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Review

Prognostic molecular markers in hepatocellular carcinoma: A systematic review

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ABSTRACT

Hepatocellular carcinoma (HCC) is the fifth commonest malignancy worldwide and its incidence is rising. Surgery, including transplantation, remains the only potentially curative modality for HCC, yet recurrence rates are high and long-term survival poor. The ability to predict individual recurrence risk and subsequently prognosis would help guide surgical and chemotherapeutic treatment. As understanding of hepatocarcinogenesis has increased, the myriad of genetic and molecular events that drive the hepatocarcinogenic disease process, including angiogenesis, invasion and metastasis, have been identified. This systematic review examines the evidence from published manuscripts reporting the prognostic potential of molecular biomarkers in hepatocellular carcinoma. In summary, a number of molecular biomarkers with prognostic significance have been identified in hepatocellular carcinoma. Not only might these molecules allow more accurate prediction of prognosis for patients with HCC, but they may also provide targets for potential therapeutic agents.

1. Introduction

Hepatocellular carcinoma (HCC) is the 5th commonest malignancy worldwide and is the third most common cause of cancer-related death. Although the prevalence is highest in Africa and Asia, the incidence in western countries is rising, mainly due to increasing rates of alcoholic liver disease and hepatitis C infection. HCC most commonly develops in patients with chronic liver disease, the aetiology of which includes alcohol, viral infection (hepatitis B and C), metabolic diseases (haemachromatosis, α-1-antitrypsin deficiency) and aflatoxin. Surgery, including transplantation, remains the only curative modality for HCC. However, the long-term prognosis of patients undergoing potentially curative hepatic resection is still poor, with reported 5-year survival rates ranging from 17% to 53%. Despite resection with curative intent, the clinical course is variable and recurrence occurs in a high proportion of cases.[1,2]

The ability to predict patients at higher risk of recurrence and with a poor prognosis would help to guide surgical and chemotherapeutic treatment according to individual risk. Attempts have been made to predict recurrence and poor prog-

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nosis in patients with HCC after hepatectomy using clinicopathological characteristics. Tumour grade, size of tumour, microvascular invasion, portal vein tumour thrombus and the presence of microsatellite lesions have all been found to predict survival.[3–7] These, however, lack sensitivity for accurately predicting individual prognosis. Serum tumour markers, particularly alpha fetoprotein (AFP), have also been found to be prognostic;[5] however, they rely on a significant tumour burden making their usefulness in operable tumours questionable. More recently, interest has been focused on the prognostic value of serum inflammatory markers as a reflection of the host response to the tumour. In particular, an elevated preoperative serum C-reactive protein (CRP) has been found to be associated with a 75% recurrence rate at 12-months following resection for HCC, as well as being associated with reduced disease-free and overall survival when compared to patients with a lower preoperative level.[8]

With advances in understanding of tumour biology, interest in molecular biomarkers of carcinogenesis has grown, both in terms of their prognostic significance and also their potential as therapeutic targets. Table 1 summarises the molecular markers which have been shown to play a role in hepatocarcinogenesis. For detailed information regarding these markers, the reader is referred to recent reviews of molecular pathways involved in HCC, particularly in relation to their association with the different aetiological risk factors for HCC.[9–11] The aim of this review was to examine the current knowledge regarding the prognostic role of these molecular biomarkers in HCC. A systematic literature review was performed of the available evidence for the role of molecular biomarkers on the prognosis of patients undergoing resection of HCC. Searches of the Pubmed, MEDLINE, and Web of Science databases were performed using the following keywords, in varied combinations: hepatocellular carcinoma, liver, hepatic, resection, hepatectomy, recurrence, prognosis, molecular markers and biomarkers. Individual biomarkers were also included in searches. Cited references in articles identified were used to find further relevant publications. The search was restricted to English Language publications and was conducted up to 31st October 2006. Studies published in abstract form only, unpublished studies and articles published in non-peer reviewed journals were not included. Animal and *in vitro* work were also excluded. Studies of molecular markers solely in unresectable HCC were also excluded. Prognostic value of the following molecular markers is discussed: tumour suppressor genes; oncogenes; cell cycle regulators; apoptotic regulators; markers of angiogenesis; markers of invasion and metastasis; growth factors and receptors; proliferation indices; telomerase; and markers of genomic instability.

2. Tumour suppressor genes

2.1. *p53*

The *p*53 tumour suppressor gene is located on chromosome 17p13.1 and is responsible for regulation of the cell cycle at the G_1/S and G_2/M interfaces, as well as induction of apoptosis in response to severe damage to cellular DNA. *p*53 has been found to be mutated in 24–69% of HCC.[12–14] Mutations of *p*53 result in unregulated replication of defective DNA, genomic instability and progression to cancer. Wild-type *p*53 has a short half-life and is therefore undetectable by immunohistochemistry (IHC). Mutations in the *p*53 gene result in stabilisation of the protein, permitting nuclear accumulation and immunohistochemical detection. Studies assessing immunohistochemical expression of *p*53 in HCC provide conflicting data (Table 2).[14–22] Possible explanations for the differences in these results include differing methodologies, including variation in antibodies and subjective analysis of staining. In addition, absence of detectable *p*53 protein is not necessarily synonymous with a normal *p*53 gene, as certain mutations, particularly frameshift and nonsense, do not result in protein stabilisation and therefore would not be detected by IHC. IHC is therefore not a reliable methodology for assessing *p*53 status. DNA mutation analysis is a better technique to assess *p*53 status. The majority of studies investigating *p*53-mutation have found it to be associated with a shorter disease-free and overall survival (Table 2).[22–26] A major limitation of the current prognostic data on p53 mutation, as assessed by both IHC and mutation analysis, is that the majority of studies report cohorts with mixed aetiologic factors. Without appropriate subanalysis it is not possible to assess the impact of p53 mutation in, for example, HBV-associated HCC. This is clearly an area on which future attention should be focused.

