Web of Science databases were searched using the following keywords in varying combinations in order to identify published works in English language up to October 2007: cholangiocarcinoma, bile duct cancer, Klatskin tumour, prognosis, biomarkers, and molecular markers. Individual biomarkers were also included in searches. All cited references were hand searched in order to identify other important published work. Peri-ampullary and gallbladder carcinoma series were excluded from the survey. Case reports, studies published in abstract form only, unpublished works and publications in non peer-reviewed journals were excluded. *In vitro* investigations and animal studies were not included.

## 2. Tumour suppressor genes:

### *p53 [Table 1]*

Located on chromosome 17p13.1, p53 is responsible for cell cycle regulation at the $G_1/S$ and $G_2/M$ checkpoints. It is known to induce apoptosis in response to severe damage to cellular DNA, through Bax activity regulation. Mutation of the p53 gene results in unchecked replication of defective DNA and possible progression to cancer(10). P53 gene mutation has been shown to be present in 28-61% of CCs(11-14). Wild-type p53 protein has a short half-life, rendering it difficult to detect by immuno-histochemical (IHC) methods. However, the mutated form is significantly more stable, possibly due to protein-stabilising conformational alteration, and this enables detection(15). Several studies of protein over-expression show no association with outcome(14, 16-24). The remainder demonstrate conflicting results. A number of groups found an association with decreased overall survival(11, 12, 25-28), but only one of these has demonstrated independent prognostic significance(29). However, in a large series Argani *et al* showed increased overall survival in their p53 positive group(30). A number of factors may account for these disparities including use of different cut-off values for positivity, antibodies and inclusion of varying anatomical and histological subtypes of CC. Indeed, two studies have noted increasing p53 expression in more distal tumours in comparison to proximal ones when a sub-analysis is undertaken(28, 30). This may indicate a more important role for p53 over-expression in extra-hepatic, distal bile duct carcinomas. The prognostic role of p53 protein over-expression therefore remains to be fully established.

Evidence from studies utilising PCR-based P53 gene mutation analysis are more consistent. Mutation appears to be associated with a decreased overall survival.

However, with only two studies available in the literature examining 18 and 41 tumours respectively(11, 12), further work to confirm this association is desirable.

### *Other tumour suppressor genes.*

DPC4 (Smad4) is a tumour suppressor gene whose protein regulates transduction of signals from the transforming growth factor-β (TGFβ) superfamily(17). TGFβ is a major cell proliferation inhibitor. Loss of DPC4 is thought to lead to progression in the cell cycle from $G_1$ to S phase and subsequent increased proliferation(31). In two studies of DPC4 protein expression, loss was noted in 55% and 60% of extra-hepatic bile duct carcinomas. However, there was no correlation with survival(17, 30).

The retinoblastoma gene encodes the Rb protein which, in its unphosphorylated state, blocks transition from $G_1$ to S phase in the cell cycle and is necessary for prevention of cell replication when DNA has been damaged(32). In 51 intra-hepatic and 34 extra-hepatic CCs no correlation between Rb expression and survival could be shown(33, 34).

The p73 gene has significant homology with p53, though mutation in human tumours has not been found. However, over-expression of the p73 protein is thought to induce inhibition of cell growth and apoptosis through activation of p53-responsive genes(35). One study of 41 resected intra-hepatic CCs found 32% of tumours over-expressed p73 protein (>10% nuclei positively stained) and this correlated with improved survival on univariate analysis, but lost significance upon multivariate analsysis(36).

## 3. Oncogenes

### *k-ras*

The ras oncogenes encode a family of signal transduction proteins downstream of growth factor receptors. Mutation in the k-ras gene can permanently activate the protein leading to increased cellular proliferation and growth(37). In five studies examining k-ras mutation in CC, four showed no significant correlation with survival(38-41), with one group demonstrating a significant decrease in survival(25). However, this study examined tissues from 12 patients with cancer arising on a background of primary sclerosing cholangitis; conceivably a different neoplastic entity.

### *KIT (CD117)*

KIT is a type three receptor tyrosine kinase which binds mast cell growth factor and can effect cellular proliferation, growth and differentiation(42). Hong *et al* examined a large series of 289 extra-hepatic bile duct carcinomas using IHC. Of these, 19% and 20% had cytoplasmic and nuclear reactivity respectively. Positive cytoplasmic expression significantly correlated with improved survival on both univariate and multivariate analysis (P=0.01 and p= 0.04)(43).

## 4. Apoptotic regulation

Evasion of programmed cell death (apoptosis) is one of the key hallmarks of malignant growth. Furthermore, loss of the normal control of cell longevity is also thought to confer increased resistance to chemotherapeutic agents, many of which utilise these pathways to induce cell death.

