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TOPIC HIGHLIGHT

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Management of hepatocellular carcinoma: Predictive value of immunohistochemical markers for postoperative survival

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Abstract

Hepatocellular carcinoma (HCC) accounts for over 90% of all primary liver cancers. With an ever increasing incidence trend year by year, it has become the third most common cause of death from cancer worldwide. Hepatic resection is generally considered to be one of the most effective therapies for HCC patients, however, there is a high risk of recurrence in postoperative HCC. In clinical practice, there exists an urgent need for valid prognostic markers to identify patients with prognosis, hence the importance of studies on prognostic markers in improving the prediction of HCC prognosis. This review focuses on the most promising immunohistochemical prognostic markers in predicting the postoperative survival of HCC patients.

Key words: Hepatocellular carcinoma; Management; Immunohistochemical; Prognostic marker; Predictive marker

Core tip: Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death worldwide. Hepatic resection is generally considered to be one of the most effective therapies for HCC patients, however, the overall post-hepatic resection survival of HCC patients remains unsatisfactory as indicated by the high recurrence rate. Therefore, there is an urgent need to identify prognostic biomarkers for the prediction of postoperative recurrence or metastasis, and to develop better strategies for HCC management. The purpose of this paper is to review the most promising immunohistochemical prognostic markers so far for predicting the postoperative survival of HCC patients.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a leading cause

of cancer-related death worldwide, with an increasing incidence[1]. The major risk factor associated with HCC is liver cirrhosis, which is predominantly caused by chronic B virus (HBV) and/or hepatitis C virus (HCV) infections, aflatoxin B1 exposure, and alcoholic liver disease. It is estimated that HBV and HCV account for approximately 75%-80% of HCC cases worldwide. In particular, chronic HBV infection is a predominant risk factor for HCC in Asia and Africa[2]. Hepatic resection (HR) is a potentially curative and popular therapy for HCC patients[3], however, the postoperative outcome remains unsatisfactory, with a 5-year post-HR recurrence rate of approximately 80%[4,5]. In fact, the high postoperative incidence of recurrence is the most frequent cause of postoperative death in HCC patients, and the main reason for the low postoperative survival rate is either intrahepatic metastasis or metachronous multicentric HCC[6].

So far, it has been still difficult to predict the probability of HCC metastasis and post-HR recurrence. There have been many studies on the risk factors contributing to post-HR recurrence of HCC where a number of prognostic factors related to clinicopathological parameters of HCC have been considered, including tumor size, stage, and grade. Due to lack of a systemic/uniformed approach, these researches results are not consistent. More investigations are required in the search for better markers for HCC prognosis, as a better prediction of postoperative recurrence or metastasis ultimately helps develop better strategies for HCC management.

Immunohistochemistry (IHC) is the most widely applied pathological technique in determining the expression status of tumor-associated proteins and in studying the prognostic and clinical relevance of biomarkers[7-9]. In spite of the paramount importance of IHC in determining the utility of a biomarker in clinical practice, the lack of universally accepted standardization guidelines has rendered the translation of promising biomarkers into clinical application. Having elaborated on nearly all the promising biomarkers so far in the main body of this review, we will discuss in conclusion the various limitations and technical challenges that need to be addressed when validating *via* IHC a predictive biomarker for clinical endpoint. More specific to HCC, although many immunohistochemical markers have been reported to have a prognostic value for HCC patients, some of which are also validated as independent prognostic markers, so far, there has been no consensus on how these markers could add prognostic value to the clinical parameters. An ideal IHC biomarker for HCC needs to be repeatable, with strong localized staining, valid across a number of patient groups and HCC subtypes, easily quantifiable, and associated with clear clinical outcome measures. Based on our extensive review of relevant literature (Table 1), this review intends to find out why no immunohistochemical markers are applicable in clinical practice, and focuses on the most promising immunohistochemical markers among existing ones in predicting the postoperative survival of HCC patients.

TUMOR SUPPRESSORS

Tumor suppressor p53

Alteration of p53 is one of the most frequent genetic changes found in HCC, and the biological function of p53 in tumor initiation and progression has been well characterized[10]. Numerous studies have investigated the prognostic value of p53 protein expression in HCC patients, but reports on the prognostic significance of p53 protein in HCC are often inconsistent and even conflicting, making it difficult to assess the clinical benefit of p53. So far, many studies have demonstrated that p53 protein expression is closely related to the occurrence, progression, metastasis, and survival of HCC. The over-expression of p53 protein is not only closely related to clinicopathological parameters, such as poorly-differentiated HCC, advanced HCC stages[11,12], but also to microvascular invasion, portal vein invasion, and high risk of tumor recurrence, and overall survival (OS) as well as recurrence-free survival (RFS) post-HR[13-16], especially within the first year post-HR in HCC patients[17]. Collectively, these findings indicate that the presence of p53 over-expression in HCC is identified as a major risk factor associated with the aggressive behavior of tumor, as well as a significant predictive marker for postoperative recurrence and survival in HCC patients[18].

Nevertheless, in some reports with either univariate or multivariate analysis, p53 protein expression in HCC has not been found to be an independent prognostic indicator of survival, despite that the over-expression of p53 protein is more frequent in tumors with poor cellular differentiation[19], > 5 cm in diameter[20], and vascular invasion[21]. Having said that, tumor differentiation and tumor size ⩾ 5 cm and vascular invasion are reported to be at high risk of HCC recurrence postoperatively[22-24], and they are independent poor prognostic factors for OS and disease free survival (DFS) in post-HR HCC patients[25,26]. These findings indicate that p53 expression in HCC may serve as a marker of a more aggressive behavior, and it could have an indirect adverse impact on survival.

Aiming at establishing whether those conclusions could provide solid grounds for applying p53 protein into prognostic clinical practice, the authors of this review carefully studied and compared the included studies. To our surprise, we have noticed several drawbacks in those studies that may affect the reliability of their own conclusions.

To begin with, variation in the immunohistochemical methods with respect to specific antibody clones, dilutions, antigen retrieval methods, as well as the cut-off values for positive expression, could have significant impact on the analysis of the prognostic value of p53 detection in HCC. Most studies used the monoclonal DO-7 antibody, with dilution ranging from 1:50 to 1:100, and citrate buffer for antigen retrieval, neither of which seems to have

Table 1 Immunohistochemical markers of hepatocellular carcinoma associated with prognosis in this review

Marker	Association with poor prognosis	Quoted literature examples
Tumor suppressors		
Mutant *p53*	Increased expression	Schöniger-Hekele *et al*[18]
Proliferation associated proteins		
Ki67 (detected by Mib1)	Increased expression	Schmilovitz-Weiss *et al*[40]
Proteins associated with angiogenesis		
CD105	Increased microvessel density	Yao *et al*[57]
Proteins involved in angiogenesis		Tseng *et al*[11]
VEGF	Increased expression	
MMPs (matrix metalloproteinases)		
MMP-2 and MMP-9	Increased expression	Xiang *et al*[74]; Nanashima *et al*[48]
Molecules involved in cell adhesion		
E-Cadherin	Decreased expression	Cho *et al*[94]
CD44 (CD44s and CD44v6)	Increased expression	Ryu *et al*[112]; Endo K *et al*[113]
OPN	Increased expression	Huang *et al*[130]
Cell cycle regulators		
p27 (Kip1)	Decreased expression	Wan *et al*[137]
DNA-binding nuclear protein		
HMGB1	Increased expression	Xiao *et al*[171]
Cancer stem cells		
CD133	Increased expression	Chan *et al*[188]
EpCAM	Increased expression	Chan *et al*[188]
CK19	Increased expression	Xu *et al*[197]
Cell surface proteins		
GPC3	Increased expression	Fu *et al*[211]
mTOR Pathway	Increased expression	Baba *et al*[223]

VEGF: Vascular endothelial growth factor; MMP-2: Matrix metalloproteinases 2; CD44s: CD44 standard isoform; CD44v6: CD44 variant isoforms; OPN: Osteopontin; HMGB1: High-mobility group box 1 protein; EpCAM: Epithelial cell adhesion molecule; CK19: Cytokeratin19; GPC3: Glypican-3; mTOR: Mammalian target of rapamycin.