Table 1 – Processes involved in hepatocarcinogenesis and associated markers (*potential prognostic value)

1. Proliferation, self-sufficiency in growth signals, insensitivity to antigrowth signals	*p53**, nm-23, Rb, *PTEN**, c-*met**, c-*myc**, cyclin A, cyclin D, cyclin E, p15, p16, p18, p19, p21, p27, p57, TGF-β, EGFR family, growth factors proliferation indices*
2. Avoidance of apoptosis	*p53**, Bcl-2, Bcl-xL, Bax, Bak, Bcl-xS, survivin
3. Limitless replicative potential	Telomerase (including TERT)*
4. Sustained angiogenesis	MVD, *VEGF**, *HIF*-1α*, NOS, bFGF, PD-EGF, tissue factor, endostatin/collagen XVIII, interleukin-8, angiopoietin
5. Tissue invasion and metastasis	*MMPs**, uPA, cadherin/catenin complex
6. Genomic instability	Chromosomal instability, aneuploidy*, microsatellite instability

nm-23, non-metastatic protein-23; Rb, retinoblastoma gene; PTEN, phosphatase and tensin homolog; TGF-β, transforming growth factor beta; EGFR family, epidermal growth factor receptor family; TGF-α, transforming growth factor alpha; HB-EGF, heparin-binding epidermal growth factor; TERT, telomerase reverse transcriptase; MVD, microvessel density; VEGF, vascular endothelial growth factor; HIF-1α, hypoxia-inducible factor-1 alpha; NOS, nitric oxide synthase; bFGF, basic fibroblast growth factor; PD-EGF, platelet-derived endothelial growth factor; MMP, matrix metalloproteases; uPA, urokinase plasminogen activator.

Table 2 – Studies examining the prognostic role of p53 in hepatocellular carcinoma

Study	Year	Patients	Marker	Method	Cutoff for positivity[a]	% Positive cases	Prognostic role
Jing et al.[46]	2005	47	p53	IHC	>10%	38.3	No significant prognostic role
Qin et al.[122]	2005	47	p53	IHC	>5%	38.3	No significant prognostic role
Lee et al.[15]	2004	35	p53	IHC	Positive immunoreactivity	71.4	p53-positivity associated with decreased DFS and OS
Osada et al.[14]	2004	153	p53	IHC	Positive immunoreactivity	41.7	p53-positivity associated with a trend towards decreased OS ($p = 0.1$)
Sheen et al.[16]	2003	79	p53	IHC	Positive immunoreactivity	81	p53-positivity associated with an increased risk of recurrence and a trend towards decreased DFS ($p = 0.08$) and OS ($p = 0.08$)
Hu et al.[17]	2003	105	p53	IHC	>10%	37.1	p53-positivity associated with decreased DFS and OS on UVA
Chen et al.[23]	2003	33	p53	DNA sequence analysis	p53 mutation	48.5	p53 mutation associated with shorter interval to development of recurrence
Qin et al.[21]	2002	222	p53	IHC	>10%	50.5	p53-positivity independently predicts decreased OS on MVA
Kobayashi et al.[128]	2002	63	p53	IHC	>10%	42.9	No significant prognostic role
Park et al.[22]	2001	20	p53	Direct DNA sequence analysis	p53 mutation	45	p53 mutation associated with a lower 1 year
Jeng et al.[18]	2000	79	p53	IHC	>10%	69.6	p53-positivity associated with a trend towards decreased DFS ($p = 0.09$) and OS ($p = 0.08$)
Endo et al.[155]	2000	107	p53	IHC	>Adjacent tissue	Not reported	No significant prognostic role
Ito et al.[36]	1999	83	p53	IHC	>5%	34.6	No significant prognostic role
Sugo et al.[26]	1999	98	p53	PCR-SSCP	p53 mutation	26	p53 mutation associated with decreased DFS and OS
Mise et al.[19]	1998	80	p53	IHC	Positive immunoreactivity	22.5	p53-positivity associated with decreased OS
Naka et al.[20]	1998	126	p53	IHC	Positive immunoreactivity	37	p53-positivity independently predicted decreased OS on MVA
Honda et al.[25]	1998	42	p53	DGGE+PCR-SSCP	p53 mutation	23.8	p53 mutation associated with decreased OS
Terris et al.[156]	1997	113	p53	IHC	>10%	22	No significant prognostic role
Soini et al.[12]	1996	33	p53	IHC	Positive immunoreactivity	24	No significant prognostic role
Hayashi et al.[24]	1995	90	p53	PCR-SSCP	p53 mutation	27.8	p53 mutation independently predicted decreased DFS on MVA
Ng et al.[157]	1995	71	p53	IHC	Not reported	31	No significant prognostic role

IHC, immunohistochemistry; PCR-SSCP, polymerase chain reaction-single-stranded conformational polymorphism; DGGE, denaturing gradient gel electrophoresis; MVA, multivariate analysis; UVA, univariate analysis; OS, overall survival; DFS, disease-free survival; 1 years, 1-year survival rate.

a Immunohistochemical cutoffs indicate the percentage of cells with positively staining nuclei unless otherwise stated.