## Bcl-2 / Bax

Members of the Bcl-2 protein family, such as the pro-apoptotic Bax, Bak, and BAD and the anti-apoptotic Bcl-2, Bcl-w and Bcl-xl, influence mitochondrial membrane permeability(44). Of this family, Bcl-2 and Bax have been investigated in CC. In a study of 28 resected tumours, no expression of Bcl-2 was found by IHC, though Bax expression was not evaluated(20). In 22 resected intra-hepatic CCs from Italy, Bax was expressed in 45%, but had no correlation with outcome and Bcl-2 was not assessed. Of note, however, Maspin, a multifunctonal protein which induces apoptosis by modulation of Bcl proteins, was reported to be expressed in 77% of cases. Low expression correlated with increased depth of invasion (P=0.043), vascular invasion (P=0.027) and was an independent prognostic factor for poor outcome on multivariate analysis(45). The role of the Bcl-2 family remains to be fully established as proteins have only been evaluated on an individual basis in CC, and need to be assessed relative to each other in order to gauge apoptotic influence. Further studies examining Bcl-2, Bax and Maspin expression together would be of value.

## DAP-kinase

Death-associated protein kinase (DAP-kinase) is a calcium/calmodulin-dependent protein which participates in several pro-apoptotic systems, with loss of expression leading to decreased cell death(46). DAP-kinase promoter hyper-methylation, and subsequent decreased protein expression, was found to be significantly associated with higher tumour grade (P=0.043), decreased overall survival (P=0.009) and an independent prognostic marker(P=0.024)(47).

### Fas/FasL

Expression of Fas / FasLigand (FasL) is thought to mediate apoptosis by interaction with T lymphocytes in the immune system. Cross-linking of the Fas receptor with either FasL or activated antibody induces apoptosis of Fas-expressing cells. Investigators have hypothesised that tumours may induce Fas mediated apoptosis in lymphocytes by expressing FasL, thus creating an immune-privileged site for increased growth(48). Two groups have assessed Fas and FasL by IHC in Japanese patients. In both study groups, tumours found to strongly express Fas had a significantly better prognosis on univariate analysis, possibly due to increased tumoural apoptosis. FasL expression did not correlate with outcome(49, 50).

### Survivin

Survivin is a strong negative regulator of apoptosis. By inhibiting or preventing the activation of caspases, it promotes cellular survival in otherwise apoptotic conditions(51). Over-expression has been associated with aggressive behaviour and poor outcome in a number of malignancies(52-54). Javle *et al* found strong nuclear expression in 17% of tumours and correlated it with poor survival (P=0.033), also finding expression to be an independent poor prognostic factor(55).

## 5. Cell cycle regulators [Table 2]

### 5.1 Cyclin and cyclin-dependent kinases

Cyclin/cyclin dependent kinase complexes are key to the progression of cells through the $G_1/S$ phase of the cell cycle by inactivating Rb through phosphorylation. Over-expression is postulated to increase cell turnover and proliferative activity(56). Two studies examining immunohistochemical cyclin D1 in a total of 110 patients have found over-expression to be associated with poor survival on univariate analysis(57,

58), with one group finding it to be an independent prognostic factor(57). Over-expression of Cyclin B1, which acts at the $G_2/M$ phase check-point of the cell cycle, has also been shown to be a poor prognostic indicator on univariate analysis. In the same study patients with decreased 14-3-3σ  expression (an inhibitor of cyclin B1 inducible by p53 in response to DNA damage) had poorer overall survival, in both univariate and multivariate analyses(16).

## *5.2 Cyclin-dependent kinase inhibitors (CKI)*

### *KIP/CIP family*

Cyclin-dependent kinase inhibitors block $G_1/S$ phase transition and decreased expression is thought to promote tumourigenesis(56). However, in CC evidence relating to their prognostic role is conflicting. Both over- and under-expression of p21$^{WAF1/CIP1}$, a p53 inducible universal CKI, have been found to be associated with decreased disease-free survival(18). Low expression of another CKI - p27, has been shown to be a negative prognostic factor in several malignancies. In five studies of CC  all utilising IHC, three demonstrated a significant association between decreased p27 expression and poor prognosis(57, 59, 60), with the other two finding no such correlation(61, 62).  The reasons for this disparity are unclear, as all these studies used similar evaluation methods, statistical analyses and involved similar numbers of patients. Further investigation is required to clarify the role of CKI's as prognostic indicators.

### *INK4  family*

p16 suppresses cyclin-dependent kinase 4-Cyclin D complexes which control phosphorylation of Rb. Lack of p16 therefore up-regulates Rb phosphorylation allowing possible inappropriate progression to S phase(63). Hyper-methylation and

subsequent silencing of the p16 promoter is common, being present in 76-83% of CCs(33, 41, 64). p16 protein expression has been assessed by IHC in CC by 4 groups, but no significant prognostic role has been found(33, 34, 41, 65).

## 6. Proliferation indices [Table 3]

An indication of rate of tumour growth and aggressiveness can be gained from measurement of tumour proliferative activity. Several experimental methods exist for this purpose, including quantification of mitotic index, S-phase cell fraction count, or immunohistochemical antibody detection of nuclear proteins (ki-67, its epitope MIB-1 or proliferating cell nuclear antigen (PCNA)).

Several studies have utilised the various nuclear proteins detectable by IHC to quantify proliferation and examine the relationship with survival (Table 3). Conflicting results exist but it is evident in several studies, including the majority of the larger study populations, that an association exists between increased proliferative activity and decreased overall survival(12, 16, 22, 23, 27, 39, 66-68). The conflicting results of some groups may be due to the small sample size investigated, between 6-9 patients in some studies(17, 19, 58), or the different criteria for proliferation indices which have been used(21, 69).