Table 2 p53 antibody used in different studies in this review

Ref.	Clone	Source	Dilution	Antigen retrieval	Cut-off value[1]
Tseng *et al*[11]	DO-7	DAKO	1:100	Citrate buffer	> 5% nuclear p53 staining
Hu *et al*[13]	DO-7	DAKO	1:1000	Citrate buffer	> 10% nuclear p53 staining
Kang *et al*[14]	DO-7	DAKO	1:100	Citrate buffer	> 5% nuclear p53 staining
Stroescu *et al*[16]	DO-7	DAKO	Not reported	Citrate buffer	< 24% nuclear p53 staining
Sung *et al*[17]	Bp53-12	Zymed	1:80	Citrate buffer	> 5% nuclear p53 staining
Qin *et al*[19]	DO-7	DAKO	Not reported	Citrate buffer	≥ 10% nuclear p53 staining
Guo *et al*[20]	CM1	SDC	1:2000	Citrate buffer	> 5% nuclear p53 staining
Umemura *et al*[21]	DO-7	DAKO	1:50	Citrate buffer	≥ 10% nuclear p53 staining

[1]Immunohistochemical cut-off value indicates the percentage of cells with p53 positively staining nuclei. DAKO: Dako Denmark A/S, Glostrup, Denmark; Zymed: Zymed Lab Inc, CA, United States; SDC: San Diego, CA, United States.

any impact on the association between p53 expression and prognosis. In the meantime, we have noticed that different researchers adopted different cut-off values for determining positive p53 expression without any explanation or justification, which has significantly affected the association between p53 expression and prognosis in HCC (Table 2). Since p53 protein expression as detected by IHC does not always reflect the presence of mutant p53 protein, the predictive value of p53 IHC in detecting *TP53* mutations is currently under debate. So far, an optimal threshold is yet to be defined.

In general, a cut-off value of > 10% p53 immunopositive cells appears to be predictive of *TP53* mutations in HCC[27].

What's more, some studies used retrospective analyses in small series of patients. Naturally, without sufficient resolution and reproducibility, it is unlikely to accurately predict disease progression by means of these study designs.

Furthermore, inappropriate proportion of important variables was included in some studies, such as tumor grade, tumor size, tumor stage. For example, too many cases for Edmondson-Steiner Grade I , tumor-node-metastasis (TNM) stage I , or tumors ≤ 5 cm in diameter were selected, which easily resulted in the comparatively low positive rate of p53. And the reliability of their conclusions suffers.

Finally, we have noticed that compared with HCV

Table 3 Clinicopathological parameters affecting the association between p53 expression and prognosis in this review (*n*)

Ref.	Number of patients	Positive rate (%)	HBsAg/HCVAb positive	Edmondson grade		TNM stage		Tumor size	
				Ⅰ + Ⅱ Well/moderate/poor (2)	Ⅲ + Ⅳ (1)	Ⅰ + Ⅱ	Ⅲ + Ⅳ	≤ 5 cm	> 5 cm
Tseng *et al*[11]	113	37.1	79/34	84	29 (1)	54	59	Not reported	
Hu *et al*[13]	124	41.9	83/30	20/38/13 (2)		61	63	Not reported	
Kang *et al*[14]	83	96.4	59/8	27	56 (1)	Not reported		57	26
Stroescu *et al*[16]	47	68	40/0	19	28 (1)	Not reported		20	27
Sung *et al*[17]	105	19	82/6	78	27 (1)	Not reported		> 3 cm	52
Qin *et al*[19]	113	22	40/25	55	58 (1)	Not reported		48	55
Guo *et al*[20]	104	34.6	14/55	18/56/31 (2)		67	37	Not reported	
Umemura *et al*[21]	90	33.3	Not reported	65	25 (1)	Not reported		37	53

TNM: Tumor-node-metastasis; HBsAg: Hepatitis B surface antigen; HCVAb: Hepatitis C virus antibody.

infection, where HCCs were caused mainly by the synergistic effect of HBV infection and aflatoxin B1, studies are more likely to confirm the over-expression of p53 and its prognostic value in HCC (Table 3). This has been partly echoed by studies on the relationship between p53 and pathogenic factors. HBV infection and exposure to AFB1 have been demonstrated to induce the point mutation of p53 in HCC tissue[28], especially exposure to AFB1 can affect the over-expression of p53 in the development of HBV-associated HCC[29]. Other studies also reported p53 protein expression in HCC has racial and regional differences[30]. Therefore, there is a higher chance of reaching a more reliable conclusion on the prognostic value of p53 protein in HCC, researchers should consider HCC cases induced by the same or similar pathogenic factors.

The detection of p53 expression by using IHC has another noteworthy problem. The p53 protein expression as detected by IHC does not always reflect the mutation status of TP53, with one cause being that not all mutations always result in stable protein formation, and another being that some tumors may also express wild-type p53. Nevertheless, in fact, lack of standardized IHC may be partly responsible for the inconsistencies in frequency of *p53* mutations and p53 protein levels. TP53 most often has missense rather than truncating mutations, and IHC antibodies will always have difficulty in detecting proteins with a small number of missense amino acid substitutions. Therefore, the studies with high p53 expression by IHC may reflect both high wild-type and mutant *p53*. Given this, when determining p53 status in HCC, we should analyze it by standardized IHC in combination with *p53* mutation analysis.

In conclusion, p53 protein expression comes short to be recommended as a universal predictive marker for survival in HCC patients, speaking from the available evidence. The prognostic value of p53 protein expression in HCC may vary according to different racial and regional groups. In area where HBV infection and AFB1 account for the major attributive risk of HCC, such as western Africa and south-east China, p53 protein tends to be high expression, and could be considered as a predictive marker for survival in HCC patients. Nevertheless, in order to identify the actual prognostic value of p53 expression in HCC, further studies are required by standardized IHC with larger populations, uniform pathological samples, homogeneous patient populations. It is also worthwhile to point out that it would help us lead to a sound conclusion the studies should include a > 10% nuclear staining as a cut-off value of p53 expression.

Due to the diversity and complexity in the research conclusions on p53, Tables 2 and 3 have been created to help with understanding. These two tables are of reference value in the following discussions on the rest of markers in this review, and hence will not be repeated.