Table 3 – Studies examining the prognostic role of cyclin-dependent kinase inhibitors in hepatocellular carcinoma

Study	Year	Patients (*n*)	Marker	Method	Cutoff for positivity[a]	% Positive cases	Prognostic role
Arzola et al.[41]	2004	41	p16	SSCP-PCR	p16 mutation	90.2	No significant prognostic role
Li et al.[42]	2004	49	p16	MSP-PCR	Promotor methylation	58	No significant prognostic role
Matsuda et al.[43]	2003	74	p16	IHC	Nuclear expression	70	p16-positivity independently predicted increased OS on MVA
Morishita et al.[44]	2004	51	p18	IHC	>8.4%	52.9	p18-positivity independently predicted increased OS on MVA
Qin et al.[45]	2001	70	p21	IHC	>21%	Not reported	No significant prognostic role
Naka et al.[20]	1998	126	p21	IHC	Positive immunoreactivity	33	No significant prognostic role
Jing et al.[46]	2005	47	p27	IHC	>10%	40.4	p27-positivity associated with increased OS on UVA
Nan et al.[47]	2004	32	p27	IHC	>Median	Not reported	p27-positivity independently predicted increased OS on MVA
Matsuda et al.[43]	2003	74	p27	IHC	>50%	54	p27-positivity associated with increased OS
Armengol et al.[48]	2003	46	p27	IHC	>50%	28.3	p27-positivity associated with increased DFS
Qin et al.[45]	2001	70	p27	IHC	>1.9%	48.6	p27-positivity associated with increased DFS (not OS)
Fiorentino et al.[49]	2000	54	p27	IHC	>50%	25.9	p27-positivity independently predicted increased OS on MVA
Tannapfel et al.[50]	2000	203	p27	IHC	>50%	51	p27-positivity associated with increased OS
Ito et al.[36]	1999	83	p27	IHC	>50%	49.4	p27-positivity independently predicted increased DFS on MVA

IHC, immunohistochemistry; MSP-PCR, methylation-specific polymerase chain reaction; SSCP-PCR, single-stranded conformational polymorphism polymerase chain reaction; OS, overall survival; DFS, disease-free survival; MVA, multivariate analysis; UVA, univariate analysis.
a Immunohistochemical cutoffs indicate the percentage of cells with positively staining nuclei unless otherwise stated.

2.2. *Other tumour suppressor genes*

The prognostic values of several other tumour suppressor genes have been investigated in HCC. Positive expression of the phosphatase and tensin homologue (PTEN) tumour suppressor gene has been identified as an independent prognostic factor for decreased overall survival following resection for HCC.[17,27] Similarly, loss of expression of the tumour suppressor gene non-metastatic protein (nm) 23 has been identified in those patients developing recurrence disease following resection[28] and has been associated with shortened overall survival following surgery.[29] No relationship has been found between expression of the tumour suppressor gene retinoblastoma (*Rb*) and prognosis following resection.[20]

3. Oncogenes

The *c-met* and *c-myc* oncogenes have both been assessed for their prognostic significance in HCC. *c-met* is commonly over-expressed by HCC, and has been associated with reduced overall survival in the only prognostic studies performed.[30,31] *c-myc* expression has been found to be increased in those patients developing early recurrence post-hepatectomy,[32] and amplification of the *c-myc* gene has been associated with reduced disease-free and overall survival following resection.[33,34]

4. Cell cycle regulators

4.1. *Cyclin and cyclin-dependent kinases*

The G_1/S transition is important for cell cycle progression. Phosphorylation of Rb by cyclin/cyclin-dependent kinase complexes inactivates it, allowing cell cycle progression. Over-expression of cyclin A, D1 and E have all been investigated for prognostic significance following resection of HCC. Chao and colleagues[35] found cyclin A expression to independently predicted decreased disease-free survival on multivariate analysis. Two studies have found elevated cyclin D1 expression to be associated with decreased disease-free and overall survival following resection,[36,37] however, others found no significant association.[38,39] No studies have found cyclin E to be significantly associated with prognosis.[36,39]

4.2. *Cyclin-dependent kinase inhibitors*

Cyclin-dependent kinase inhibitors are potent negative regulators of the cell cycle by inhibiting the G_1/S transition. Two

families of CDK inhibitors exist, the INK4 family, which contains p15, p16, p18 and p19 and specifically inhibits D-type cyclin-CD4/CDK6 complexes, and the KIP/CIP family, which contains p21, p27 and p57 and binds to and inhibits a number of cyclin/CDK complexes.