## 7. Growth factors and receptors

### *Epidermal Growth Factor Receptor (EGFR) family*

The trans-membrane tyrosine kinase receptors of the EGFR family which include EGFR (ErbB-1, HER-1), HER-2/neu (ErbB-2), HER-3 (ErbB-3) and HER-4 (ErbB-4) play a significant role in cellular growth and proliferation signalling(70).

EGFR expression was assessed by IHC in 24 patients with CC and was unrelated to survival(71). Similarly, Gwak and colleagues performed DNA sequencing for EGFR mutation and they also failed to find any correlation with outcome(72).

In advanced breast cancer, HER-2/neu over-expression has attracted considerable interest due to correlations with prognosis and has subsequently been successfully targeted with monoclonal antibodies(73). In CC, however, four groups have examined HER-2/neu expression using IHC and found no correlation with survival(61, 62, 74, 75).

### Tenascin

The extra-cellular matrix glycoprotein Tenascin may directly bind the EGFR receptor, activating the EGFR kinase cascade and subsequently increasing the proliferative and migratory activity of tumour cells(76). Aishima and colleagues found expression of Tenascin at the invasive front of tumours to correlate with decreased overall survival, though this was not significant on multivariate analysis(77).

### CCN proteins

The CCN protein family (named by the first letters of the first three proteins discovered – Cyr61, CTGF and Nov) includes connective tissue growth factor (CTGF), a highly fibrogenic protein, which may promote desmoplastic reactions, possibly decreasing the ability of tumours to invade and metastasise. However, over-expression has been shown in several epithelial and non-epithelial malignancies, but prognostic implications remain to be confirmed(78). In the only study in CC, Gardini *et al* found over-expression in 60% of tumours, demonstrating a significantly

improved disease-free and overall survival in this group compared with low-expressors. On multivariate analysis, CTGF expression independently predicted disease-free and increased overall survival(79).

The Wnt-inducible secreted proteins (WISP1, WISP2 and WISP3) are three further members of the CCN family(80). These proteins appear to have complex roles in cell signalling and their prognostic implications in cancer remain to be fully established(81). In CC the WISP1v splice variant has been to shown to be expressed in 49% of intra-hepatic tumours at the mRNA level and this correlated with reduced overall survival on univariate analysis(82).

## 8. Invasion and Metastasis

### Cadherins

Mikami *et al* examined expression of E-cadherin and the alpha- and beta-catenins in 55 extra-hepatic CCs. These important molecules in calcium-dependent intercellular adhesion have been shown to be down-regulated in several epithelial malignancies(83) and are a hallmark of the epithelial-mesenchymal transition thought to play a pivotal role in cancer invasion and metastasis(84). Decreased E-cadherin expression in this group of CCs was significantly associated with poor prognosis on univariate, but not multivariate, analysis. Catenin expression was significantly lower in carcinoma tissues when compared to normal epithelium, but was unrelated to prognosis(85).

### Syndecan-1

The trans-membrane heparin sulphate proteoglycan molecule Syndecan-1 (CD138) is a mediator of cell-cell and cell-matrix adhesion and communication(86). Loss of

expression was found to be an independent predictor of poor outcome in the only study to express the prognostic role of this factor in CC to date(87).

### CEACAM6

Over-expression of carcino-embryonic antigen-related cell adhesion molecule 6 (CEACAM6), a member of the immunoglobulin superfamily, has been associated with a variety of malignancies. Over-expression is thought to enhance metastatic and invasive ability via its ability to influence intra-cellular signalling cascades(88). The only study in CC demonstrated over-expression of CEACAM6 to correlate with decreased disease-free, but not overall, survival on univariate analysis(89).

### CD24

CD24 is a heavily glycosylated, mucin-like, cell-surface protein which can act as an alternative ligand for P-selectin. Binding of P-selectin may enhance the metastatic potential of CD24-expressing tumour cells by allowing them to "roll" on the endothelium(90, 91). Su *et al* found CD24 expression to be an independent predictor of shortened survival in 70 patients undergoing resection of CC  (P=0.0113)(92). Agrawal *et al* also found over-expression of CD24 to correlate with decreased overall survival on univariate analysis (P=0.02)(93).

### CD44

The polymorphic transmembrane glycoprotein CD44 is a cell adhesion molecule which may be expressed in several isoforms due to alternate splicing. It appears to have tissue-specific roles in inflammation and tumourigenesis(94). In a study of 25 extra-hepatic bile duct tumours, the CD44v6 splice variant was found to be absent in tumours with poor prognosis on univariate analysis(95).

### *Matrix Metalloproteinase 7 (MMP7)*

Abnormal secretion of matrix metalloproteinases enables tumour invasion and metastasis(96). In the only study to examine matrix metalloproteinase expression in CC, MMP7 over-expression was found to correlate with decreased survival (P=0.003) on univariate analysis(97).

## 9. Angiogenesis

The ability to stimulate new blood vessel formation and so increase nutrient supply is an essential characteristic of any growing tissue, not least malignancy. However, only two studies have investigated this area in relation to its prognostic implications in CC. Park *et al* found high vascular endothelial growth factor-C (VEGF-C) expression to correlate with shortened overall and disease-free survival in 36 patients undergoing resection of intra-hepatic CC (P=<0.01 and <0.05 respectively), with strong VEGF-C expression representing an an independent prognostic indicator (P=0.028)(98). IN contrast, Tang *et al* investigated several angiogenesis-related factors including VEGF, micro-vessel density, angiopoietin-1, angiopoietin-2 and thrombospondin-1 expression with IHC in CC, but found no correlation with survival(99).