PROLIFERATION MARKERS

The proliferative activity of tumor cells is an important indicator for assessing aggressiveness and could be useful for predicting clinicopathological and prognostic significance. Many antigens, such as proliferating cell nuclear antigen (PCNA) and Ki-67, have been used as proliferation markers for cancer cells. Compared with assessments by Ki-67, cell growth fraction is often overestimated when assessed by PCNA. Thus Ki-67 is considered a more accurate marker for the proliferative stage of tumor cells than PCNA[31,32].

Ki67

Ki-67 is a nuclear non-histone protein initially expressed in cell-cycle phases G_1, S, G_2 and mitosis, and absent in the G_0 phase. The expression of the Ki-67 protein in humans is closely associated with cell proliferation. Naturally, Ki-67 is an excellent marker for proliferating cells[33]. MIB-1 is a monoclonal antibody that identifies Ki-67 protein in paraffin-embedded tissue. Numerous studies have shown that Ki-67 immunohistochemical staining is an effective method to predict prognosis in various tumors.

Ki-67 expression is significantly associated with histological grade of HCC patients[34,35], in other words, the increased expression of Ki-67 in poorly differentiated tissues implies that the single fact of tumor cells losing growth control in hepatocarcinogenesis is a reflection of malignant behavior of tumor cells. Therefore, Ki-67

is an objective indicator of the proliferative ability of HCC cells, and can serve as an important index of the proliferation and differentiation of HCC cells. In addition, Ki-67 expression is significantly higher in HCC cases with shorter DFS; The same applies to the HCC cases with biologically aggressive features such as advanced stages, portal invasion and intra-hepatic metastasis[36]. Therefore, Ki-67 expression could serve as a useful marker for evaluating the progressive activity and predicting DFS in HCC patients. Furthermore, multivariate analysis shows that Ki-67 expression is an independent prognostic factor for DFS and OS[35]. Hence it's been concluded that the expression of Ki-67 is an independent prognostic indicator for post-HR HCC patients[37-40].

In short, Ki-67 expression is an objective factor for predicting survival for post-HR HCC patients, and it could be considered a promising independent prognostic immunohistochemical marker in HCC patients. Therefore, Ki-67 should be taken into consideration when making decisions on adjuvant therapy. HCC patients with high expression of Ki-67 protein may need intensive surveillance and adjuvant therapy.

In spite of the above discussions, lack of standardized IHC and cut-off value has hindered Ki-67 from routine clinical application. Different studies use different methods of antigen retrieval, antibodies concentrations; In addition, the time of incubation varies from study to study; as to cut-off value, some studies have chosen median values while others an arbitrary value (*e.g.*, 10%, 20% and so on) without any explanation or justification. All of these significantly influence the final results. Ironically, the choice of the cut-off value has a major impact on clinical practice, simply because it determines which patients are classified as "high Ki-67 expression"-those who in turn have a poorer prognosis should generally receive more aggressive therapy. We believe future researchers should work towards a standardized IHC and validated cut-off level before Ki-67 could be established as a reproducible and robust prognostic factor in HCC.

To throw in some light, a study has demonstrated that when determining the clinically relevant threshold for immunohistochemical tumor positivity, receiver operating characteristic (ROC) curve analysis could be a reproducible and reliable alternative in selecting and validating cut-off scores[41]. The term "ROC" came from tests of the ability of World War II radar operators to determine whether a blip on the radar screen represented an object (signal) or noise. At present, ROC curve analysis is a well established analytic tool and has been widely applied in various fields, including Medicine. Applications in a number of cancers have proved that cut-off scores based on ROC curve analysis guarantee maximum sensitivity and specificity, and therefore allow the greatest number of tumors to be correctly classified as carrying or not carrying the clinical outcomes[42,43].

Therefore, we propose that Ki-67 cut-off value should be set up according to ROC curve analysis.

MARKERS OF ANGIOGENESIS

Markers of Microvascular Density

Angiogenesis is critical for the growth, invasion and metastasis of cancers. Microvascular density (MVD) is commonly used to assess tumor neovascularization. This is especially true in HCC, characteristically a highly vascular tumor. However, there are conflicting reports in regard to whether MVD in HCC is associated with prognosis. This could be explained by the fact that different studies use different antibodies to calculate MVD.

The evaluation of MVD is generally identified by immunohistochemical staining of endothelial cells with the so-called pan-endothelial cell markers, such as CD34, CD31, and von Willebrand factor. Among them: Firstly, MVD appears to be better assessed by CD31 than by von Willebrand factor (vWF)[44]. Secondly, antibody against CD31 fails to stain sinusoid endothelial cells in many HCC cases, therefore the prognostic value of CD31 could at most be used as a marker of vascular changes in the liver[45]. Thirdly, although CD34 has proven to be a more sensitive and specific endothelial cell marker for microvessels in HCC[46], MVD determined by CD34 appears to be closely correlated with the prognosis of HCC[11,47] in some studies, while such correlation is not identified by others[48,49]. Differences in methodology, *i.e.*, different counting techniques, selection of microvessels, *etc.*, contribute to the conflicting results. The non-specificity in CD34 determines that CD34 can not be an ideal marker for neovascularization. In addition, all the above mentioned markers react with not only newly formed vessels but also normal vessels trapped within tumor tissues.

The conclusion is the MVD identified by anti-pan-endothelial antibodies is not an ideal prognostic marker[50].

The good news is MVD assessment using CD105 as marker (CD105-MVD) has demonstrated a higher MVD specificity in tumor tissues, and it has been more widely adopted, compared with vWF, CD31, or CD34[51-53], as a predictor for progression and prognosis in a variety of cancers.

Endoglin

Endoglin (CD105) is a transforming growth factor-β co-receptor mainly expressed in the endothelium of tissues' blood vessels, particularly in de novo formed blood vessels within tumor. It has been used as a marker for tumor angiogenesis, with a potential for prognostic prediction[54,55].

In HCC, some studies have demonstrated CD105 excels CD34 in marking new microvessels in HCC[56,57]. When median scores of MVD are used as cut-off points, patients with higher score of MVD-CD105 have a significantly poorer prognosis in either DFS or OS analysis, whereas similar prognostic significance of MVD-CD34 is

observed only in DFS analysis[57]. One study reveals that no prognostic significance is observed when median values are used as cut-off points using either IMVD-CD105 or IMVD-CD34, however, the presence of the diffuse pattern of CD105 expression in the adjacent non-tumorous liver tissues can predict a poorer DFS[58]. Collectively, compared with CD34-MVD, CD105-MVD is a significant and independent prognostic indicator for recurrence and metastasis in HCC patients. Having said that, some study found that MVD-CD105 did not show prognostic influence in a cohort of predominantly large HCCs (> 5 cm)[58]. Further studies need to be conducted in larger cohorts of patients with a longer follow-up period.

In summary, CD105, by specifically staining newly formed tumor vessels, is a promising and independent prognostic marker for HCC, which could in turn lead to future therapeutic trials with antiangiogenic therapy. To date, however, the lack of commonly accepted objective criteria in counting microvessels under light microscopy has hampered the clinical use of tumor MVD for prognostication. The authors of this review propose that, before other better microvessel counting methods have been established, microvessel counting should be performed in accordance with Weidner's standards[59].