4.2.1. *INK4 family*

Methylations of promotor regions of p15 and p16 leading to gene silencing have been identified in HCC. Patients with p15 and p16 methylation have been found to be more likely to develop recurrent disease following resection.[40] Whilst no studies have examined the prognostic role of p15, three have looked at p16 expression, however only one identified any prognostic role (Table 3).[41–43] Loss of p18 expression has also been shown to independently predict overall survival on multivariate analysis.[44]

4.2.2. *KIP/CIP family*

None of the studies investigating the prognostic role of p21 identified any significant correlation with survival (Table 3).[20,45] However, the majority of studies investigating the prognostic role of p27 expression in patients with HCC have found it to be associated with improved disease-free and overall survival (Table 3).[36,43,45–50] In addition, three separate studies have found loss of p57 expression to independently predict reduced overall and disease-free survival following hepatectomy for HCC.[51–53]

5. Apoptosis

5.1. *Bcl-2 family*

The Bcl-2 family is one of the best-characterised groups of apoptosis-mediating factors. Amongst the members of this family, Bax, Bak and Bcl-xS act as promoters of apoptosis, with Bcl-2 and Bcl-xL acting as apoptotic inhibitors. Several of these have been investigated for prognostic significance following resection for HCC. Bcl-2 expression was not found to be associated with prognosis following resection.[54] However, Bcl-xL over-expression has been found to independently predict decreased overall- and disease-free survival on multivariate analysis.[54,55] Garcia and colleagues also found expression of the pro-apoptotic protein Bax to independently predict increased overall survival following resection of HCC.[54]

5.2. *Survivin*

Survivin is a member of the inhibitor of apoptosis protein (IAP) family of antiapoptotic proteins. It inhibits apoptosis mainly by targeting the terminal effectors caspase-3 and -7 in the apoptotic protease cascade. Survivin expression is associated with a poor prognosis following resection of HCC. Nuclear survivin expression has been shown to be associated with shortened disease-free survival.[56] Patients with tumours expressing survivin mRNA suffered higher rates of recurrence and poorer disease-specific survival rates than those with tumours not-expressing survivin mRNA.[57,58] A high survivin/GADPH mRNA ratio has also been shown to independently predict tumour recurrence after hepatectomy and to be associated with reduced disease-free survival.[59]

6. Angiogenesis

6.1. *Microvessel density*

Microvessel density (MVD) is a commonly used index of angiogenesis in tumours, involving immunohistochemical staining of endothelial cell markers. Commonly used markers include CD34, CD31 and von Willebrand factor (vWF). MVD using CD34 has been found to be independently prognostic for decreased disease-free survival on multivariate analysis in patients undergoing resection of HCC under 5 cm in size.[60,61] Another study found CD34 to predict decreased overall survival on multivariate analysis.[62] However, two more recent studies using CD31 and CD34 as markers suggest no prognostic value.[63,64] MVD assessed using vWF expression has been shown to independently predict decreased disease-free survival,[65] however in a larger study, Poon and colleagues[61] found no prognostic association using the same marker. These differing results may reflect the differing endothelial markers, sampling site, and selection bias in choosing hotspots to define MVD however, it remains debatable whether MVD offers any prognostic information following resection for HCC.

6.2. *Vascular endothelial growth factor*

Vascular endothelial growth factor (VEGF) is a key angiogenic stimulator and has been extensively studied for its prognostic significance in HCC. VEGF over-expression has been associated with a greater risk of metastatic recurrence[66] and to be higher in those patients developing early recurrence.[67] Increased expression of VEGF, as assessed by immunohistochemistry, has been associated with a decreased overall survival following resection for HCC,[68,69] however, other studies identified no prognostic role.[65,70] Jeng and colleagues[71] analysed the expression VEGF165 isoform mRNA in resected HCC specimens using reverse transcriptase-polymerase chain reaction (RT-PCR) and found positive expression of VEGF165 mRNA to independently predict the development of recurrence and recurrence-related mortality. Serum levels of VEGF have been found to correlate with tumour VEGF expression in HCC[72] and therefore may allow assessment of prognosis pre-operatively. Elevated pre-operative serum VEGF levels have been shown to independently predict decreased disease-free and overall survival following resection on multivariate analysis.[73,74] In addition, Jeng and colleagues[75] found pre-operative serum levels of VEGF165 isoform mRNA to be independently prognostic for disease recurrence and recurrence-related mortality.

6.3. *Hypoxia-inducible factor*

Hypoxia-inducible factor-1 (HIF-1) is a basic-helix–loop–helix-PAS transcription factor that plays a critical role in angiogenesis. HIF-1α, the active subunit, is expressed in response to hypoxic conditions, but rapidly degraded in normoxic conditions due to continuous ubiquitination and proteosome-mediated degradation. Hypoxia inhibits this degradation. HIF-1α binds to hypoxia response elements and activates transcrip-

tion of several genes, including erythropoietin, iNOS, PD-EGF and VEGF. HIF-1α is expressed by several human malignancies and over-expression has been associated with resistance to chemotherapeutics and poor prognosis in some cases. Several studies have addressed the prognostic value of HIF-1α expression following resection of HCC. Wada and colleagues[70] found >1% nuclear HIF-1α expression to be significantly associated with reduced disease-free survival following resection and this was found to have borderline significance on multivariate analysis. Huang and colleagues[69] also found high HIF-1α expression to be associated with shorter overall survival, although this did not reach statistical significance. In addition, Lee and colleagues[76] utilised gene expression profiling using oligo microarray analysis to predict survival following resection for HCC. They found certain gene expression profiles to be associated with survival. HIF-1α mRNA was found to be over-expressed, while egl nine homologue 2 mRNA, an inhibitor of HIF-1α, was reduced in the poor prognosis group.