## 10. Mucins [Table 4]

Mucins are large proteins synthesised by epithelial cells in many organs. They may be secreted to form a protective barrier at the mucosal surface or may act as transmembrane proteins(100). There is considerable interest in their possible role as targets for chemotherapy(100). Table 4 displays identified studies in CC.

## MUC1

MUC1 is a trans-membrane mucin thought to be particularly important in the invasive and metastatic potential of CC. Of the nine studies that have examined the prognostic value of MUC1 in CC, all except one have demonstrated a significant correlation between MUC1 expression and decreased survival, both on univariate and multivariate survival analyses(61, 62, 101-107).

## MUC2

In contrast to MUC1, MUC2 acts as a protective protein and is associated with tumours of mucinous type, both in the biliary and pancreatic systems, which carry a more favourable prognosis. The majority of studies in CC show a significantly better prognosis in MUC2 expressing tumours, and a tendency for MUC2 expression to be associated with extra-hepatic rather than intra-hepatic tumours(101, 103, 105, 108, 109).

## MUC4

MUC4 is a trans-membrane mucin which may act as a ligand for receptor tyrosine kinase ErbB2, and is thought to play a role in the regulation of p27 which is involved in control of the $G_1/S$ phases of the cell cylcle. High expression has been shown by two groups to significantly correlate with poor survival and act as an independent prognostic factor on multivariate analysis(61, 62).

## MUC5AC

This gel-forming secreted mucin has been studied by three groups. Following IHC analysis of 100 resected tumours, a combined mucin phenotype including MUC2, MUC5AC and MUC6 expression, was found to be an independent prognostic factor on multivariate analysis (P=0.0042)(69). Another  analysis of 179 serum samples

taken pre-operatively from CC patients demonstrated MUC5AC positive patients to have a 2.5-fold higher risk of death on multivariate analysis (P<0.001)(110).


## 11. Serum markers

### Carbohydrate Antigen 19-9 (CA19-9, sialyl Lewis[a] serum antigen)

Carbohydrate antigens may be expressed on the tumour cell membrane and are thought to play an important role in extravasation by mediating adhesion to endothelial cells expressing E-selectin(111). Elevation of serum levels has been demonstrated in several malignancies(112). Of the studies relating to prognostic implications in CC, three groups have demonstrated a significant difference in survival when a high cut-off level (>1000ng/ml(113), >100ng/ml(114, 115)) is used to compare groups. Wahab $et\ al$ also found elevated CA19-9 to be associated with poor survival after resection, both on univariate and multivariate analysis, but did not define their cut-off level(116). At lower cut-off values (>37ng/ml) two studies found no significant association with survival(21, 97). Indeed, Miwa $et\ al$, in a further analysis after their 2002 paper, found CA19-9 to be a significant prognostic factor on univariate analysis, but found jaundice confounded elevated CA19-9 on multivariate analysis(117). The conflicting evidence within these studies is likely to be related to the non-standardised cut-off values used in the analyses. In addition, biliary obstruction, whether due to benign or malignant disease, is known to cause elevation of CA19-9 and few groups have allowed for this in multivariate analysis. Overall, high levels of CA19-9 appear to be a poor prognostic indicator.

### Carcinogenic Embryonic Antigen (CEA)

Serum CEA levels have been examined in five studies. Four of these found that a raised pre-operative CEA had no significant relationship with survival(21, 97, 115, 117). In the remaining study, which examined 35 resected intra-hepatic CCs, there was a significant correlation between high CEA level and decreased survival on univariate and multivariate analysis (P=0.021 and P=0.029 respectively)(118). Overall, the evidence suggests serum CEA measurement is less useful than CA19-9 as a significant prognostic marker.

### Bilirubin

Elevated serum bilirubin has been evaluated as a prognostic indicator and seems to indicate a worse overall survival in the groups analysed(116, 117, 119). This should perhaps be interpreted with caution as, in at least one study, jaundiced patients had a tendency to receive more palliative rather than resection procedures, which may obviously influence survival(119). Further evaluation of serum bilirubin as a prognostic indicator is needed.

### C-reactive protein (CRP)

C-reactive protein (CRP) has been shown to be of prognostic value in many malignancies(120). In 98 peri-hilar CCs, Gerhardt *et al* found an elevated CRP to be an independent predictor of worse survival (P=0.029)(121).

### Sialic acid

In a single study of serum sialic acid concentration, Wongkham *et al* found elevated levels in 75% of CC patients from Thailand, but this had no prognostic association(122).

## 12. Sialyl Lewis antigens in tissue

In addition to measurement of serum sialyl Lewis[a] (CA19-9) IHC examination of tissue specimens for various sialyl Lewis antigens has been performed. Further supporting the role of serum sialyl Lewis[a] as predictive of prognosis, Juntavee *et al* found sialyl Lewis[a] expression in 60% of the 110 specimens they examined, finding it to be an independent prognostic indicator on multivariate analysis (P=0.001)(123). In contrast, Takao *et al* examined the sialyl Lewis[x] and sialosyl-Tn antigens in 73 extra-hepatic bile duct carcinomas and found no relationship with survival(104).