MARKERS OF PROTEINS INVOLVED IN ANGIOGENESIS

Vascular endothelial growth factor

Angiogenesis is crucial for tumor growth and metastasis, and could be stimulated by several regulators, among which vascular endothelial growth factor (VEGF) seems to be most important[60]. The VEGF family comprises six glycoproteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor. These major VEGF subtypes are in the nature of multiple isoforms. The best representative of VEGF family is VEGF-A (commonly referred to as VEGF). VEGF mediates its angiogenic effects *via* several different receptors, including VEGFR1 and VEGFR2[61].

VEGF plays an important role in tumor angiogenesis and progression, including HCC, and elevated VEGF levels in serum and tissues are related to poor prognosis in HCC patients[62]. So far, numerous studies have explored and confirmed the prognostic value of VEGF for survival in HCC patients. Some studies found the VEGF over-expression was closely correlated with MVD, high alpha-fetoprotein levels, tumor size, dedifferentiation, advanced TNM stage, vascular invasion, capsular invasion, intrahepatic metastasis, and lymph node metastasis (LNM)[63,64]. These findings suggested that VEGF over-expression was useful in predicting progression, metastasis, and recurrence of post-HR HCC[65,66]. In addition, survival analyses indicated that VEGF over-expression was an independent factor for poor-prognosis DFS and OS[11]. Therefore, VEGF expression in HCC tissues could be regarded as a valuable indicator in estimating prognosis of HCC patients[20].

More recent studies suggested that co-expression of platelet-derived growth factor receptors-α, PDGFR-β and VEGF could be considered an independent prognostic biomarker for predicting DFS and OS in HCC patients, and that this co-expression could be used to identify patients at a higher risk of tumor recurrence and poor prognosis, and to select therapeutic schemes for HCC treatment[67]. In addition, the co-index [VEGF/platelet-derived endothelial cell growth factor (PD-ECGF)] was an independent prognostic factor for OS and RFS; Furthermore, the co-index of VEGF and PD-ECGF was a promising independent predictor for recurrence and survival of alpha-fetoprotein (AFP)-negative HCC patients after curative resection[68].

In spite of all the research efforts in establishing VEGF expression status as a promising prognostic marker, there is still a long way to go before the findings could have any impact on clinical practice.

Metalloproteinase

Matrix metalloproteinases (MMPs) comprise a large family of zinc- and calcium-dependent proteolytic enzymes that have been repeatedly implicated in invasion and metastasis. MMPs are capable of degrading most components of the extracellular matrix (ECM), including the basement membrane which serves as a barrier between tissue compartments[69]. Type Ⅳ collagen (Col Ⅳ) is a major component of the ECM and basement membrane, and plays an important role in regulating and limiting tumor invasion and metastasis[70]. Among MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are of particular importance as far as tumor invasion and metastasis are concerned, because they are capable of degrading ColⅣ[71,72]. Furthermore, high MMP-2 or MMP-9 expression in tumor or stromal cells might serve as a poor-prognosis predictor in various cancers[72,73]. This applies to HCC according to some researches.

MMP-2: Quite a few researches concluded that high intratumoral MMP-2 expression in HCC was correlated with high Edmondson grade, advanced TNM stage, and barcelona clinic liver cancer stage[66,74], and that MMP-2 was related to HCC invasion, metastasis and recurrence[75]. As a result of these research findings, it is widely acknowledged that MMP-2 expression could serve as a predictive marker for HCC progression, metastasis, and recurrence, and that MMP-2 expression is an independent prognostic factor for DFS and OS in HCC patients with LNM[74].

MMP-9: The expression of MMP-9 in HCC was proved by a number of researches to be closely correlated to tumor nodule, vein invasion, advanced TNM stage, extrahepatic metastasis, and the formation of portal vein tumor thrombus[48,76,77]. These researches suggest that the expression of MMP-9 reflects the biologically aggressive behavior of HCC, and that MMP-9 is an important molecule which participates in the progression,

metastasis and invasion of HCC. Some studies demonstrated that MMP-9 expression was up-regulated in HBV-associated HCC compared with HCV-associated HCC[78]. Other studies concluded that MMP-9 expression was a significant predictive factor for post-HR recurrence in HCC patients with the background of HBV[79]. Still other studies found that increased expression of MMP-9 protein was an independent prognostic factor after HCC resection[48].

It is worthwhile to note that, when both MMP-2 and MMP-9 were analyzed in the same set of patients, MMP-2 was predominantly involved in hepatocarcinogenesis and progression, while MMP-9 was predominantly involved in the capsular infiltration and portal vein invasion[80]; and that the MMP-2 expression only had weak correlations to HCC recurrence, while positive MMP-9 expression was an independent recurrence-risk factor[25]. Moreover, multivariate analysis confirmed that MMP-9 expression was an independent predictor of time to recurrence (TTR) and OS, whereas high MMP-2 expression was only correlated with TTR[81]. This suggests that MMP-9 is superior to MMP-2 in predicting tumor recurrence and survival in post-HR HCC patients.

One study concluded that high expression of MMP-9 and MMP-2 in peritumoral stromal cells was related to poorer prognosis in HCC patients[82]; However, this was overturned by another study[83]. Still, one study found that MMP-2 or MMP-9 expression was not related with the histological differentiation of HCC[84]; And yet another study claimed that MMP-2 and MMP-9 protein could serve as independent prognostic factors for poor survival regardless of the age, tumor size, tumor grades, TNM classification[85].

The question is: What has contributed to the discrepancies in those research findings? It could be a long list that includes the differences in pathological samples, antibodies used, different IHC methods, different patient populations and different cut-off values. It is advisable that further studies enroll larger scale of clinical HCC samples and use standardized IHC. This has an add-on value of ultimately benefiting the clinical application of MMP inhibitors as chemopreventive and antiangiogenetic drugs.

ADHESION MOLECULES

E-cadherin

Tumor progression is characterized by loss of cell adhesion and increase of invasion and metastasis. Cell adhesion molecules play a significant role in cancer progression and metastasis[86]. E-cadherin is a key molecule for the maintenance of intracellular adhesion, and down-regulation of this protein has been associated with tumor progression in diverse human cancer types[87,88].

Many researches have concluded that E-cadherin expression is very weak in HCC tumors but very strong in the cell membranes of non-tumor tissues, and E-cadherin expression is significantly correlated inversely with histological grade, *i.e.*, with the highest in well-differentiated[89] as put in one study, or the increased loss of E-cadherin expression is observed particularly in poorly-differentiated[90] as put in another; In addition, low expression of E-cadherin in HCC is also related to pathological stage and later TNM stage[91]. Therefore, it is safe to say that low expression of E-cadherin is a strong indicator of malignant HCC progression. There are also some researches that suggest low expression of E-cadherin is significantly associated with intrahepatic metastasis and regional lymph node metastasis[92,93]. When you combine the findings of these two types of researches, it seems natural to conclude that loss of E-cadherin expression in HCC could predict a high risk of post-HR recurrence[94]. Taken together, these findings indicate that detection of E-cadhein expression could be useful in predicting HCC prognosis.

On the opposite side, two studies revealed low expression of E-cadherin had no direct correlation with the post-HR recurrence[95], and it did not predict poor survival even when there was increased loss of E-cadherin in tumors of higher histologic grade[96]. The researchers themselves admitted insufficient number of and lack of homogeneity in the included patients could have contributed to the opposite findings[95]. Another two studies confirmed atypical cytosolic expression of E-cadherin or high E-cadherin membrane/cytoplasm ratio was correlated with a poorer patient prognosis[97,98].