6.4. *Nitric oxide synthase*

Nitric oxide synthase (NOS) exists as three isoforms: endothelial NOS, neuronal NOS and inducible NOS (iNOS). NOS is the rate-limiting step in the production of nitric oxide (NO), which is thought to play an important role in tumour angiogenesis. Over-expression of iNOS has been identified in several human malignancies and increased expression has been associated with an aggressive phenotype and a poor prognosis. In HCC, positive iNOS-expression has been associated with an increased risk of tumour recurrence.[57] Rahman and colleagues[77] found no prognostic value of iNOS expression alone. However, when in conjunction with COX2 expression, combined negative expression independently predicted improved disease-free and overall survival following hepatectomy with curative intent on multivariate analysis.

6.5. *Basic fibroblast growth factor*

Basic fibroblast growth factor is a soluble heparin-binding polypeptide with a potent mitogenic effect on endothelial cells. Two studies have assessed the prognostic significance of serum bFGF following resection for HCC; Poon and colleagues[78] found that levels of bFGF above the median of >10.8 pg/mL independently predicted decreased disease-free survival on multivariate analysis in a series of 88 patients. However, Chao and colleagues[74] failed to identify any association with prognosis using a lower cutoff (>2.1 pg/ml).

6.6. *Platelet-derived endothelial growth factor*

Platelet-derived endothelial growth factor (PD-EGF), also known as thymidine phosphorylase, is a pro-angiogenic factor known to play a role in tumour angiogenesis. No studies investigating PD-EGF activity in HCC tissue have found a correlation with survival.[79,80] However, increased PD-EGF activity in normal liver adjacent to the HCC has been associated with a reduction in disease-free survival[81] and more frequent long-term recurrence (>24 months post-hepatectomy).[80]

6.7. *Tissue factor*

Tissue factor (TF) is a transmembrane glycoprotein involved in triggering the extrinsic coagulation pathway by binding with factor VII. TF expression has been shown to correlate with metastatic potential in several human tumours, and also to be associated with tumour angiogenesis. High TF expression has been found to independently predict decreased disease-free and overall survival following resection of HCC.[82,83]

6.8. *Endostatin/collagen XVIII*

Endostatin is a potent angiogenesis inhibitor that specifically inhibits the proliferation and migration of endothelial cells. A study of 105 resected HCC has shown higher endostatin/collagen XVIII expression in adjacent non-tumour tissue to be associated with significantly shorter overall and disease-free survival following resection and to independently predict tumour recurrence.[84]

6.9. *Interleukin-8*

Interleukin (Il)-8 is a multifunctional chemokine that has been shown to be important in inducing angiogenesis. Il-8 is expressed in both the tumour and the serum of patients with HCC.[85] Overall and disease-free survival have been shown to be significantly improved in patients with low pre-operative serum Il-8 compared to those with higher levels.[86] Il-8 was found to be an independent prognostic factor for overall survival on multivariate analysis.[86]

6.10. *Angiopoietins*

Angiopoietins are endothelial cell growth factors which act as ligands for the tyrosine kinase receptor, Tie2. Angiopoietin-1 (Ang-1) promotes vascular maintenance and maturation, whereas angiopoietin-2 (Ang-2) acts as an antagonist of Ang-1 and induces angiogenesis in the presence of VEGF. Over-expression of Ang-2 has been associated with a poor prognosis in several human cancers, including HCC. High Ang-2 expression has been associated with reduced disease-free survival following resection of HCC,[70] and Mitsuhashi and colleagues[87] found the ratio of Ang-2:Ang-1 to be an independent prognostic factor for overall survival on multivariate analysis.

7. Invasion and metastasis

7.1. *Matrix metalloproteases*

The matrix-degrading metalloproteinases (MMP) are a family of proteolytic enzymes characterised by their ability to degrade the extracellular matrix, and are considered to play an important role in cancer invasion and metastasis. Elevated levels of MMP-2 (gelatinase A), a MMP which degrades collagen IV, a chief component of the basement membrane, have been found in patients who suffered recurrence post-resection[88] and to be associated with early recurrence.[89] Concomitant over-expression of MMP-7 with MMP-2 was associated with recurrence within the first postoperative year.[90] Expres-

sion of human macrophage metalloelastase (MMP-12) has also been found to independently predict improved overall survival on multivariate analysis.[91]

7.2. Urokinase plasminogen activator

Urokinase plasminogen activator (uPA) is a serine protease that converts plasminogen into the protease plasmin, which subsequently degrades the extracellular matrix and activates MMP. Expression of uPA and its receptor (uPAR) is associated with tumour growth and invasion. Increased activity of uPA, as assessed by ELISA, has been found to be associated with reduced disease-free survival following resection.[92] In addition, Zheng and colleagues[93] showed combined expression of uPA, uPAR and PAI-1 in resected specimens to be associated with reduced overall survival.