## 13. Genetic Factors

### Chromosomal instability / anueploidy

Chromosomal instability and aneuploidy are characteristic features of most aggressive human malignancies(124). Defining features include the loss or gain of the whole or a part of a chromosome and / or gross chromosomal rearrangements(125). Two studies in CC have examined tumours using DNA flow-cytometry, both confirming a significant association between aneuploid tumours and poor survival(126, 127). Iachino *et al* found aneuploidy to be an independent prognostic marker(127).

### Loss of heterozygosity (LOH) and microsatellite instability (MSI)

LOH at genetic loci has been investigated in CC, but the prognostic implications remain unclear. In three studies, no correlation with survival has been shown(128), though Muenphon and colleagues demonstrated a significant association between allelic imbalance and survival at three individual loci(129).

Microsatellites are short tandem repeat sequences found throughout the human genome. Differences in the lengths of repeat sequences between normal and tumour DNA from the same patient are used to assess the instability of these microsatellites(130). Increased MSI is thought to be due to abnormalities in the function of DNA mismatch repair genes. Studies examining a single chromosomal locus in CC have found varying MSI rates, though only MSI at chromosomal locus 1p36 D1S228 has been shown to correlate with prognosis(128, 130). However, when more loci are taken together or combined with LOH, the representation of overall tumour genetic instability may give a more accurate indication of aggressive behaviour(14). As in other malignancies(131), newer techniques for genome wide evaluation of damage, such as array comparative genomic hybridisation, may assist in assessing the prognostic implications of genomic instability as a whole.

### *Genetic repair*

As part of the mismatch repair pathway, $O^6$-Methylguanine-DNA methyltransferase (MGMT) participates in repair of DNA which has been incorrectly transcribed during replication(132). In CC, MGMT expression has been found to be a good prognostic indicator on univariate analysis(133). In a further study, methylation of the MGMT promoter (which closely correlated with decreased protein expression) was significantly associated with poor survival(134). Loss of MGMT expression is thought to be one of the factors responsible for increased MSI and overall genetic instability.

### *Genotype*

The GNAS1 locus encodes the Gαs subunit of G protein, which is involved in the cAMP pathway, participating in control of apoptosis and proliferation. Schmitz *et al*

determined the GNAS1 T393C genotype in 87 resected intra-hepatic CCs and found the TT genotype to significantly associate with decreased survival on univariate and multivariate analysis(135). The proliferation indices in TT genotype tumours were significantly elevated, suggesting a reduced function of the G protein in these patients. Further studies are needed to fully clarify the implications of these results.

## 14. Other markers

### *Cytokeratins*

Cytokeratins are intermediate keratin filaments which form part of the intra-cytoplasmic cytoskeleton of epithelial cells and there is increasing interest in their application as serum biomarkers(136). Keratin 903 monoclonal antibody can be used to stain tissues to assess expression of the 1, 5, 10 and 14 cytokeratins. In a study of 73 resected mass-forming CCs, patients with Keratin 903 reactivity were found to have significantly improved survival on univariate and multivariate analyses(137).

### *Neuroendocrine differentiation*

Neuroendocrine differentiation has been shown to be of prognostic importance in several malignancies(138). In CC synaptophysin has been found to be a significant independent negative prognostic marker(139).

### *Host immune response*

One study of dendritic and T-cell localisation in CC found a significant improvement in survival of patients with positive dendritic cell markers (CD83 and CD1a) and T cell markers (CD8 and CD4), as opposed to their negative counterparts, presumably denoting an increased anti-tumour response(140)

### *Pancreatic secretory trypsin inhibitor (PSTI)*

Having previously been shown to be an independent prognostic marker in bladder(141), ovarian(142) and renal(143) carcinomas, PSTI expression was investigated in CC specimens. High expression was associated with decreased recurrence-free survival on univariate analysis(144).

## Cyclooxygenase-2 (COX-2)

The involvement of COX in cancer is now well established and its potential as a target for chemo-preventive and / or chemotherapeutic inhibitors is under investigation(145). In CC the role of COX-2 remains unclear, with one study suggesting a significant association between over-expression and decreased overall survival(146), but two others finding no relationship(71, 147). Different methods of quantifying COX-2 expression may account for the discrepancies between these studies. Without further investigation the prognostic value of COX-2 remains a matter for debate.

## 15. Conclusions

Research in CC has yielded a multitude of molecular markers which have a significant role in predicting outcome. These biomarkers hold promising application for the future, both in terms of patient counselling with stratification of risk and individualised treatment strategies, and the potential to target some of these molecules with novel therapeutic agents. Individualised treatment holds obvious advantages, allowing patients at high risk of recurrence to receive aggressive adjuvant therapy, and those with very low risk to avoid the possible morbidity associated with chemotherapeutic drugs. Follow up, normally based around a generalised protocol for

all patients, may also be individualised according to the molecular profile of the tumour, potentially allowing serum monitoring alone to guide imaging and further therapy.