Decreased expression of E-cadherin has been found in all three types of epithelial-mesenchymal transition (EMT) and is thought to be the prototypical marker of EMT[99]. EMT has been shown to be a pivotal mechanism contributing to cancer invasion and metastasis, as epithelial cells lose their polarity and acquire the migratory properties of mesenchymal cells. The characteristic changes during EMT include the downregulation of epithelial markers such as E-cadherin and the upregulation of mesenchymal markers such as vimentin[100]. The EMT of HCC cells is thought to be a key event in intrahepatic dissemination and distal metastasis[101]. A recent study suggests that the loss of E-cadherin followed by the overexpression of vimentin may play a vital role in the invasive and metastatic phenotype and in the process of EMT, leading to unfavorable outcomes in patients with HCC[102].

The authors of this review carefully studied all the related articles, in the course of which differences in antibodies, cut-off values, or race stood out. Further investigation is necessary for assessing these discrepancies.

CD44

CD44, is a transmembrane glycoprotein and has been implicated in numerous biological processes, including cell-cell interactions, cell adhesion, and cell migration[103]. Through alternative mRNA splicing, cells produce numerous CD44 protein isoforms: standard isoform (CD44s) and variant isoforms (CD44v). CD44s is a cell adhesion molecule known for mediating cellular adhesion

to the extracellular matrix-a prerequisite for tumor cell migration. Some researchers argue CD44s is involved in invasion and metastasis of various cancers[104]. Among the CD44 variant isoforms, CD44v6 has reportedly been associated with increased invasion, metastasis, and poor prognosis[105,106]. Recent studies also suggest CD44 is one of the cancer stem cell markers associated with poor prognosis[107].

CD44s: Many studies have indicated that high CD44s expression in HCC is correlated with high AFP level, large tumor size, multiple tumors, poor tumor differentiation, advanced tumor stage, portal vein tumor thrombus, and early tumor recurrence or metastasis[108-110]. These findings suggest that CD44s expression may serve as a predictive marker for HCC progression, metastasis, and recurrence. However, it is not always the case. For example, one study found that there was a significant correlation between CD44s expression and the presence of vascular invasion, but not between CD44s expression and tumor grade, from which the author concluded that high CD44s expression may have implications relating to metastasis and prognosis in HCC patients[111]. Another study found that statistically Edmondson grades had a significant correlation with CD44s expression, and yet such correlation did not exist between CD44s expression and vascular invasion, from which the conclusion is CD44s expression was significantly correlated with DFS and independent factor in multivariate analysis[112].

Some other studies suggested either high CD44s expression was a poor prognostic factor following curative HR of primary HCC, including reduced DFS and OS[108,109], or high CD44s expression was an independent factor for OS[113]. One study failed to present a significant correlation between patient survival and CD44s expression, however , it did show expression of CD44s as a significant predictable marker for LNM[110].

The inconsistency in CD44s expression and clinicopathological parameters is obvious, however, all relevant studies endorse that CD44s expression could serve as a predictive marker for HCC metastasis and survival.

CD44v6: Some studies suggest that high expression of CD44v6 is related to aggressive clinical behavior in HCC, more specifically it is correlated with high tumor grades, advanced TNM stage[114,115]; In addition, CD44v6 overexpression presents a positive correlation with HCC metastatic potential[116]. These findings indicate that high expression of CD44v6 may serve as a predictive marker for HCC progression and metastasis. As to its relationship with vascular invasion, some studies concluded that high CD44v6 expression significantly correlated with the presence of vascular invasion[113,115], while one study demonstrated that a low expression level of CD44v6 tended to be associated with vascular invasion[117]. These studies adopted different scoring systems and cut-off values, which could have contributed to the discrepancy in the results.

In multivariate survival analysis, some studies demonstrated high expression of CD44v6 was significantly correlated with OS and TTR[115], or that it was an independent factor for OS[113]. Thus, detection of CD44v6 expression could be useful in predicting prognosis of HCC.

The authors of this review would tentatively recommend that, for CD44v6 expression evaluation, cut-off value be selected on the basis of ROC curve analysis. In addition to a valid cut-off value, future studies should consider a larger sample and a longer follow-up period. Only then could relevant studies add clinical value to CD44v6 expression in HCC.

Osteopontin

Osteopontin (OPN) is a multifunctional secrete phosphorylated glycoprotein that belongs to the small integrin-binding ligand N-linked glycoprotein family, and it is implicated in promoting malignant cell proliferation, detachment, invasive and metastatic progression of many carcinomas[118-120]. The expression level of OPN is elevated in a variety of human cancers, particularly those that metastasize preferentially to the skeleton[121]. Recent studies have indicated that OPN is involved in HCC progression and metastasis.

It is widely acknowledged that OPN expression is localized predominantly in the cytoplasm, and OPN expression in HCC is stronger than those in paracarcinoma tissues and normal liver tissues[122]; And that higher expression of OPN in HCC is closely associated with poor differentiation and advanced tumor stage[123,124]; And that it is positively correlated with tumor size, capsular invasion, portal vein tumor thrombus, lymph node metastasis[122,125,126]. Therefore, it is safe to say OPN could serve as novel biomarker for monitoring HCC progression and metastasis.

In addition, numerous studies have suggested OPN could serve as a useful marker for predicting early recurrence in HCC patients[122,127], and that OPN could help determine whether individual patient needs adjuvant therapy to prevent early post-HR recurrence[128], and that OPN expression is an independent prognostic factor either for DFS in HBV-positive small HCC (< 5 cm)[129], or for OS and DFS in patients with the TNM stage I HCC[127]. These findings suggest that OPN could be solely identified as an independent prognostic biomarker for post-HR HCC patients[130].

Recent studies have suggested that the combination of OPN and some other markers seem promising for HCC prognosis. For example, the combinations of tumor OPN with either caspase-3, or Bcl-2, or CD44, have all been announced as promising independent predictors of tumor recurrence and survival in HCC patients[130,131]; It is especially true for those with early-stage disease when tumor OPN is combined with microenvironment-associated peritumoral macrophages[132]. Nevertheless, the interaction between tumor OPN and these markers, which facilitates tumor progression and metastasis, still

remains unclear in clinical practice. Further large-scale studies are required to confirm their clinical value[133].

CELL CYCLE REGULATORS

p27

The functional alterations of cell-cycle regulators, such as Cyclin Dependent Kinases (CDK) and their inhibitors, occur frequently in cancers. As a critical CDK inhibitor, p27 (Kip1) is involved in G1 phase progression, and is widely regarded as adverse prognostic biomarker for various types of cancers, since decreased or absent expression of p27 (Kip1) is frequently observed in various types of human cancers with poor prognoses[134,135]. It has been reported that p27 (Kip1) is exclusively inactivated by proteasome-mediated protein degradation[136]. p27 (Kip1) is frequently inactivated in HCC and is considered a potent tumor suppressor.