7.3. The cadherin/catenin complex

E-cadherin is a transmembrane glycoprotein that mediates cell-cell adhesion. β-Catenin is responsible for linking E-cadherin to α-Catenin, which is directly connected to the actin cytoskeleton of the cell. β-Catenin acts both as an intracellular adhesion molecule and as an intracellular effector of the Wnt pathway. Pathway activation results in nuclear translocation and stimulation of transcription of cell proliferation, anti-apoptotic and pro-angiogenic genes. Loss of E-cadherin expression is closely related to the progression and invasiveness of several cancers. In HCC, loss of expression of E-cadherin or its mRNA has been associated with the early development of recurrent disease following resection.[94] Loss of E-cadherin expression has also been associated with reduced overall survival in patients with HCC following resection,[95] although Inagawa and colleagues[38] found no association between E-cadherin expression and survival following resection of HCC under 3 cm in size. Several studies have examined the prognostic role of β-catenin expression in HCC. These studies however demonstrate conflicting results, with nuclear expression shown to predict both increased[96,97] and decreased[38] survival on multivariate analysis. Possible explanations for these discrepancies include differing antibodies, scoring systems and areas of tumour tissue analysed.

8. Growth factors and receptors

8.1. Transforming growth factor-β

Transforming growth factor betas (TGF-βs) belong to a superfamily of polypeptide signalling molecules involved in regulating cell growth, differentiation, angiogenesis, invasion and immune function. TGF-β1 is the predominant form in humans, promoting angiogenesis and suppressing immune function. Higher expression of TGF-β1 has been found to be an independent prognostic factor for reduced survival in patients with inoperable HCC.[98] However, the only study investigating the prognostic value of TGF-β expression post-hepatectomy found no relationship with survival.[99] Elevated urinary TGF-β1 has been found to be prognostic for shortened survival in patients with HCC, although levels were measured at the time of diagnosis, and not all patients underwent surgery.[100]

8.2. Epidermal growth factor receptor family

The epidermal growth factor receptor (EGFR) family consists of four closely related transmembrane tyrosine kinase receptors EGFR (erbB-1), c-erbB-2 (HER-2/neu), c-erbB-3 (HER-3), and c-erbB-4 (HER-4). These bind ligands of the EGF family, including EGF, TGF-α and heparin-binding EGF. EGFR is expressed by HCC and high levels of expression have been associated with early recurrence[101,102] and reduced disease-free survival following resection.[102] C-erbB-2 (HER-2/neu) over-expression is uncommon in HCC. Confusion exists regarding its prognostic role; patients with HER-2 amplification as assessed by fluorescent in situ hybridisation had a lower 2-year overall survival rate following resection,[103] however an immunohistochemical study failed to find similar results.[102] This disparity may reflect the differing methodologies. Ito and colleagues[102] also investigated c-erbB-3 and c-erbB-4 expression, finding c-erbB-3 expression to be associated with reduced disease-free survival following resection; however, c-erbB-4 did not have any association with survival.

8.3. Leptin receptor

Leptin, a circulating hormone secreted by adipocytes, is involved in the modulation of cell growth, differentiation and angiogenesis and has been implicated in the pathogenesis of several human malignancies. Leptin and its receptor Ob-R are over-expressed in HCC. Increased expression of both has been found to be independent prognostic factors for improved overall survival on multivariate analysis.[63,104]

9. Proliferation indices

The proliferative activity of a tumour provides an indication of its rate of growth and correlates with an aggressive phenotype. Proliferation may be assessed by mitotic index, S-phase cell fraction, or immunohistochemical assessment of proliferation-associated nuclear proteins (Ki-67, its epitope MIB-1, proliferating cell nuclear antigen (PCNA) or argyrophilic nucleolar organizer regions (AgNOR)). A higher mitotic index has consistently been found to predict decreased disease-free and overall survival.[105–109] High expression of AgNOR has also been shown to be significantly associated with decreased overall survival in patients undergoing hepatic resection for HCC.[109,110]

A large number of studies have utilised immunohistochemical assessment of proliferative activity, using either Ki67 or its epitope MIB-1, or PCNA, in patients with HCC. High expression has been correlated with the development of recurrent disease following resection,[32,67,111] particularly early recurrence.[112] Studies investigating the prognostic value of these markers have generally shown high levels of expression of these markers to be associated with a poor prognosis, although the evidence is more conclusive for Ki67/MIB-1 (Table 4).[14,21,38,43,47,55,56,63,68,109,112–123] Two studies have combined Ki67 expression with ssDNA, a marker of apoptosis, to create a growth index (=Ki67–ssDNA). They both found high scores to independently predict prognosis on multivariate analysis.[124,125]

Table 4 – Studies examining the prognostic role of markers of proliferation in hepatocellular carcinoma