In this review we have identified several biomarkers of particular interest which appear to carry prognostic significance. Of these, p53 mutation, cyclins, proliferation indices, mucins, CA19-9, CRP, and aneuploidy appear to hold potential as predictors of outcome. In other types of hepatobiliary malignancy angiogenesis factors, growth factors and cyclo-oxygenase have been shown to be of value and these should be evaluated as a priority in CC(148-150). Few studies have examined circulating serum markers in CC. The use of simple serum analysis holds obvious benefits to the patient over the need to obtain tissue samples by biopsy or at operation and would facilitate earlier prediction of outcome. However, given the continual increase in the plethora of potential molecular markers in CC, application of genomic and proteomic micro-array technology should allow measurement and analysis of a number of these markers simultaneously in the future. Application of these techniques will produce gene or protein profiles, allowing much more accurate stratification of individual risk and the recognition of utility of each biomarker in individual cases. The first steps have already been taken in this area and although direct prognostic implications have not been assessed, initial studies indicate correlations with increased stage and presence of lymph node metastases; well recognised markers of poor outcome(151, 152).

The majority of studies to date in CC have utilised retrospective analyses, with differing methodologies. These factors may explain inconsistencies in results from individual markers. In addition, CC is a complex, heterogeneous disease with a wide

variety of aetiological factors in different parts of the world. A large proportion of the available literature comes from studies in south-east Asian populations. Biological behaviour of these tumours at the molecular level may be significantly different to other populations due to the increased incidence of pro-inflammatory conditions, such as hepatitis and liver fluke infestation. However, given the higher incidence of CC in these populations, these studies investigate significant numbers of patients and provide some of the best evidence available to date.

Allied to this idea of varying epidemiological conditions in different parts of the world is the predisposition of some patients with chronic conditions, such as primary sclerosing cholangitis, to CC. Patients with presumed sporadic CC may represent a different biological and pathological type of disease to those in whom CC arises on a background of chronic disease. It remains to be elucidated whether or not the patients in these sub-groups express different biomarkers which may indicate more or less aggressive disease.

It is widely accepted in the literature that CC from different anatomical locations may represent different sub-groups of biological disease. Indeed, due to the relatively low incidence of CC, many studies have grouped these tumours together; though in studies where sub-group analyses have been performed clear differences are apparent. In future investigations the authors would suggest that it is desirable to standardise the approach to studying molecular prognostic markers in CC. However, given the relatively low incidence of CC, multiple sub-groups are undesirable. One strategy, as utilised in several studies, would be to clearly divide CC into intra-hepatic and extra-hepatic tumours for analyses, placing hilar tumours in the intra-hepatic group. In

addition, the intra-hepatic group appears to contain three distinct histological types (mass-forming, intra-ductal growth and peri-ductal-infiltrating types) and sub-analyses of these groups would be of interest.

In summary, we have reviewed the available evidence with regard to prognostic molecular biomarkers in CC. Several of these biomarkers appear to hold significant potential both as prognostic indicators and as targets for future therapeutic agents. The application of these markers in clinical practice possibly holds the key to significant advances in this disease.

## Conflict of interest statement

None declared.

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| Study | Year | Patients (n) | Method | Cut-off for positivity[a] | % Positive cases | Prognostic role |
|---|---|---|---|---|---|---|
| Kuroda et al(16) | 2007 | 93 | IHC | >10% | 37 | No significant prognostic role |
| Cheng et al(29) | 2007 | 112 | IHC | >30% | 33 | p53-positivity associated with decreased OS on UVA and independently predicted decreased OS (P=<0.01) |
| Limpaiboon et al(14) | 2002 | 55 | DNA-PCR and electrophoresis | p53 MSI<br>p53 LOH | 0<br>32 | No significant prognostic role |
| Hoang et al(17) | 2002 | 9 | IHC | >30% | 50 | No significant prognostic role |
| Argani et al(30) | 2001 | 116 | IHC | >30% | 51 | p53-positivity associated with **increased** OS on UVA, but NS on MVA |
| Li et al(18) | 2000 | 34 | IHC | >20% | 50 | No significant prognostic role |
| Tannapfel et al(12) | 2000 | 41 | IHC<br>PCR-SSCP | Not reported<br>p53 mutation | -<br>37 | p53 mutation associated with decreased OS on UVA, NS on MVA |
| Havlik et al(11) | 2000 | 18 | PCR-SSCP | p53 mutation | 28 | p53 mutation associated with decreased OS on UVA |
| Ahrendt et al(25) | 2000 | 12 | IHC | >10% | 54 | p53-positivity associated with decreased OS on UVA |
| Albores- Saavedra et al(19) | 2000 | 9 | IHC | >30% | 42 | No significant prognostic role (Papillary carcinomas) |
| Rijken et al(26) | 1999 | 47 | IHC | >30% | 40 | p53-positivity associated with decreased OS on UVA |
| Arora et al(20) | 1999 | 28 | IHC | >1% | 86 | No significant prognostic role |
| Kim et al(21) | 1999 | 28 | IHC | >10% | 36 | No significant prognostic role |
| Shrestha et al(22) | 1998 | 32 | IHC | >10% | 56 | No significant prognostic role |
| Suto et al(23) | 1998 | 54 | IHC | >5% | 44 | No significant prognostic role |
| Takamori et al(27) | 1997 | 17 | IHC | >5% | | p53-positivity associated with decreased OS on UVA |
| Washington et al(24) | 1996 | 27 | IHC | >30% | 38 | No significant prognostic role |
| Diamantis et al(28) | 1995 | 45 | IHC | >10% | 64 | p53-positivity associated with a trend towards decreased OS |

IHC, immunohistochemistry; OS, overall survival; UVA, univariate analysis; MVA, multivariate analysis; NS, not significant; PCR-SSCP, polymerase chain reaction-single-stranded conformational polymorphism; MSI, microsatellite instability; LOH, loss of heterozygosity.