So far, many studies have reported that decreased p27 expression is significantly lower in HCC than those in the adjacent noncancerous tissues or in normal liver tissues, and it is a risk factor in HCC[137-139]. Furthermore, some studies have indicated decreased p27 expression is closely related to the aggressive HCC tumor behaviors[139,140]. In addition, some studies indicated that p27 expression was decreased in advanced cases in a series of curatively resected HCCs[141], and the p27 labeling index was significantly decreased in the cases with advanced tumor stages, portal invasion, poor differentiation, larger size, and intrahepatic metastasis[142,143]. As concluded by a researcher, p27 expression in HCC could act as an independent predictor of post-HR recurrence[142].

It has been reported that in multivariate analysis, p27 expression could be recognized as an independent prognostic marker for OS[144], and OS and loco-regional recurrence-free[145], which suggests low expression level of p27 is associated with significantly worse prognosis in HCC patients[137,146]. Similar findings have been reported that high expression of p27 is a favorable independent prognostic parameter[147]. Taken together, p27 could be regarded as a powerful clinical indicator for prognosis prediction in individual HCC patient.

An interesting point is that it is in both nucleus and cytoplasm that tumor cells were found to have expressed p27 protein[148]. The significance of cytoplasmic p27 protein is still under debate, and cytoplasmic p27 protein is rarely considered in assessing p27 IHC score. Decreased or absent expression of p27 (Kip1) in nucleus is frequently observed in various types of human cancers with poor prognoses[149-153]; however, some researchers argue over-expression of cytoplasmic p27 may also serve as a marker for poor prognosis in several types of human cancers[154-156]. Further studies suggest that the nuclear localization of p27 is essential for its growth-inhibiting function[157]. When narrowing down to HCC, the expression of p27 is mainly found in nucleus and cytoplasm[144]. It has been generally accepted that low expression of nuclear p27 protein is associated with poorer prognosis, while cytoplasmic expression of p27 is positively associated with poor cellular differentiation-the higher the expression, the higher incidence in HCC patients[140]. This is echoed by a study that concluded cytoplasmic localization of p27 could be an early event during hepatocarcinogenesis[158].

It remains unclear whether the cytoplasmic staining represents a methodological artifact or a finding of biological and/or prognostic importance. In view of this uncertainty, the authors of this review propose that only nuclear p27 (kip-1) staining for HCC survival analyses be considered in staining evaluation.

Taken together, IHC detection of p27 on routine tissue sections could be useful in predicting survival of individual HCC patient and in determining future therapeutic strategies. Therefore, p27 is worthy of further evaluation as a potential prognostic marker in clinical trial samples of large cohorts.

DNA-BINDING NUCLEAR PROTEIN

High-mobility group box 1 protein

High-mobility group box (HMGB) proteins are non-histone nuclear proteins with different functions in the cell[159]. HMGB1, HMGB2, and HMGB3 are the members of the HMGB protein family, with HMGB1 being the most important one. While the expressions of HMGB2 and HMGB3 are limited, HMGB1 plays a role in cancer progression, angiogenesis, invasion, and metastasis development[160]. The function of HMGB1 is complicated by its cellular localization. In nucleus, HMGB1 binds with DNA and serves as a structural component[161]. Cytoplasmic localization of HMGB1 is associated with the proliferation and metastasis of different tumor types. The process could be dramatically sped up when cytoplasmic localization of HMGB1 binds with the receptor for advanced glycation end products[162]. As for the "sped up" process, one study has deduced that the interaction between receptor for advanced glycan endproducts and HMGB1 activates mitogen-activated protein kinases, nuclear factor kappa B, and phosphoinositide 3-kinases (PI3K)/AKT signaling pathways to promote cellular proliferation and metastasis[163].

There are many relevant studies that focus on HCC and their findings include: In HCC cells, downregulation of HMGB1 could remarkably inhibit the growth of HCCLM3 cells, as well as their migration and invasion ability[164]; HMGB1 knockdown inhibited the proliferative activities and metastatic potential of SMMC-7721 cells. That is to say, the expression of HMGB1 was closely correlated with pathological grade and distant metastases of liver cancer, and HMGB1 knockdown inhibited liver cancer growth and metastasis[165]. In addition, HMGB1-siRNA could inhibit the invasion and migration abilities of human hepatoma cell line HepG2[166]. In the liver tumor model, stable knockdown of HMGB1 suppressed HCC invasion and metastasis[167]. In detection of serum

HMGB1, serum HMGB1 was positively correlated with clinicopathological features in HCC patients, higher serum HMGB1 level was correlated with bigger tumor size, poor Edmondson grade and advanced TNM stage[168]. Collectively, these findings suggest that HMGB1 in HCC is significant in tumor progression, invasion and metastasis.

In recent years, many studies have explored the clinical significance of HMGB1 expression in various human tumors, including HCC. Some study reported that over-expression of HMGB1 was significantly associated with HCC incomplete encapsulation and advanced TNM stage[169]; similarly, another study demonstrated that, by detecting fresh samples, over-expression of MGB1 mRNA was correlated with HCC high Edmondson grade, advanced TNM stage, vascular invasion and capsule invasion[170]. These findings indicate that over-expression of HMGB1 is associated with HCC tumor growth and invasion.

Recent studies have also demonstrated the expression of HMGB1 could serve as an independent prognostic factor for poor OS and DFS for post-HR HCC patients; more importantly, subgroup analysis showed the expression of HMGB1 was significantly associated with poor prognosis in HCC patients > 5 cm, but not in HCC patients ≤ 5 cm[169]. This trend suggests that HMGB1 could be an important prognostic marker for late stage HCC; in addition, multivariate analysis has also concluded that HMGB1 expression is a key independent prognostic factor that could be associated with OS of HCC patients[171]. Therefore, HMGB1 expression could be taken as an independent predictor of prognosis for post-HR HCC patients. However, further studies are necessary before we could tell for sure whether HMGB1 is a reliable clinical predictor of survival for individual post-HR HCC patient.

STEM CELL MARKERS

In recent years, many findings have suggested that tumors are comprised of heterogeneous cell populations, only a small fraction of which are tumorigenic with the ability to self-renew and produce phenotypically diverse tumor cell populations[172]. Cells in this fraction are called cancer stem cells (CSCs) or tumor-initiating cells or cancer progenitor cells, and they have the ability to self-renew, proliferate, and maintain the neoplastic clone. Accumulating evidence has shown that these CSCs have long-term proliferative potential and the ability to regenerate tumors with phenotypically heterogeneous cell types, and that these CSCs are important mediators of tumor metastasis and cancer relapse[173].

So far, various cell surface and transmembrane proteins expressed by CSCs have been identified, including CD44, CD47, CD123, epithelial cell adhesive molecule (EpCAM) (CD326), CD133[174]. In HCC, the three major types of liver CSCs (LCSCs) are dedifferentiated hepatocytes, hepatic oval cells, and bone marrow cells. To date, CD133, CD90, and EpCAM, CD44, CD24, and CD13 have been identified as specific antigenic markers for HCC stem cells[175]; The oval cell-specific marker (OV6) is identified as a marker for hepatic oval cells[176], in addition, cytokeratin 7 (CK7) and CK19 are identified as markers for dedifferentiated hepatocytes[177].