Study	Year	Patients (*n*)	Marker (method)	Cutoff for positivity[a]	% Positive cases	Prognostic role
Wang et al.[63]	2006	66	Ki-67 (IHC)	>50%	22.7	No significant prognostic role
Nakanishi et al.[112]	2005	49	MIB-1 (IHC)	>50%	30.6	High MIB-1 independently predicted decreased OS in patients with early recurrence (within 6 months) on MVA
Qin et al.[122]	2005	47	PCNA	>30%	46.8	High PCNA associated with a trend towards decreased OS ($p = 0.052$)
Fields et al.[56]	2004	72	MIB-1 (IHC)	>10%	Not reported	High MIB-1 independently predicted decreased DFS on MVA
Watanabe et al.[55]	2004	33	Ki-67 (IHC)	T/N >10	66.6	High Ki-67 associated with decreased OS on UVA
Nan et al.[47]	2004	32	PCNA (IHC)	>Median	Not reported	No significant prognostic role
Claudio et al.[68]	2004	21	PCNA (IHC)	>50%	23.8	No significant prognostic role
Osada et al.[14]	2004	153	PCNA (IHC)	>50%	Not reported	High PCNA associated with decreased 5 years
Hu et al.[121]	2003	105	PCNA (IHC)	>mean	Not reported	High PCNA associated with decreased DFS and OS on UVA
Xu et al.[120]	2003	59	PCNA (IHC)	>49%	47.5	High PCNA independently predicted decreased OS on MVA
Matsuda et al.[43]	2003	40	Ki-67 (IHC)	>20%	35.0	High Ki-67 associated with decreased OS on UVA
Aoki et al.[119]	2003	142	Ki-67 (IHC)	>20%	49.3	High Ki-67 associated with decreased DFS on UVA
Inagawa et al.[38]	2002	51	Ki-67 (IHC)	>10%	Not reported	High Ki-67 associated with decreased OS on UVA
Qin et al.[21]	2002	244	PCNA (IHC)	Not reported	Not reported	No significant prognostic role
Zeng et al.[118]	2002	51	PCNA (IHC)	>15%	35.3	High PCNA associated with decreased OS
Tannapfel et al.[109]	1999	193	MIB-1 (IHC)	Nuclear staining	Not reported	High MIB-1 associated with decreased OS on UVA
			PNCA (IHC)	Nuclear staining	Not reported	High PCNA associated with decreased OS on UVA
Ito et al.[117]	1999	76	MIB-1 (IHC)	>20%	59.2	High MIB-1 independently predicted decreased DFS on MVA
King et al.[116]	1998	67	Ki-67 (IHC)	>10%	70.1	High Ki-67 independently predicted decreased DFS and OS on MVA

IHC, immunohistochemistry; T/N, tumour to adjacent normal tissue ratio; OS, overall survival; DFS, disease-free survival; MVA, multivariate analysis; UVA, univariate analysis; 5 years, 5-year survival rate.
a Immunohistochemical cutoffs indicate the percentage of cells with positively staining nuclei unless otherwise stated.

10. Telomerase

Human telomerase is a ribonuclear protein composed of telomerase RNA and human telomerase reverse transcriptase (hTERT). Shortening of telomeres in human cells to a critical length limits the growth to a finite number of divisions before senescence or cell death occurs and it is thought that telomerase activation stabilises telomere length, preventing this. In patients with HCC, studies investigating the prognostic value of telomerase activity have found high telomerase activity, as assessed by the TRAP assay (telomeric repeat amplification protocol assay), to be associated with lower 5-year survival rates following resection[126] and to consistently independently predict decreased disease-free survival on multivariate analysis.[127–129]

11. Genomic instability

11.1. *Chromosomal instability/aneuploidy*

Aggressive tumours are characterised by genomic instability which is thought to favour cancer progression and adaptation. Chromosomal instability (CIN) is the loss or gain of a chromosomal segment during cell division. As in other human malignancies, HCC demonstrates a high incidence of CIN. CIN leads to an increase in aneuploidy, which in turn

drives further mutations, enhancing tumour progression. Two studies have used array comparative genomic hybridisation (aCGH) to assess the prognostic role of genomic damage in HCC.[130,131] Katoh and colleagues[130] found chromosomal losses on 4q, 8p, 13q, 14q and 17p, and gains on 8q and 17q, to be associated with shorter survival, although only loss on 17q13.3 and gain on 8q11 were independent prognostic factors. Kusano and colleagues[131] correlated the total number of chromosomal alterations with increasing tumour stage. A gain of 8q24 was preferentially observed in well-differentiated HCCs, however a loss of 13q13–14 and amplification of 11q13 were associated with poorly differentiated tumours. Losses of 8p and 13q and amplification of 11q13 were associated with a poor prognosis. However, only total number of chromosomal alterations was independently prognostic on multivariate analysis. Loss of 13q and total number of alterations were also associated with an increased risk of recurrence.

Aneuploidy is common in HCC, with reported rates varying between 35% and 84%.[19,118,132–135] The majority of studies investigating the prognostic role of aneuploidy in HCC have found it to be associated with a reduced overall survival following resection.[19,118,132,134,135] Ng and colleagues[133] found tumour aneuploidy to be associated with reduced overall survival following resection, however when subanalysed with respect to tumour size, aneuploidy was found to independently predict reduced overall survival on multivariate analysis in tumours under 5 cm in size.

11.2. *Microsatellite instability*

Microsatellites are small tandem repeats throughout the genome. Instability of these microsatellites (MSI) is caused by mutations in DNA mismatch repair genes. Reported rates of MSI at one chromosomal locus range between 34% and 68%,[136–138] however other studies report much lower rates (0–11%[139–141]). These differences may be explained by differing aetiology of HCC, and differences in the number and type of markers examined. In addition, expression of BAT26, a microsatellite marker that reliably predicts high-rate MSI (MSI-H – defined as MSI in >30% of markers examined) is rarely altered.[138,139] Despite this, reported rates of MSI-H range between 16% and 18%.[142,143] Chiappini and colleagues[143] found patients with MSI-H to have a significantly lower disease-free survival than those without (17% cf. 47% at 36 months). Salvucci and colleagues[137] found no association between overall MSI and prognosis, but did find the presence of a specific alteration (*D16S402*) to be associated with a reduced disease-free survival rate.