[a] Immunohistochemical cut-offs indicate the percentage of cells with positively staining nuclei unless stated otherwise.

**Table 1 - Studies examining the prognostic role of p53 mutation and protein expression in cholangiocarcinoma.**

| Study | Year | Patients (n) | Marker (Method) | +ve(+) / -ve(-) regulator | Cut-off for positivity[a] | % Positive cases | Prognostic role |
|---|---|---|---|---|---|---|---|
| Kuroda et al(16) | 2007 | 93 | Cyclin B1 (IHC) | + | >4% | 82 | High Cyclin B1 associated with decreased OS on UVA. |
| | | | 14-3-3σ (IHC) | - | >30% | 68 | Low 14-3-3σ associated with decreased OS on UVA and independently prognostic on MVA |
| Hui et al(57) | 1999 | 34 | Cyclin D1 (IHC) | + | >5% | 68 | High Cyclin D1 associated with decreased OS on UVA and independently prognostic on MVA |
| | | | p27$^{KIP1}$ (IHC) | - | >50% | 44 | Low p27$^{KIP1}$ associated with decreased OS on UVA and independently prognostic on MVA |
| Sugimachi et al(58) | 2001 | 66 | Cyclin D1 (IHC) | + | >10% | 42 | High Cyclin D1 associated with decreased OS on UVA |
| Li et al(18) | 2000 | 34 | p21$^{WAF1/CIP1}$ (IHC) | - | >30% | 15 | p21$^{WAF1/CIP1}$ –ve and high expressors associated with increased recurrence and decreased DFS on UVA |
| Fiorentino et al(59) | 2001 | 47 | p27 (IHC) | - | >50% | 38 | Low p27 associated with decreased OS on UVA and independently prognostic on MVA |
| Taguchi et al(60) | 2001 | 62 | p27$^{KIP1}$ (IHC) | - | >50% | 73 | Low p27$^{KIP1}$ associated with decreased OS on UVA and independently prognostic on MVA |
| Shibahara et al(61) | 2004 | 27 | p27 (IHC) | - | >50% | 63 | No significant prognostic role |
| Tamada et al(62) | 2006 | 70 | p27 (IHC) | - | >20% | 57 | No significant prognostic role |
| Tannapfel et al(33) | 2002 | 51 | INK4a(MS-PCR) | - | Methylation status | 76 | No significant prognostic role |
| | | | ARF (MS-PCR) | - | | 25 | |
| Tannapfel et al(41) | 2000 | 41 | INK4a(MS-PCR) | - | Methylation status | 83 | No significant prognostic role |
| | | | p16 (IHC) | - | Not reported | 17 | No significant prognostic role |
| Hong et al(64) | 2006 | 90 | INK4a(MS-PCR) | - | Methylation status | 77 | No significant prognostic role |
| | | | p16 (IHC) | - | Any nuclear staining | 48 | No significant prognostic role |
| Ichikawa et al(65) | 2002 | 99 | p16 (IHC) | - | Diffuse staining | 19 | No significant prognostic role |
| Cui et al(34) | 2000 | 34 | p16 (IHC) | - | >1% | 35 | No significant prognostic role |

IHC, immunohistochemistry; MS-PCR, methylation specific-polymerase chain reaction; OS, overall survival; DFS, disease-free survival; UVA, univariate analysis; MVA, multivariate analysis.

[a] Immunohistochemical cut-offs indicate the percentage of cells with positively staining nuclei unless stated otherwise.