LCSCs can be observed by IHC and electron microscope. In HCC, the phenotypes of LCSCs express as OV6, CK7, CK19, CD133 and EpCAM[178]. There have been a number of studies reporting that the expression of LCSCs markers in HCC is associated with poor clinical outcome after surgical resection[179,180]. Among them, the expression of EpCAM, CK19 and CD 133 has demonstrated association with intrahepatic recurrence in HCC patients[181].

To our knowledge, EpCAM, CK19, and CD 133 have been so far the most widely studied LCSCs markers in HCC using IHC.

CD133

Prominin 1 (CD133) is a pentaspan transmembrane glycoprotein with uncertain physiological function, and it is often expressed by various epithelial and non-epithelial cells, notably by stem and cancer stem cells. CD133 is currently recognized as a marker for LCSCs[182-184]. A number of studies have demonstrated *via* IHC that CD133 expression is associated with poorer tumor grade and advanced tumor stage[185]; Moreover, CD133 expression is associated with the absence of tumor capsule; and CD133 tends to be expressed in tumors showing stronger potential for invasion and metastasis[186]. These findings suggest that CD133 expression is associated with HCC progression, invasion and metastasis. In addition, several studies have demonstrated that CD133 expression is a significant risk factor for the OS of HCC patients, especially patients with Stage Ⅲ and ⅣA HCC[187]; And that Cox proportional hazard model has shown that CD133 expression is an independent predictor for DFS[177]; and the multivariate survival analysis has demonstrated that CD133 expression is an independent adverse prognostic factor for OS and DFS, especially for patients with early-stage HCC[188]. All the above mentioned studies agree to the basic point that increased CD133 expression could serve as an independent prognostic factor for survival in HCC patients.

EpCAM

EpCAM, also known as 17-1A, GA733-2, KSA, ESA, and EGP-40, is a type I transmembrane glycoprotein and acts as a homotypic calcium-independent cell adhesion molecule. It is expressed in almost all carcinomas. EpCAM is currently recognized as a marker for LCSCs[189-191]. Many studies have demonstrated *via* IHC that EpCAM expression is associated with younger age[181], poorer histological differentiation, vascular invasion and/or more advanced stage[180,188,192]. These findings suggest that EpCAM expression is associated with HCC progression. Furthermore, several studies have demonstrated that EpCAM expression could serve as an independent factor

for DFS in HCCs at all stages[188]; And the multivariate survival analysis has demonstrated that EpCAM expression is a significant predictor for shorter survival time in HCC patients[186], especially patients with T1 HCC[180]. Taken together, increased EpCAM expression could serve as an independent prognostic factor for survival in HCC patients.

CK19

CK19 has been considered as a marker for the biliary phenotype[193], and it is not expressed in normal hepatocytes[194]. CK19 is currently recognized as a marker for LCSCs[181,183,195,196]. Increased CK19 expression is correlated with high histological differentiation, advanced BCLC stage, TNM stage[197], tumor non-encapsulation[198], the presence of satellite lesions[74], number of tumor foci, and vascular tumor embolism[199]. These findings dictate that increased CK19 expression could serve as a new biomarker predicting HCC progression and recurrence. In addition, some studies have identified association between CK19 expression in HCC and increased vascular invasion, lymph node metastasis, and intrahepatic spread[200,201], dictating that CK19 expression is an independent risk factor for developing LNM, and that it is an important risk factor for early tumor recurrence. In addition, increased CK19 expression has also been found to be both an independent poor prognostic factor for OS, DFS, and RFS in post-HR HCC patients[74,197], and an independent prognostic factor for HCC with LNM[202]. However, other studies have come to a different conclusion. Some studies have demonstrated that CK19 is an independent prognosticator for OS, but not for DFS[194,199]. Still some studies have suggested that CK19 expression has prognostic significance for DFS, though CK19 fails to offer independent prognostic value[188].

Taken together, the expressions of CD133, EpCAM and CK19 could be readily assessed by IHC and they are clinically significant biomarkers for surgically resected HCCs. However, predictive values of single LCSCs markers remain controversial and further validation is required in independent cohorts ahead of any clinical utilization[203]. More importantly, because of high degree of HCC heterogeneity, the predictive range of a single marker is limited to a very small subpopulation. A combination of several LCSCs markers may provide greater specificity and reliability in predicting HCC prognosis[178].

CELL SURFACE PROTEINS

Glypican-3

Glypican-3 (GPC3) is an oncofetal protein considered as a relatively specific HCC biomarker that is not detectable in hepatic para-carcinomatous and cirrhotic tissues[204], and it is over-expressed in HCC using IHC[205,206]. Recently, much evidence has shown that GPC3 could be a useful tool to identify early HCC development. More recently, GPC3 has been reported to be a new prognostic factor after curative hepatectomy in HCC patients.

In addition to being a marker for HCC, GPC3 plays a role in the progression of the disease[207]. GPC3 expression has been less frequently observed in well-differentiated HCC than in moderately and poorly differentiated HCC[205,208,209]; furthermore, it has been found significantly correlated with serum AFP level, tumor number and presence of satellite nodules, and TNM stage[210,211]; in addition, GPC3 expression has also been found to be associated with postoperative metastasis/recurrence in HCC patients[129,208,212]. These findings indicate that GPC3 expression might be a valuable marker closely related with post-operative progression, metastasis/recurrence in HCC patients. Multivariate analysis has identified GPC3 expression as an independent prognostic factor for OS[129]. However, in other studies, for HCC patients with HCV infection in particular, the high membranous GPC3 immunoreactivity has been identified as an independent prognostic factor for DFS[213]; one study has even suggested that over-expression of GPC3 is an independent prognostic factor for DFS in HBV-positive small HCC (< 5 cm)[129]. Recently an extensive study has shown that high GPC3 expression is an independent risk factor for poor postoperative tumor recurrence, DFS, and OS[211], again suggesting that GPC3 expression is a potential and reliable biomarker for predicting tumor recurrence and OS in post-HR HCC patients.

Overall, these studies indicate that GPC3 expression has the potential to serve as a valuable predictive marker for survival in post-HR HCC patients. Further studies are required to confirm GPC3 is one of the reliable clinical predictors of survival for individual post-HR HCC patient.

MAMMALIAN TARGET OF RAPAMYCIN PATHWAY

Currently there is evidence suggesting that phospho-specific antibodies could serve as potential biomarkers for HCC. These markers provide useful reagents for analysis of signaling pathways in clinical samples, and therefore has the potential for actionable targets[214]. So far, the molecular biology of hepatocarcinogenesis and HCC progression has been widely investigated. Many studies have indicated that signaling pathways dysregulated in HCC are important steps towards understanding HCC pathogenesis and developing new therapeutic approaches. Over recent years, several molecular pathways have been identified contributing to the molecular pathogenesis of HCC, among which the mammalian target of rapamycin (mTOR) signaling pathway has been identified to play a critical role in the pathogenesis of HCC[215]. And many studies have investigated the relationship between mTOR pathway and HCC prognosis.

mTOR pathway, an important regulator of multiple cellular functions including proliferation, differentiation, tumorigenesis, and apoptosis, is up-regulated in many cancers[216]. Deregulation of the mTOR signaling pathway has been reported in many malignancies, and some of the

signaling molecules in this pathway could predict prognosis in different cancers. PI3K/AKT is considered a critical upstream mediator of the mTOR signaling pathway. Recent data from a genomic sequence of HCC samples identified mutations in PIK3CA, an upstream regulator of AKT, in 50% of patients with poor prognosis and survival length of < 3 years following partial liver resection, whereas only 10% of the HCC patients with a good prognosis had a mutation in PIK3CA[217]. Activation of AKT is a risk factor for early disease recurrence and poor prognosis in patients with HCC[218]. Activated AKT positively modulates mTOR function. mTOR is a key component of PI3K and AKT pathways that activate downstream kinases required for G1 to S phase transition[219]. mTOR acts by directly activating p70S6 kinase (p70S6K/S6K1) and inhibiting 4E binding protein 1 (4E-BP1)[220], both regulating the translation of important factors involved in cell proliferation (such as c-myc, cyclic D1 and pRb) and angiogenesis (such as HIF1-α)[221]. The p70S6 kinase and 4E-BP1 have shown to be independent predictors of prognosis in several types of solid tumors including liver[216,222,223]. Therefore, the expression of mTOR pathway could be used as prognostic indicator in HCC.