12. Conclusions

Research into the molecular biology of hepatocarcinogenesis has identified a multitude of molecular biomarkers with potential prognostic significance. Markers of particular interest include *p*53-mutation, *PTEN*, *c-met*, *c-myc*, p18, p27, p57, serum VEGF, HIF-1α, MMP-2, -7, and -12, as well as proliferation indices, telomerase activity and aneuploidy (Table 1). The majority of studies have focused on individual markers in retrospective studies, and differing methodologies between studies may explain inconsistencies in results. In addition, most studies include HCC of several aetiologies, and variation in the prevalence of these between different populations may also account for inconsistent results. HCC is a complex and heterogenous disease involving several aetiological agents that exert differential effects on the molecular pathways involved. It may be that a particular marker, prognostic for HBV-related HCC, is not relevant for HCV-related HCC. Therefore, a study containing a mixed population may fail to take account of this unless appropriate subanalysis is performed. Realistically, such analysis is often not possible as the majority of patients are likely to have been exposed to a combination of aetiological factors.

There is a need for both a standardised approach to assessment of molecular biomarkers, and validation of these potential markers in larger cohorts of patients, in order to formulate panels of biomarkers into an accurate tool, with which a clinician can predict early disease progression and prognosis. Combining panels of molecular biomarkers with more traditional histopathological characteristics may enable more accurate prediction of those at high risk of disease progression and more appropriate targeting of resources. In addition to biomarker expression in resected specimens or biopsy samples, further emphasis should be placed on the role of circulating serum biomarkers. Assessment of molecular biomarkers in serum (for example pre-operative serum VEGF), as well as other bodily fluids including urine, may allow formulation of pre-operative prognostic criteria to identify patients most likely to benefit from particular therapies, such as hepatic resection and transplantation, as well as predict those most likely to respond to different chemotherapeutic agents. It may be that high-risk patients achieve no advantage in undergoing hepatic resection compared to a less invasive treatment modality, such as tumour ablation, with its reduced morbidity, mortality, and cost. In addition, the ability to stratify patients' prognoses pre-operatively would improve provision of patient information when obtaining informed consent, allow assessment of the need for adjuvant therapies, and facilitate comparative studies and clinical trials. Serum and urinary biomarkers may also have a potential role in screening for recurrent disease following treatment.

Realisation of these aims in a clinical setting has been made more viable by microarray gene profiling. Although much data have been gleaned from aggregating separate studies focusing on molecular targets of a specific (usually topical) pathway, this methodology provides only a brief snapshot of the carcinogenic process. Microarray-based technology allows the study of multiple genes simultaneously, and several important findings relating to HCC have been made. Ho and colleagues[144] used microarray to identify 14 genes that could discriminate between those patients with vascular invasion from those without. They subsequently tested the prognostic value of this finding on a separate group, finding a significantly poorer disease-free survival in those patients predicted to have vascular invasion, and therefore to be at higher risk of recurrence. Work by Iizuka and colleagues based on microarray analysis identified a group of genes that could predict intrahepatic recurrence with a positive predictive value of 88% and a negative predictive value of 95%.[145] Subsequent work by the same group identified separate signature gene profiles for patients developing early

intrahepatic recurrence compared to those developing extrahepatic recurrence.[146] Whilst this is important in determining prognosis, with the shortage of donor organs, it may also help target those patients most likely to benefit from transplantation. In addition, microarray analysis of tissue from individual patients may enable us to predict which patients are likely to respond to specific chemotherapeutic agents, or combinations, thus creating individualised treatment regimes.[147]

Another new technology that holds considerable promise in terms of biomarker discovery is proteomic analysis. To date, proteomics studies in HCC have focused mainly on identifying proteins differentially expressed between malignant and benign tissue (reviewed in Ref. [148]). In addition, serum proteomics has been employed in the attempt to discover novel serum tumour markers; heat-shock protein (HSP)27 being one example of this,[149] however no prognostic data exist. A recent study by Luk and colleagues[150] using tissue proteomic profiling identified three proteins (HSP27, HSP70 and glucose-regulated protein (GRP)78) which were consistently over-expressed in resected HCC compared to adjacent non-tumourous tissue and normal liver. Although they did not specifically investigate prognostic value, they did find raised HSP27 and GRP78 expression to be associated with increased alpha-fetoprotein level and venous invasion respectively, both of which have previously been associated with aggressive phenotype and poor prognosis. Proteomic analysis has also shown HSP27 to be overexpressed in HCC with a metastatic phenotype.[151] Li and colleagues used proteomics to identify differential expression between HCC cell lines with high and low metastatic potential; cytokeratin 19 was found to be over-expressed in the highly metastatic cell line.[152] Based upon this, they demonstrated that positive expression of cytokeratin 19 in resected HCC specimens was associated with increased tumour stage, and the presence of satellite nodules and portal vein tumour emboli.[153] More recent work has shown elevated serum CYFRA 21-1, a fragment of cytokeratin 19, to be associated with increased tumour stage and the presence of portal vein tumour emboli in patients with HCC undergoing resection.[154] This series of experiments provides an eloquent example of how large-scale mapping techniques, such as proteomics and microarray analysis, may be used to generate hypotheses for future work.

In summary, several molecular markers with prognostic significance have been identified in HCC. Not only may these molecules allow accurate prediction of prognosis of patients with HCC and allow targetting of therapy, but they may also represent novel targets for therapeutic agents.

Conflict of interest statement

None declared.

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