**Table 2 - Studies examining cell cycle regulators in cholangiocarcinoma and their prognostic significance.**

| Study | Year | Patients (n) | Marker (Method) | Cut-off for positivity[a] | % Positive cases | Prognostic role |
|---|---|---|---|---|---|---|
| Kuroda et al(16) | 2007 | 93 | MIB-1 (IHC) | >10% | 69 | High MIB-1 LI associated with decreased overall survival on UVA, NS on MVA |
| Aishima et al(69) | 2006 | 100 | Ki-67 (IHC) | >18% | 51 | No significant prognostic role |
| Hoang et al(17) | 2002 | 9 | MIB-1 (IHC) | >30% | 89 | No significant prognostic role |
| Sugimachi et al(58) | 2001 | 6 | Ki-67 (IHC) | Nuclear staining | Not reported | High ki-67 LI independently predicted OS on MVA (P=0.0036) |
| Albores-Saavedra et al(19) | 2000 | 9 | MIB-1 | Not reported | Not reported | No significant prognostic role (Papillary carcinoma) |
| Tannapfel et al(12) | 2000 | 41 | MIB-1 and PCNA (IHC) | Nuclear staining | Not reported | No significant prognostic role |
| Kim et al(21) | 1999 | 28 | Ki-67 (IHC) | Nuclear staining | 100 | No significant prognostic role |
| Rijken et al(68) | 1998 | 35 | MIB-1 (IHC) | >20% | 34 | High MIB-1 LI associated with decreased OS on UVA |
| Shrestha et al(22) | 1998 | 32 | MIB-1 (IHC) | >57% | Not reported | High MIB-1 LI associated with decreased OS on UVA |
| Suto et al(23) | 1998 | 54 | MIB-1 (IHC) | >29% | 46 | High MIB-1 LI associated with decreased OS on UVA, NS on MVA |
| Nishida et al(66) | 1997 | 35 | PCNA (IHC) | >45% | 29 | High PCNA associated with decreased OS and independently predicted decreased OS on MVA (P=0.012) |
| Takamori et al(27) | 1997 | 17 | Ki-67 (IHC) | >20% | 62 | High ki-67 LI at tumour margin associated with decreased OS on UVA |
| Yamada et al(67) | 1995 | 21 | MIB-1 (IHC) | >10% | Not reported | High MIB-1 LI associated with decreased OS on UVA |

IHC, immunohistochemistry; OS, overall survival; UVA, univariate analysis; MVA, multivariate analysis; NS, not significant; LI, labelling index.
[a] Immunohistochemical cut-offs indicate the percentage of cells with positively staining nuclei unless stated otherwise.

**Table 3 - Studies examining proliferation indices in cholangiocarcinoma and their prognostic significance.**

| Study | Year | Patients (n) | Marker (Method) | Cutoff for positivity[a] | % Positive cases | Prognostic role |
|---|---|---|---|---|---|---|
| Shibahara et al(61) | 2004 | 27 | MUC1 (IHC) | >5% | 81 | MUC1 positivity associated with decreased OS on UVA |
| | | | MUC4 (IHC) | >5% | 37 | MUC4 positivity associated with decreased OS on UVA and independently prognostic on MVA |
| Tamada et al(62) | 2006 | 70 | MUC1 (IHC) | >5% | 87 | MUC1 positivity associated with decreased OS on UVA |
| | | | MUC4 (IHC) | >5% | 51 | MUC4 positivity associated with decreased OS on UVA and independently prognostic on MVA |
| Matsumura et al(102) | 2002 | 50 | MUC1 (IHC) | >5% | 76 | MUC1 positivity associated with decreased OS on UVA and independently prognostic on MVA |
| Takao et al(104) | 1999 | 73 | MUC1 (IHC) | >25% | 69 | MUC1 positivity associated with decreased OS on UVA and independently prognostic on MVA |
| Yuan et al(106) | 2005 | 37 | MUC1 (IHC) | >25% | 71 | MUC1 positivity associated with decreased OS on UVA (includes HCC, no subgroup analysis) |
| Higashi et al(101) | 1999 | 39 | MUC1/DF3 | >50% | 44 | MUC1/DF3 or MUC1/MY.1E12 positivity associated with decreased OS on UVA |
| | | | MUC1/MY.1E12 | >50% | 56 | |
| | | | MUC2 (all IHC) | >50% | 18 | MUC2 positivity associated with increased OS on UVA |
| Suh et al(103) | 2002 | 30 | MUC1 (IHC) | >5% | 80 | No significant prognostic role |
| | | | MUC2 (IHC) | >5% | 40 | MUC2 positivity associated with increased OS on UVA |
| Tamada et al(105) | 2002 | 60 | MUC1/DF3 | >5% | 77 | MUC1/DF3 or MUC1/MY.1E12 positivity associated with decreased OS on UVA |
| | | | MUC1/MY.1E12 | >5% | 78 | |
| | | | MUC2 (all IHC) | >5% | 19 | MUC2 positivity associated with increased OS on UVA |
| Boonla et al(107) | 2005 | 87 | MUC1 (IHC) | >25% | 39 | MUC1 positivity associated with decreased OS on UVA and independently prognostic on MVA |
| | | | MUC5AC (IHC) | >25% | 53 | No significant prognostic role |
| Hong et al(108) | 2005 | 193 | MUC2 (IHC) | >5% | 42 | No significant prognostic role |
| Yonezawa et al(109) | 1997 | 16 | MUC2 (mRNA) | >1% | 38 | MUC2 positivity associated with increased OS on UVA |
| Aishima et al(69) | 2006 | 100 | MUC2 (IHC) | >1% | 9 | Combined mucin phenotype associated with OS on UVA and independently prognostic on MVA. |
| | | | MUC5AC (IHC) | >10% | 40 | |
| | | | MUC6 (IHC) | >10% | 21 | |
| Boonla et al(110) | 2003 | 179 | MUC5AC (serum) | >1% | 63 | MUC5AC serum positivity associated with decreased OS on UVA and independently prognostic on MVA |

IHC, immunohistochemistry; OS, overall survival; UVA, univariate analysis; MVA, multivariate analysis; NS, not significant; HCC, hepatocellular carcinoma.
[a] Immunohistochemical cutoffs indicate the percentage of cells with positively staining cytoplasm unless stated otherwise.

**Table 4 - Studies examining mucins in cholangiocarcinoma and their prognostic significance.**