In addition, one study has indicated that c-Jun N-Terminal Protein Kinase 1 (JNK1) activation contributes to poorer HCC prognosis, and there is similarity in gene expression patterns between the HCC with abnormal mTOR signaling and JNK1 HCC[224], which further supports the assumption that HCCs with abnormal mTOR signaling are tumors of a highly aggressive nature and with poorer prognosis.

Recently, mTOR has emerged as an exciting target for cancer therapy including HCC. mTOR inhibitors have been tested successfully in clinical trials for their antineoplastic potency and good tolerability[225]. A second generation of mTOR pathway inhibitors has been utilized in preclinical HCC models[226] and the results suggest that mTOR inhibitors in HCC treatment could have a bright future.

Noticeably, although phospho-specific antibodies used in IHC are expected to detect phosphorylated proteins[227-229], some preanalytic variables (such as fixation technique and duration) may critically affect the signal[230], and in some cases these antibodies may also cross-react with nonphosphorylated proteins[231]. Therefore, it is of ultimate importance to standardize preanalytic variables and to employ a control in determining whether the staining pattern is specific.

CONCLUSION

In this review, we give an overview of the literature published on immunohistochemical prognostic markers in HCC. Out of 17 markers that have been investigated by ten groups (summarized in Table 1), there are twelve markers (over-expression of Ki67, VEGF, MMP-2, MMP-9, CD44s, CD44v6, OPN, HMGB1, CD133, EpCAM, CK19, GPC3 and mTOR pathway, and increased microvascular density of CD105) that have shown to be independent prognostic factors for survival in HCC patients. However, studies on some markers, such as p53, E-cadherin and p27, have all reported inconsistent results. Lack of standardized IHC has contributed to these discrepancies; other possible contributors include small sample sizes, pathological differences in samples, heterogeneous patient populations, various follow-up periods of the patients, and different racial and regional groups.

So far, numerous investigations have demonstrated many immunohistochemical markers could be potential prognostic/predictive indicators of HCC. However, their clinical utilization is severely hindered by the lack of standardized IHC methodology.

Although IHC is the most widely applied technique in pathology to determine the expression status of tumor-associated proteins and to study the clinical prognostic relevance of biomarkers, IHC results are subject to a variety of pre-analytical variables (*e.g.*, fixation method or the duration of fixation, methods of tissue processing), analytic variables (*e.g.*, antibodies, dilutions, antigen retrieval, time of incubation), and post-analytic variables, most importantly, subjectivity in determining scoring system for protein expression (cut-off values, *i.e.*, thresholds for positivity and interpretation criteria). Throughout IHC, each and every variable may greatly affect the accuracy and reliability of IHC results.

In view of the urgent demand from clinical practice, it is prerequisite to rigorously standardize IHC methodology, and this standardization should include all aspects of pre-analytical, analytic and post-analytic variables.

It sounds like a mission impossible to exercise full control over all pre-analytic variables, not to mention a complete standardization. Having said that, the collaboration among laboratories in Europe and the States has proven to be effective in tackling them. Analytic variables could to some degree be compensated for by using a large sample series. It is worthwhile to highlight that, because polyclonal antibodies have higher chances to cross-react with other antigens, it is important to further validate if the results presented in the study are specific by comparing staining patterns obtained with polyclonal antibodies with staining patterns generated by monoclonal antibodies. In addition, in order to improve reliability and interpretability of immunohistochemical markers, it has been advocated that standardized reporting criteria be used for biomarker studies[232]. A wide-spread adoption of these recommendations will help overcome some of these methodological issues.

Nevertheless, subjectivity in applying a scoring system for protein expression is probably the biggest obstacle for the pathology laboratories. Therefore, we put strong emphasis on post-analytic variables, *i.e.*, cut-off values and interpretation criteria.

Prognostic significance of immunohistochemical marker fluctuates sharply with different cut-off values,

which in itself makes it difficult to determine a valid cut-off value for clinical use. ROC curve analysis could be used as an alternative method in the selection and validation of cut-off scores for determining the most clinically relevant threshold for immunohistochemical tumor positivity[41]. Where contradictory results have been yielded from researches on established biomarkers, this tool should be adopted to re-evaluate protein expression. In addition, the authors of this review would tentatively recommend future investigations on novel tumor markers use ROC curve analysis.

No IHC scoring methods have been strictly agreed on. Researchers have been relying on percentage of positivity or intensity of positive staining, or a combination of these two, to estimate protein level. The intensity of positive staining in liver tissue sections could be easily affected by such pigments as iron deposition or brown granules in Kuppfer cells, and therefore is not a valid indicator of specific immunostaining. Additionally, IHC is a technique that detects specific antigens present in the target cells by labeling them with antibodies against them which are tagged with enzymes to convert a soluble colorless substrate to a colored insoluble precipitate which can be detected under the microscope. The intensity of positive staining is easily affected by individual researcher's skill and experience both in operating IHC and in reading slides, as well as technical conditions for IHC operation. Therefore, IHC intensity is not an appropriate criterion to be used in HCC research. The authors of this review would tentatively recommend that, for protein positive expression evaluation in liver tissue sections, percentage of stained area/field be selected as a quantitative method for IHC results. To ensure objectivity, the scoring methods of immunohistochemical markers should be assessed by independent observers.

It is worthwhile to highlight that IHC in itself could never tell us about the mutation status of these proteins. That is to say, in order to better understand the relevance between immunohistochemical markers and clinical outcomes, standardized IHC should be combined with gene mutation analysis using polymerase chain reaction methods in the same patients.

A number of studies have demonstrated that although single marker could provide useful information on the prediction of patients' survival and treatment outcomes, and could monitor efficacy of individualization of therapy, the heterogeneity of HCC tumors requires a combination of biomarkers in order to yield better clinical performance. In the foreseeable future it is likely that multiple markers need to be integrated into a prognostic signature to accurately predict outcomes. In fact, the HCC biomarkers in combination are increasingly becoming part of surveillance protocols in United States clinics[233]. Still a further long way to go before their routine use in clinical practice becomes an reality, which requires immunohistochemical markers of prognosis and prediction to be validated in carefully designed large-scale, prospective clinical trials, using standardized IHC techniques. Then, and only until then, could the validation of prognostic and predictive markers eventually guide our clinical decision making in regard to follow-up scheduling and treatment choice.

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